

# Estrogen differentially regulates expression of P-38 and hemeoxygenase-1 in mouse cervix

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**Key Words:** P-38, hemeoxygenase-1, cervix, VEGF, cervical remodeling

**Author's Summary:** The research was conducted to determine the specific pathway(s) in mice that estrogen utilizes in order to induce cervical inflammation. A variety of qualitative and quantitative techniques were used in order to delineate the pathway(s) used by estrogen. The results showed that the P-38 pathway was up-regulated by estrogen to induce the inflammation and that the expression level of the anti-inflammatory protein, HO-1, was concomitantly decreased. These findings support the need for further research into developing methods or drugs to inhibit the estrogen-activated P-38 pathway to potentially decrease the cervical inflammation and also increase the anti-inflammatory HO-1 expression to achieve reduction in preterm labor.

## Abstract

Recent research has shown that estrogen stimulates vascular epithelial growth factor (VEGF) expression in mouse cervix, and that VEGF induces inflammation, as well as proliferation of cervical epithelial cells. However, the pathway that mediates estrogen's effects on VEGF in the cervix is unknown. P-38 is a signaling molecule that has been shown to mediate estrogen's up-regulatory effects on VEGF in other tissues. Further, hemeoxygenase-1 (HO-1) has been shown to reduce inflammation in the placenta, thus preventing preterm labor. However, estrogen's role in the cervix and the effects of estrogen on HO-1 expression have not been studied. Here, I examine the effect of estrogen on the expression of P-38 and HO-1 in the mouse cervix using H&E staining, confocal immunofluorescence microscopy, and western blotting. The results of this study show that estrogen up-regulates P-38 protein expression but down-regulates HO-1 protein expression. Based on these findings, I conclude that estrogen up-regulates the P-38 protein expression, which in turn mediates estrogen's downstream effects, likely including the expression of VEGF, a mediator of cervical inflammation. Also, reduction in HO-1 protein expression in response to high estrogen levels during late pregnancy may lead to increased inflammation, contributing to preterm labor.

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## Introduction

Normal pregnancy and the birth process are considered to be physiological inflammatory processes [1]. Since inflammation is the leading cause of preterm labor, factors that may either abnormally increase or decrease the tone of inflammation may influence the timing of birth, i.e., abnormally high inflammation may induce premature labor and vice versa [2]. Sex steroid hormones play varied and significant roles in inflammation by regulating a number of genes. For example, overall, estrogen promotes the expression of pro-inflammatory factors, such as interleukin 6 and vascular endothelial growth factor (VEGF) in the uterus and cervix (T. Ohashi, Masters Thesis, Appalachian State University, 2013). However, estrogen's inflammatory effects are complex, i.e., it can be pro- or anti-inflammatory in different tissues and also be different under physiological or pathological conditions within the same tissue. Here, I studied two proteins that may possibly mediate estrogen's pro- and anti-inflammatory actions in the cervix,

i.e. the expression of P-38 protein and of the anti-inflammatory enzyme, heme-oxygenase-1 (HO-1). Cervical remodeling (CR) is a complex process that occurs during pregnancy and is regulated by multiple factors, including pro-inflammatory factors and, possibly VEGF. Collectively these factors promote a timely opening of the cervix during labor. Some of the notable cervical changes induced by exogenously administered VEGF in non-pregnant mice include increased edema, marked induction of cell growth, as well as increase in inter-epithelial paracellular space [3]. The process of cervical remodeling can be considered similar to inflammation where some of the foremost events include angiogenesis, vasodilation, and an increase in vascular permeability [2].

VEGF stimulates various vascular processes, including angiogenesis, vascular permeability and vasculogenesis. It has also been shown to up-regulate levels of pro-inflammatory cytokines, such as interleukin-6 (IL6) and tumor necrosis factor (TNF- $\alpha$ ) in the mouse cervix [2]. Since VEGF increases the tone of inflammation in the cervix of non-pregnant mice, abnormal concentration

of this protein during pregnancy can potentially induce a precocious or premature remodeling of cervix, and thus, could induce preterm labor. Previous studies have shown that 17- $\beta$  estradiol ( $E_2$ ) up-regulates the expression of VEGF in microvascular endothelial cells (MEC) of the human myometrium [4], most likely via the estrogen receptor- $\alpha$  [5]. Recently, it has been shown that VEGF induces proliferation of cervical epithelial cells in mice and that estrogen promotes the expression of VEGF in the same tissue [3]. Although, it is known that in breast cancer cells, P-38 mediates estrogen's up-regulatory effects on VEGF expression [6], the exact signaling pathways that mediate estrogen's effects in the cervix are currently unclear.

Estrogen is also known to activate or down-regulate the expression of anti-inflammatory factors such as HO-1 in several tissues in a tissue-dependent fashion. For instance, administration of estrogen to the lungs following trauma hemorrhage increases HO-1 expression and decreases cleaved executioner caspase-3 levels. [7]. Further, in the rat prostate, estrogen-induced increase in HO-1 protein expression in the stroma, and the resultant carbon monoxide release by HO-1 is believed play a vital role in the regulation of smooth muscle activity [8]. HO-1 is normally induced by oxidative stress or nitric oxide, and its activity inhibits oxidative damage and apoptosis, as well as inflammatory events. Carbon monoxide is a byproduct of HO-1 activity and it modulates the anti-inflammatory characteristics of this enzyme [9]. Specifically, HO-1 plays a significant role in the catabolism of heme, which generates biliverdin, carbon monoxide and iron as the products of the reaction [10]. Contrary to the activation of HO-1 reported in the studies above, but more relevant to the cervical tissues, a previous study has indicated that HO-1 is down-regulated through activation of the P-38 pathway following the transfection of the iNOS gene into chorion trophoblast cells [11]. The apoptosis of the cells observed in this experiment could have a causal role in the subsequent rupture of fetal membranes, especially since rupture did not occur when the iNOS gene was transfected to the amnion membrane. This rupture is thought to be related to inflammation. Interesting and relevant to the above discussion, expression of the iNOS gene is up-regulated by estrogen [12], implying that estrogen may have a role in membrane rupture. Further, since VEGF is known to be a potent inducer of iNOS, it is likely that estrogen activates VEGF, which, in turn, induces iNOS. Thus, my long-term hypothesis is that estrogen up regulates VEGF expression in the cervix via the P-38 pathway and that the resulting increased inflammatory tone together with the simultaneous reduction of HO-1 activity by estrogen, contributes to inflammation-induced preterm labor. Therefore, I examined estrogen's effects on P-38 and HO-1 protein expression levels in mouse cervix in vivo because the results of this study may provide further insights into the prevention of growing global epidemic of preterm labor.

## Materials and Methods

The hormone treatments of mice and harvesting of tissues used in this study were performed prior to this study in the laboratory of Dr. Mowa as described below.

**Animals:** All procedures were performed in accordance with the regulations established by the Animal Care and Use Committee, the *Guide for the Care and Use of Laboratory Animals* of the Appalachian State University, and NIH Publication No. 86-23. Non-pregnant ovariectomized mice (C57BL6, Charles Rivers) were used in the current study. Prior to estrogen treatment, all animals were ovariectomized under general anesthesia with a mixture of ketamine (43-129 mg/kg body weight) and xylazine (8.6-26 mg/kg body

weight). Immediately after surgery, all animals were administered the antibiotic Baytril® (Bayer, Leverkusen, Germany) in order to prevent postsurgical infection. To allow for complete excretion of residual endogenous ovarian sex steroid hormones, animals were allowed to rest for at least seven days following ovariectomy prior to estrogen treatment. Before tissue harvest, animals were euthanized by lethal injections of Sleepaway® (concentrated sodium pentobarbital, Fort Dodge Laboratories Inc., Burlingame, CA). Uterine cervical tissues from treated mice were harvested, processed, and analyzed. Harvested tissues were analyzed using morphological techniques (H & E staining and confocal immunofluorescence microscopy) and protein expression (western blot) in order to determine the effects of estrogen on P-38 and HO-1 expression. Three animals (n=3) were used per treatment group.

**Treatment with 17 $\beta$ -estradiol:** The ovariectomized mice were divided into three treatment groups and a control group (n=3). The mice were treated with 17 $\beta$ -estradiol dissolved in sesame oil once a day for four days with an injection of 50 microliters into the intra-abdominal cavity; the dosages were as follows: 2 mg/kg body weight for the "low dose" group, 4 mg/kg body weight for the "medium dose" group, and 8 mg/kg body weight for the "high dose" group. The control group was treated with sesame oil alone.

**Tissue harvest:** 24 hours following the last hormone treatment, the animals were euthanized with lethal injections of Sleepaway® (concentrated sodium pentobarbital, Fort Dodge Laboratories Inc., Burlingame, CA). Following lethal injection, animals were perfused intra-cardially with 0.9% sodium chloride to flush out blood from the tissues. Uterine cervical tissues were then carefully dissected under a stereomicroscope. Tissues were immediately weighed and those designated for western blot were stored at -80°C until further processing. Tissues designated for morphological studies were fixed in 4% paraformaldehyde for a minimum of 24 hours, followed by fixation in 0.1 M PBS at 4°C for at least 24 hours, and then gradually dehydrated by step-wise immersion in ethanol-water mixtures of increasing ethanol concentration (70%, 95%, 95%, 100%) for a minimum of 2 hours per dilution followed by two changes of xylene for 2 hours each. The fixed tissues were embedded in paraffin and stored at room temperature until sectioning.

**Hematoxylin and Eosin (H&E) staining:** Basic histology of cervical tissues was performed using H&E staining. The paraffin blocks were cut into 5  $\mu$ m-thick sections using a microtome (Leica RM 2125 RTS, Leica microsystems, USA) and incubated at 37°C overnight in a dry oven. The sections were deparaffinized using two changes of xylene, rehydrated by step-wise immersion in aqueous alcohol solutions (100%, 100%, 85%, 70% and 50%), and washed with distilled water followed by 0.1 M PBS, for 10 min each. Tissues were stained with standard H & E staining procedures (VWR international LLC, USA) and imaged using an Olympus DSU IX81 (Olympus, USA).

**Protein quantification and localization:** Cellular localization and tissue quantification of P-38 and HO-1 protein expressions were performed using confocal immunofluorescence microscopy and western blot analysis as described below.

- i) **Confocal immunofluorescence microscopy:** The paraffin-fixed sections prepared as described previously were incubated with primary antibodies, as described by the manufacturer (Santa Cruz Biotechnology, Santa Cruz, CA). After the paraffin removal, non-specific protein bindings were blocked using 10% normal

goat serum in 0.1 M PBS (NGS) or 10% bovine serum albumin in 0.1 M PBS (BSA) for 30 minutes at room temperature. Thereafter, the sections were incubated overnight with the appropriate primary antibody (HO-1 or P-38- $\alpha/\beta$ , 0.5  $\mu\text{g}/\text{ml}$ ) in humidified chamber at 4°C. The sections were then washed three times in 0.1 M PBS for 5 minutes each. Control tissue sections were incubated with either 10% NGS or 10% BSA. All sections were incubated with fluorescent secondary antibodies (0.5  $\mu\text{g}/\text{ml}$ ) for 1 hour and then washed 3 times for 5 minutes each in 0.1 M PBS. The nuclei were counterstained with 5  $\mu\text{M}$  Sytox<sup>®</sup> Green for 5 minutes and then washed for 5 minutes in 0.1 M PBS. All images were obtained using a confocal microscope (Zeiss LSM510, Thornwood, NY).

- ii) *Western blot analysis:* Quantitative rather than qualitative analysis of protein expression was performed in order to complement data gathered from confocal immunofluorescence imaging. Total protein was isolated from the harvested tissue using standard protein isolation protocol[13]. Concentrations of protein samples were determined by the Pierce BCA assay (Thermo Scientific, USA) at an absorbance of 562 nm in a Spectrophotometer (Thermo Scientific, USA). For western blotting, the proteins were separated by gel electrophoresis at 125 V for 90 minutes and then transferred to PVDF membranes. The membranes were then incubated in blotto (5.0 g of nonfat dry milk and 100 ml of 1X TBST) for 24 hours at 4°C. Membranes were incubated overnight at room temperature with appropriate primary antibodies (P-38 [rabbit] and HO-1 [goat]) at 1:500 dilution as described by the manufacturer. They were then washed 3 times with 1x TBST for 5 minutes each and then incubated with the appropriate secondary antibody (anti-rabbit and anti-goat) for 1 hour at room temperature. They were then washed with three 5-minute washes of 1X TBST followed by a 5 minute wash with 1X TBS. The membranes were then coated with luminol-enhancer/peroxidase solution (VWR, USA) and the luminescent image was analyzed using ImageJ (NIH). The specific bands of interest were identified using a standard

ladder of a known molecular weight of  $\beta$ -actin (Santa Cruz Biotechnology, USA).

*Statistical analysis:* Western blot data was analyzed by ANOVA and values with  $P < 0.05$  were considered significant.

## Results

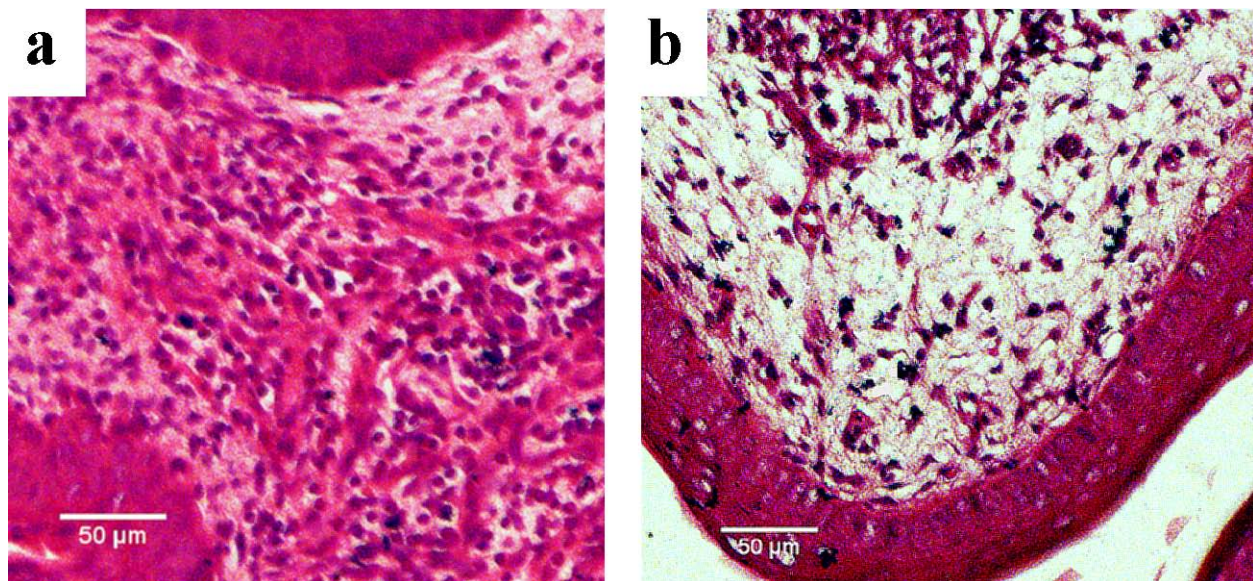
### Estrogen induces histological changes in ovariectomized mouse cervix

*Basic histological changes:* H&E of the negative control showed densely populated cervical stromal cells as compared to the group treated with high estrogen (Figure 1). Furthermore, the layer of cervical epithelial cells of estrogen-treated animals was thicker than that of the negative control (Figure 1b). The control section is more densely populated with cells in the stroma compared to the high estrogen treated cervix. In the epithelium of the high estrogen treated cervix, there are unidentified cells which are absent in the control. The integrity of the cervical epithelium observed in the sections indicated that these tissues were suitable for molecular studies that follow.

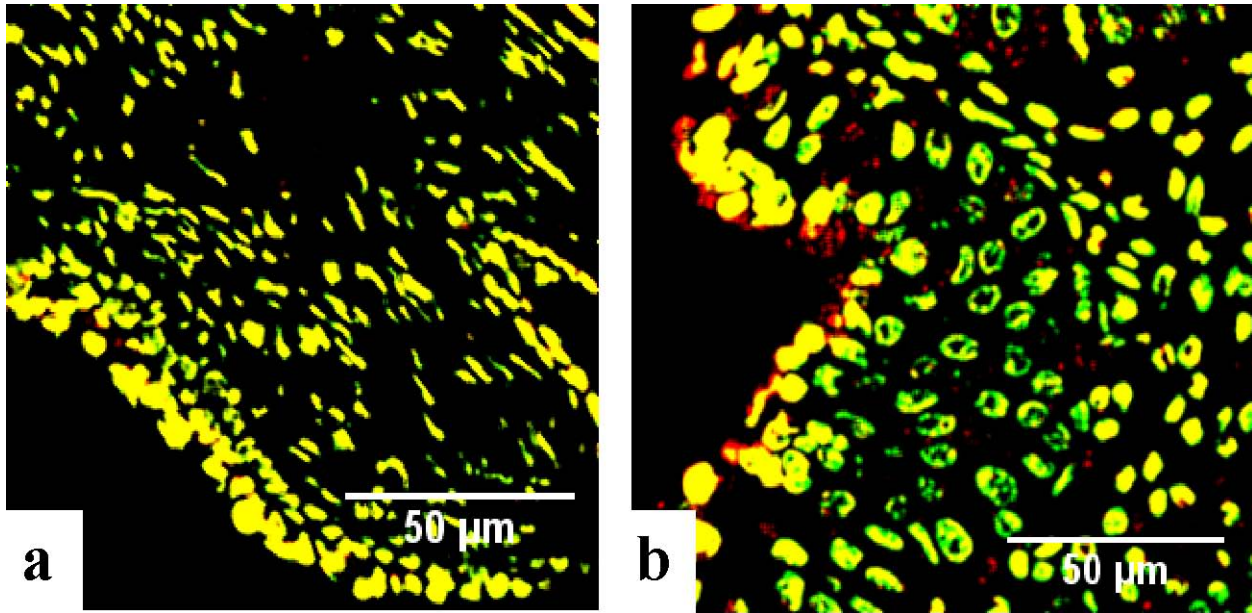
### Estrogen up-regulates P-38 (MAPK) protein expression in the cervix

The data from the confocal immunofluorescence showed strong P-38 immunoreactivity in cervical epithelial cells, with scattered weak to moderate signals from stromal cells, in animals estrogen-treated as compared to the control group (Figure 2), demonstrating the increase in P-38 upon estrogen treatment. Figure 2b displayed more p38  $\alpha/\beta$  protein expression (red/yellow) than Figure 2a in the stroma as well as the epithelial lining of the cervical cells.

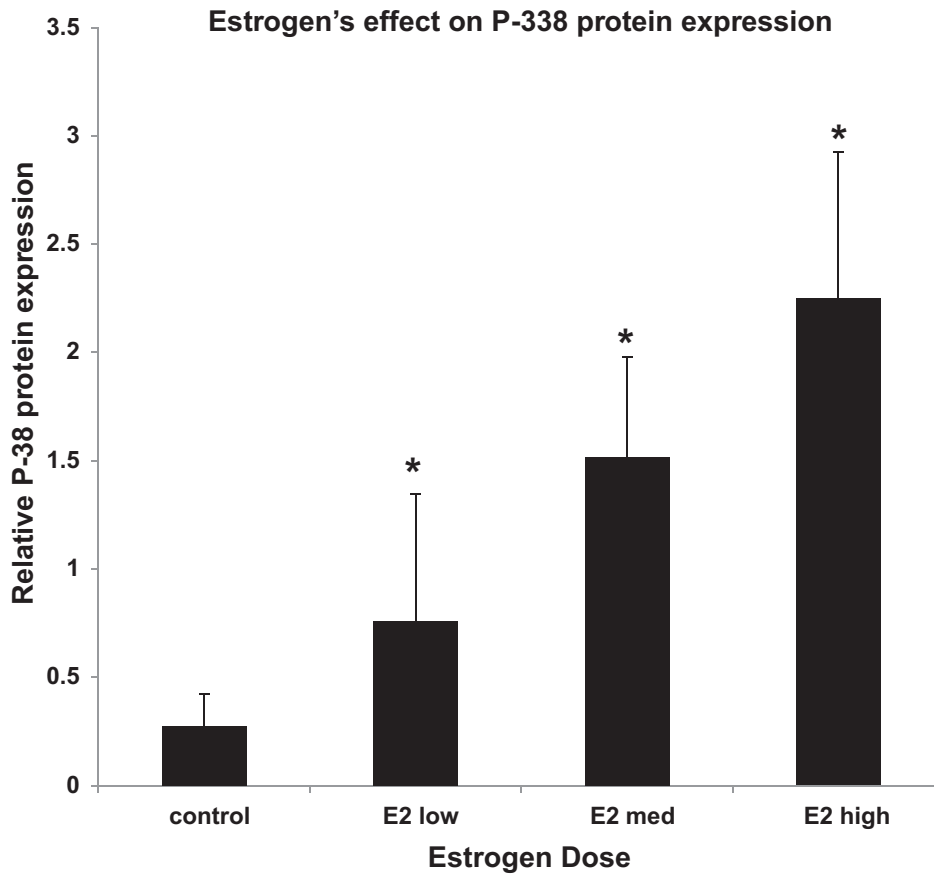
As depicted in Figure 3, the western blot data showed a dose-dependent increase in the expression of the P-38 protein in the



**Figure 1.** Effect of estrogen on the histology of mouse cervix. H&E staining method was used to determine the integrity and cytological alterations in the mouse tissues. Figure 1a (20x) control, sesame oil; Figure 1b (20x) high estrogen, 8mg/kg. The control section is more densely populated with cells in the stroma compared to the high estrogen treated cervix. In the epithelium of the high estrogen cervix, there are unidentified cells which are absent in the control.



**Figure 2.** Effect of estrogen on the immunohistochemistry of P-38 protein expression in mice cervix.: The cervical sections were immunostained with p38 $\alpha/\beta$  primary antibodies (red fluorescence) and the nuclei were stained with Systox@green (green). Figure 2a (40x) negative control; Figure 2b (40x) high estrogen dose group, 8mg/kg). Cells in Figure 2b display more P-38  $\alpha/\beta$  protein expression (red/yellow) than those in Figure 2a, both in the stroma as well as the epithelial lining.



**Figure 3.** Effect of estrogen on P-38 protein expression on mice cervix: Estrogen treatment up-regulated the P-38 protein expression significantly. Error bars show standard errors of mean. Statistical significance ( $P < 0.05$ ) relative to the controls was clearly observed at all doses by ANOVA analysis.

cervix upon estrogen treatment relative to the negative control ( $P < 0.05$ ).

### Estrogen down-regulates HO-1 protein expression in the mouse cervix

Confocal immunofluorescence microscopy detected strong signals of HO-1 immunoreactivity in the cervical epithelial cells, with scattered weak to moderate signals from stromal cells in the control group, which was significantly reduced in the animal treated with estrogen (Figure 4) showing a decrease in HO-1 levels upon estrogen treatment. The section in Figure 4a displayed more HO-1 protein expression (red/yellow) than the one in Figure 4b in the stroma as well as the epithelial lining of the cervical cells.

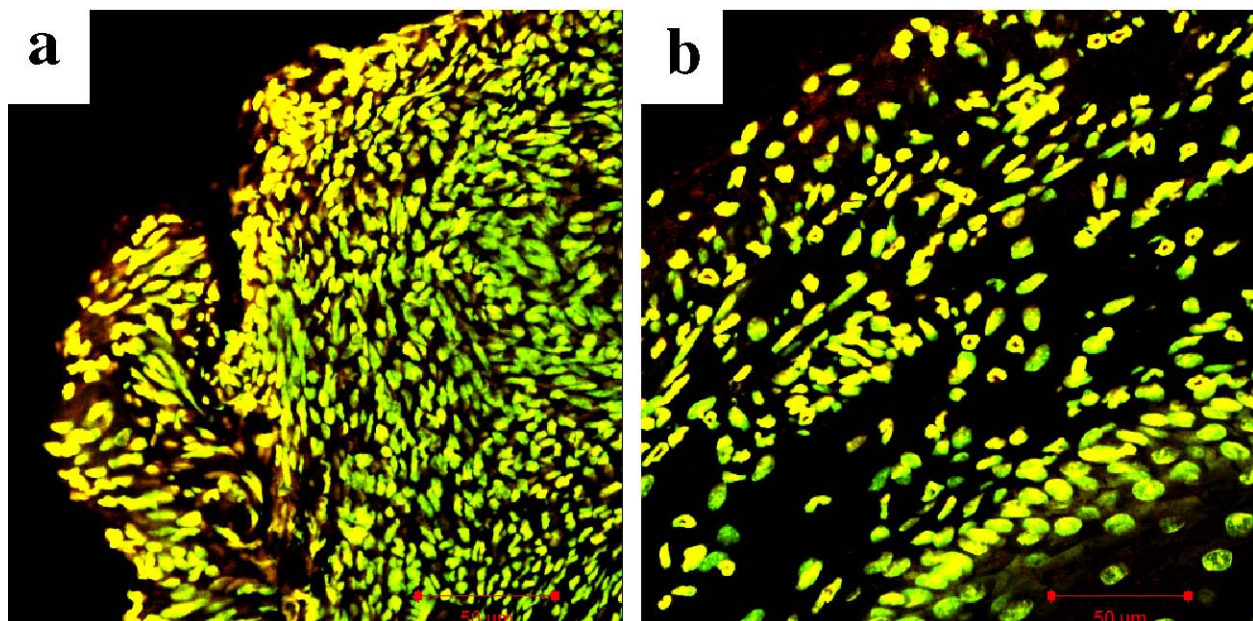
In agreement with the immunofluorescence data, the Western blot data (Figure 5) showed that estrogen down-regulated the expression of HO-1 protein in the cervix in a dose-dependent manner when compared to the negative control ( $P < 0.05$ ). Although the data in Figure 5 show statistically significant suppression of HO-1 expression relative to the control, the differences between the three dose groups are not statistically significant by ANOVA analysis of the data, thus not revealing a dose response.

### Discussion

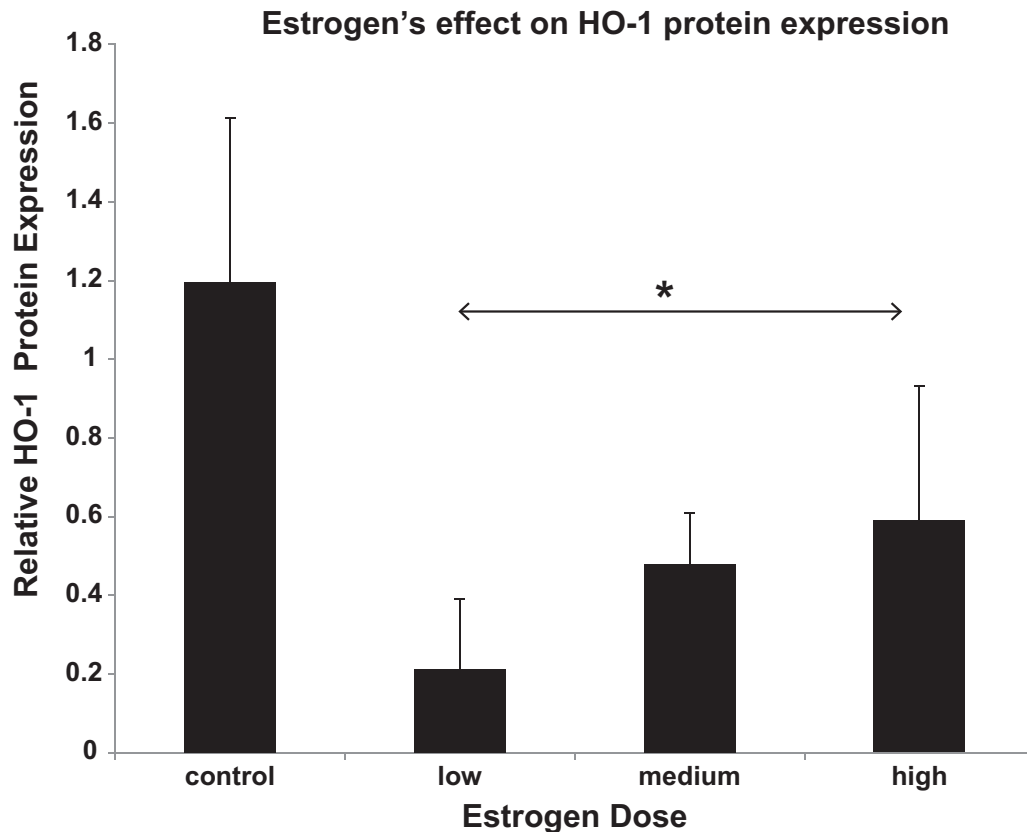
The aim of this study is to identify the pathway(s) that estrogen uses to activate the expression VEGF in the cervix well as estrogen's effects on HO-1 expression. The key findings in our study are that estrogen 1) up-regulates the expression P-38 protein in the cervix of non-pregnant mice, and 2) down-regulates the expression of HO-1 protein in the same tissues. The physiological effects of estrogen are mediated by estrogen receptors (ER), notably ER- $\alpha$  and ER- $\beta$ . ER- $\alpha$  chiefly act in the uterus and mammary glands while ER- $\beta$  chiefly acts in the nervous, immune, and cardiovascular systems [4]. Estrogen uses the Wnt signaling pathway to induce breast

cancer [14]. Further in porcine trophoblast cells, prostaglandin E<sub>2</sub>, has been shown to induce aromatase, resulting in estrogen secretion and also the activation of the MEK/MAPK signaling pathway to induce cell adhesion during implantation [15]. In addition, studies strongly suggest that estrogen can influence growth hormone-regulated endocrine and metabolic function in the human liver through the GHR-STAT5 pathway [16]. Furthermore, estrogen utilizes the PI3K and the MAPK pathways to induce retinal endothelial cell proliferation [17].

The P-38/MAPK pathway is also among the many signaling pathways that are utilized by estrogen. Specifically, the P-38/MAPK pathway has been shown to regulate the effects of estrogen on human corneal epithelial cells [18], as well as up-regulate the LMP1 oncogene [19]. Although no specific studies have implicated the P-38/MAPK pathway in estrogen action in the cervix, P-38 activity has also been demonstrated in the cervical tissue. For example, *in vitro* studies have been shown that the P-38/MAPK pathway is activated by the flavonol, quercetin via the ER- $\alpha$ -dependent mechanisms, ultimately leading to the apoptosis of human cervical epithelial carcinoma HeLa cells [20]. Further, in studies *in vitro*, the flavanone naringenin (Nar) also induces apoptotic effects on the human hepatoma HepG2 cells by activating the P-38/MAPK pathway through the ER- $\alpha$  receptor [21]. In addition, the P-38 pathway mediates apoptosis *in vitro* in human colon carcinoma DLD-1 cells through ER- $\beta$  [21]. Further, the P-38 pathway was also activated by diosgenin, which bears structural similarities to estrogen and hence has been hypothesized it to embody estrogenic effects. Diosgenin has been shown to up-regulate VEGF-A to stimulate angiogenesis in pre-osteoblast like cells [22]. Collectively these studies show that the P-38 pathway plays a role in cell proliferation under physiological conditions, but induces cell death (apoptosis) under pathological conditions, and that estrogen can modulate the P-38 pathway achieve some of these effects. These *in vitro* studies suggested a possible role for P-38



**Figure 4.** Effect of estrogen on immunocytochemistry of HO-1 protein expression in mice cervix. The cervical sections were immunostained with HO-1 primary antibodies (red fluorescence) and the nuclei were stained with Systox®green (green). Figure 4a (40x) negative control; Figure 4b (40x) mice treated with 4mg/kg estrogen. Cells in Figure 4a display more HO-1 protein expression (red/yellow) than Figure 4b in the stroma as well as the epithelial lining of the cervical cell.



**Figure 5.** Effect of estrogen on HO-1 protein expression in the mouse cervix. Estrogen treatment down-regulated the p38 protein expression significantly. Error bars show the standard errors of the mean. Statistical significance ( $P < 0.05$ ) relative to the control was clearly observed for the treatment group, but not among the treated groups, by ANOVA analysis.

pathway in estrogen action in cervical cells *in vivo*. In the present study, which mimics physiological aspects of estrogen action (in contrast to the carcinogenic studies cited earlier), the data demonstrate the activation of the P-38 pathway by exogenous estrogen. It is likely that this induction of P-38 by estrogen may induce biological events such as VEGF expression which further induces inflammation in the cervix demonstrated by Nguyen et al. [2]. This hypothesis would imply that abnormally high estrogen during pregnancy can lead to excessive inflammation in the cervix, which in turn can lead to preterm labor. HO-1 is present in multiple tissues in the body and is involved in various processes, notably in counteracting oxidative stress and in inflammation. Estrogen has been shown to increase HO-1 expression in the lungs and protects them from trauma-induced hemorrhaging through the Akt pathway [7]. Further, the HO-1 protein expression is also increased when estrogen up-regulates the Nrf2-Keap1 defense pathway in homocysteine (Hcy) treated dopaminergic SH-SY5Y cells to reduce the neurotoxicity of Hcy [23]. Additionally, estrogen down-regulates HO-1 protein expression while inducing stress and cell death in estrogen-deprived breast cancer cells since HO-1 is an indicator of oxidative stress [24]. Based on these observations, I hypothesized that estrogen likely suppresses expression of HO-1 in the cervix during pregnancy further leading to “run-away” pathological inflammation, which could induce preterm labor.

Currently, no studies exist that relate the effect of estrogen on HO-1 protein expression and the functional implications. The data indicate that estrogen down regulates HO-1 protein expression.

This obtained result indicates that the down-regulation of the anti-inflammatory activity of HO-1, combined with increased expression of pro-inflammatory factors such as VEGF by abnormal levels of estrogen could lead to “runaway” inflammation in the cervix, possibly causing preterm labor. Further studies are needed to elaborate further on these mechanisms.

Infants born prematurely are susceptible to hearing and vision impairment as well as to other neurological and behavioral disorders. If induction of P-38 does indeed play a role in preterm labor as suggested by this study, it would be reasonable to attempt to inhibit P-38 using appropriate inhibitors to pre-treat the mice in order to suppress estrogen-mediated VEGF expression and hence avert preterm labor.

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