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The Role of G protein-coupled estrogen receptor in vascular function and hypertension

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THE ROLE OF G PROTEIN-COUPLED ESTROGEN RECPTOR IN VASCULAR FUNCTION AND HYPERTENSION

by

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DISSERTATION

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DEDICATION

To Duane, Margaret and Nicholas Fredette

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THE ROLE OF G PROTEIN-COUPLED ESTROGEN RECEPTOR IN VASCULAR FUNCTION AND HYPERTENSION

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Doctorate of Philosophy, Biomedical Sciences

ABSTRACT:

Regulation of arterial tone relies heavily on molecular crosstalk between endothelial and smooth muscle cells. Although it has been demonstrated that estrogen (E2) exerts protective effects against cardiovascular disease (CVD), the exact mechanisms and balance of E2 signaling in endothelial and smooth muscle cells remain unresolved. Here we characterize the G protein-coupled estrogen receptor (GPER) in the vasculature by elucidating its pathway in endothelial nitric oxide (NO) production and vascular smooth muscle maintenance of reactive oxygen species (ROS) by NADPH oxidases (NOX). NO is thought of as a vasoprotective entity, while the overproduction of ROS by NOX can lead to vascular dysfunction. These two forces counteract each other to maintain arterial tone, and understanding their mediation by GPER will provide significant insights in sex differences in vascular function.

In endothelial cells, we demonstrated that GPER activation induces phosphorylation of eNOS at ser1179, and contributes to ER-dependent NO production. Inhibition of GPER through pharmacologic antagonism (G36) or genomic deletion (GPER KO) reduced NO production and vasorelaxation to

E2. We also found that NO production by selective estrogen receptor modifiers and downregulators (SERMs/SERDs), known to antagonize classic ERs and agonize GPER, was only partially abrogated by GPER inhibition, suggesting that there may be E2- independent targeting effects by these compounds.

In the smooth muscle we were surprised to find that the arteries of GPER KO animals exhibited a reduced contraction response to Ang II. We characterized a novel pathway in which GPER mediates genomic expression of the NOX1 subunit in smooth muscle cells. Critically, we report that GPER inhibition or deletion reduces or blocks the hypertensive response to Ang II in mice.

Taken together, these data describe a counterintuitive paradigm in which GPER signaling is important for E2-mediated NO production at the endothelium through acute eNOS activation, but conversely is involved in ROS generation at the smooth muscle though expression of NOX1. In lieu of the conflicting CVD reported for E2 replacement in post-menopausal women, we propose that the signaling pathway(s) of GPER may increase ROS production through smooth muscle NOX1 expression, which may override endothelial NO production, resulting in vascular dysfunction.

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CHAPTER 1

INTRODUCTION

1.1 Estrogen Synthesis & Biochemistry:

Estrogen is a steroid hormone synthesized in the ovaries of sexually mature females, and in the testes and adrenal glands of males. Estrogen is also synthesized at comparatively lower levels in peripheral tissues of both sexes, such as adipose, vascular endothelium and liver by the aromatase enzyme. Estrogen is synthesized through a multi-step process (**Fig 1.1**). First, cholesterol undergoes a multistep conversion into the androgen intermediate adrostenedione. Adrostenedione is converted to testosterone, which is then converted by the cytochrome P450 enzyme aromatase into estrone. Estrone undergoes a hydroxyl addition by the enzyme 17β HSD to become 17β estradiol. There are three naturally occurring forms of estrogen: estrone (E1), estradiol (E2), and estriol (E3), which are named based on their number of hydroxyl groups (1, 2 or 3, respectively) (**Fig 1.2**). The predominantly active form of estrogen is 17β-estradiol, which has a second hydroxyl group located at the beta position of the 17^{th} carbon, and will be referred to in this thesis as E2.

The ovaries begin to synthesize estrogen around 12-15 years of age and continue until the 5^{th} or 6^{th} decade of life. Aromatase continues to synthesize estrogen from testosterone at low levels in both sexes throughout an individual's life. In particular, low levels of E2 are secreted by the adipose tissue. The primary form of estrogen in post-menopausal women is the metabolite estrone (1). Circulating E2 has a half-life of approximately 3-10 hours in women and is metabolized in the liver by cytochrome p450 (CYP)

enzymes into the less metabolically active estrone and estriol, which are passed in urinary excretion. Small amounts of estriol are reabsorped by the liver into circulation, which partly contributes to basal estrogen levels.

E2 was originally described as the primary signaling molecule of sexual maturation by Doisey *et al.* in 1923 (2). Doisey and colleagues crystallized purified E2 from rat uterus, and reported that crystallized E2 induced morphological changes in reproductive tissues associated with sexual maturity, such as increased uterine wet weight, thickening of the vaginal wall, and ovulation in rodents.

In circulation, E2 molecules are bound to albumin and globulin with approximately 2 to 2.5% in an unbound or “biologically active” state. In females, detectable serum E2 levels fluctuate with the menstrual cycle. Serum concentrations of E2 typically range from 100-200 pM prior to cycling (follicular phase), and peak at approximately 1 nM in the preovulatory phase. E2 levels peak a second time post-ovulation (luteal phase) at concentrations up to 500 pM. Post-menopausal E2 serum levels typically range from 30 to 100 pM. In sexually mature males, plasma E2 levels range from 50 to 100 pM, similar to post-menopausal women. In females, reproduction-related functions of E2 include ovulation, endometrial development, breast and milk duct development, as well as uterine and vaginal wall growth. In males, reproductive functions of E2 include development of testes and epididymis as well as spermatogenesis and sperm cell maturation in these tissues. In non-reproductive tissues, E2 has been implicated in osteocyte activity for bone health, brain development

and function, immune cell activation, production of clotting factors, adipose tissue activation and metabolism, as well as multiple functions in cardiovascular tissues, which will be a focus topic of this thesis.

1.2 Estrogen Receptors and Signaling

Experiments using tritiated E2 uncovered a specific binding pattern within reproductive tissues (particularly the uterus), leading to the description of the first “estrogen receptor” in 1967 by Jensen et al. (3) (4). These experiments suggested that the effects of circulating E2 are mediated by its interaction with a specific steroid receptor. Soon after, the isolation of a macromolecule from the human uterus with specific binding to E2 was reported by McGuire *et al.* in 1972 (5). Since these initial reports, almost 40,000 studies have characterized the structure, localization and function of these receptors.

1.2.1 Receptor structure and localization:

There are three primary receptors now accepted as mediating estrogen’s physiologic effects: estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), and the more recently described G protein-coupled estrogen receptor (GPER). For over 20 years only one estrogen receptor (ER) was known, until the discovery of a second homologous receptor in rat prostate in 1996 (6). These receptors were ultimately named ERα and ERβ, respectively. ERα and ERβ share a 58% homology within the ligand binding domain and 96% homology in the DNA binding domain (7-9). The third estrogen receptor, now named G protein-coupled estrogen receptor GPER (formerly GPR30) was

cloned from shear stress-stimulated endothelial cells and breast cancer cell lines in the late 1990s. Its signaling activities were described by several laboratories in the early 2000s (10, 11). Expression of ERα and GPER has been reported in multiple tissues and cell types including breast, uterus and endometrium, adipose, kidney, brain, heart, immune cells and vascular tissues with various physiologic effects (12).

Like other soluble steroid receptors, ERα and ERβ lack a plasma membrane domain and reside predominantly in the nucleus and cytoplasm. The lipophilic nature of its cholesterol-derived ligand E2 allows diffusion of E2 across plasma and other cellular membranes. The structure of ERα/β consists of 5 domains (**Fig 1.3**). The activation function 1 "AF-1" A/B domain is located at the amino terminus and is responsible for binding co-factors and other proteins. The C domain is responsible for DNA binding and the D domain or "hinge" domain provides flexibility of the receptor, allowing conformational changes upon ligand interaction. The E domain contains the ligand (E2) binding domain, and the AF2 F domain at the carboxyl terminus binds transcriptional co-factors in a ligand-dependent manner. Within each of these domains, multiple post-translational modifications such as phosphorylation and palmitoylation (13) (14) regulate trafficking and signaling in a tissue-specific manner (15) (16). Palmitoylation targets a small proportion of ERs to the plasma membrane to scaffold with caveolin-1 (17) (18), whereas phosphorylation of ERα can induce ligand-independent activation.

1.2.2 Signaling pathways: genomic and rapid

The first described and most studied signaling pathway of ERs is the "classical" or "genomic" pathway (**Fig 1.4 A**). Upon ligand binding, ERs shed Heat shock protein (HSP) and dimerize with a second ER as a homo or hetero dimers (α/α, β/β, or α/β). The dimerized unit can then bind to multiple transcription or co-factors, depending on cell and tissue type. Three general mechanisms of genomic activation have been described. In "direct activation", ligand-activated, dimerized ERs bind DNA directly to estrogen response elements (EREs) as a dimer via the C domain. In the second, "indirect activation" (or "tethered activation") mechanism, ligand-activated dimerized ERs binds indirectly as a complex with other transcriptional co-factors at other (non-ERE) gene promoters (19). The third mechanism of ER binding is ligand-independent, where kinases such as MAPK/ERK phosphorylate ERα in the absence of E2 binding, resulting in receptor binding to EREs and other promoter regions. Co-factor recruitment by ERs is tissue and cell type-specific, depending on the molecular cues and post-translational modifications (16) (12).

In contrast to these now classic genomic signaling activities, early studies by Szego *et al*. reported increases in uterine cyclic AMP (cAMP) concentrations within minutes of E2 treatment (20). Szego *et al*. followed these functional studies with reports identifying estrogen binding to isolated plasma membranes of the uterine endometrium (21). These rapid cAMP increases in response to E2 were later confirmed in vascular tissues treated with E2 (22, 23). Such reports indicated a role for rapid signaling induced by E2, and

suggested that ERs were functionally diverse in mediating both genomic and rapid signaling responses. Additional rapid effects mediated by E2 have been demonstrated in multiple cell types, including EGF release, ERK and Akt activation, calcium mobilization and vascular nitric oxide production, the latter in vascular endothelial cells (24, 25) (**Fig 1.4.B**). One mechanism of rapid signaling by ERs has been proposed to occur through a small subpopulation of membrane-bound ERs (26) (mERs). Membrane association of ERs requires post-transcriptional palmitoylation within the E domain and tethering to the membrane in caveolae lipid rafts by the scaffolding protein Caveolin 1 (14, 27, 28) (29, 30). Ligand activated mERα subsequently activates heterotrimeric G proteins, such as Gq, which in turn activate PLC to cleave PIP3 into IP3 and DAG downstream targets, or Gi/s proteins that regulate adenylyl cyclase activity to decrease (αi) or increase (αs) cAMP. Studies with pertussis toxin, a Gi protein inhibitor, have been shown to abrogate ERα rapid signaling, revealing the role of this G protein in estrogen signaling (24, 31).

1.3 GPER discovery & mechanisms:

The extensive cloning of GPCRs in the late 1990s yielded a plethora of receptors with no known ligand, resulting in their designation as orphan receptors, and heralded decades of research aimed at identifying endogenous ligands for these receptors and describing their physiologic functions (16180917 9884064). The orphan GPCR ‘GPR30’ was cloned from human endothelial cells exposed to shear stress (among other sources), implicating a function in vascular tissues (32). Soon after its cloning, studies

by Filardo *et al.* characterized GPR30 as a receptor that mediated rapid signaling in response to E2 (33) (10). This was based on the observation of rapid ERK1/2 phosphorylation in response to E2 in SKBr3 cells, a GPR30-expressing breast cancer cell line that lacks expression of both ERα and ERβ, as well as in MDA-MB-231 breast cancer cells when transfected with GPR30 (34). However, these early reports of rapid estrogen-mediated signaling in GPR30-expressing cells did not establish commensurate binding of E2. Only in 2005 did separate studies by Revankar *et al.* and Thomas *et al*. report specific E2 binding in GPR30-expressing cells. Experiments by Thomas *et al.* utilized tritiated E2 in SKBr3 (ERα/β-negative, GPER-positive) cells, and in HEK293 cells (GPER/ERα/β-negative) transfected with GPR30, and observed tritiated E2 binding only in GPR30-expressing cells. They calculated a binding affinity of E2 for GPER of 3.3 nM, suggesting E2 as a ligand for GPR30.

In a study using transfection of a GFP-GPR30 fusion protein into COS7 cells (GPER/ERα/β-negative), Revankar *et al.* demonstrated that GPER predominantly localized to an intracellular tubuloreticular network, including the endoplasmic reticulum and Golgi apparatus, with negligible expression at the plasma membrane or in the nucleus (11). A novel Alexa633-conjugated E2 compound was found to colocalize with GFP-fused GPER in transfected COS7 cells and demonstrated a binding affinity of 6 nM, similar to the experimental values reported by Thomas *et al*. in HEK293 cells. These

reports provided convincing evidence that the orphan receptor GPR30 was likely a receptor for binding and mediating the rapid signaling effects of E2.

Filardo and colleagues as well as others (34) (35) (11) first described rapid signaling of GPER via EGFR transactivation by employing a series of inhibitors to investigate increased phosphorylated ERK levels in MCF7 (ERα+/GPER+) and SKBr3 (ERα-/GPER+) breast cancer cell lines (33). This increase was abrogated by the ERK inhibitor PD98059 and the EGFR inhibitor AG1478, and was also inhibited by pertussis toxin, an inhibitor of the Gαi proteins (36). Inhibition of c-Src kinase signaling using PP2 also blocked GPER-mediated ERK1/2 phosphorylation (37). Furthermore, neutralizing antibodies to HB-EGF also blocked GPER-mediated ERK1/2 phosphorylation. Thus, the following mechanism of GPER in rapid cell signaling was proposed (**Fig 1.5**). First, ligand binding of E2 to GPER leads to activation of G protein subunits and phosphorylation of c-Src, which in turn activates matrix metalloproteases (MMP). MMPs cleave extracellular pro-heparin-binding epidermal growth factor (pro HB-EGF) into it its activated form HB-EGF. HB-EGF then binds with the EGFR receptor tyrosine kinase at the plasma membrane, which initiates a cascade of multiple enzyme activation pathways, including most notably ERK1/2 phosphorylation, PI3K/AKT activation and/or c-Src activation. GPER has also been shown to activate adenylyl cyclase (AC) and increase cAMP through Gαs, which was downregulated ERK1/2 phosphorylation (38). Characterization of this additional GPER mechanism suggests that there is a

tight regulation of ERK1/2 activity by GPER to prevent overstimulation of adenylyl cyclase.

Another downstream target of cross-activated EGFR is PI3K/AKT. Our laboratory first reported that GPER activation by E2 also induces PI3K signaling, which results in the production of PIP3 and the recruitment and activation of AKT. Experiments from our laboratory utilized the monomeric red fluorescent protein (mRFP)-fused PH domain of AKT (mRFP-PH) to assess PIP3 production following GPER activation with E2 or 4-hydroxy tamoxifen (4OHT) in COS7 cells transfected with mRFP-PH and GFP-fused GPER. These experiments demonstrated that GPER activation resulted in translocation of mRFP-PH to the nucleus, suggesting production or at least accumulation of PIP3 in the nucleus following GPER activation. GPER-mediated nuclear translocation of mRFP-PH was blocked by LY294004, an inhibitor of PI3K/AKT signaling, demonstrating the involvement of PI3K in this process. Finally, intracellular calcium increases in response to GPER activation have been reported in cells exogenously and endogenously expressing GPER (34) (39) (40) (41). Experiments revealed that GPER activation with E2 resulted in increased intracellular calcium levels using the calcium-sensitive dye Indo1-AM in GPER-transfected COS7 cells. Calcium increases by GPER were abrogated both by EGFR inhibitor AG1478 and by the Gi protein inhibitor pertussis toxin (33) (36), indicating GPER increases intracellular calcium through Gαi and EGFR signaling pathways. Taken together, these data demonstrated a novel non-classical estrogen receptor

(GPER) can mediate several forms of rapid estrogen signaling through a G protein-coupled mechanism, resulting in EGFR transactivation for pathways promoting ERK1/2 activation, Ca^{2+} increase, and PI3K/AKT activation (42).

In addition to rapid signaling of GPER, several genomic effects of GPER have also been described. Activation of GPER has been linked to increased expression of cell cycle regulation proteins such as cyclin A, cyclin D1, cyclin E, and early growth response-1 factor (43) (44) (45) (46). Most of these genomic influences are mediated by the GPER pathway components EGFR, ERK and PI3K. Interestingly, a direct genomic mechanism of GPER/EGFR complex binding to the promoter region of cyclinD1 gene has also been reported (46). A mechanism of GPER nuclear localization has been suggested to occur through the nuclear trafficking protein importin (47). GPER-mediated genomic signaling has been specifically implicated in vascular tissues and targets, including early studies in rat aortic smooth muscle showing that E2 treatment leads to increased expression of c-fos in a GPER-dependent manner, (45), and increased expression of vascular endothelial growth factor (VEGF). It should be noted that the delineation of genomic and rapid signaling effects may be considered arbitrary, since rapid activation of secondary kinases can initiate downstream transcription factor activation and changes in gene expression, making these actions inseparably linked. In recent years, GPER signaling has been studied in multiple systems and pathologies using uniquely designed synthetic compounds.

1.4 GPER-targeting compounds:

To advance investigations into GPER-mediated cell signaling and its physiological consequences, our laboratory with collaborators at New Mexico State University developed a GPER-specific agonist (G-1) and two antagonists (G15 and G36)(48) (49) (**Fig.1.5**). The GPER specific agonist G-1 is based on a tetrahydro-3H-cyclopentoquinolone (50, 51) structure with an ethanone moiety that may be involved in binding to GPER within the ligand pocket and may provide for steric hindrance limiting cross reactivity with ERα and β (50). The binding affinity of G-1 to GPER was determined to be approximately 10 nM (49). G-1 specificity for GPER is approximately 10,000-fold higher than for ERα/β. Weak biological effects of G-1 binding towards ERα were observed at concentrations of 10 μM, with the transcriptional activity through ERα being less than that observed at 10 pM E2 (49). Studies using G-1 have demonstrated a variety of GPER-mediated effects, including endometrial and breast epithelial cell proliferation, modulation of immune cell activity, and vasodilaton in epicardial coronary arteries (52) (53) (54).

Two GPER antagonists have been developed, G15 and G36 (**Fig 1.6**). The G15 compound lacks the ethanone moiety, but still binds to GPER with an affinity similar to G-1 (~2-fold reduced). *In vivo* administration of G15 in mice resulted in reduced uterine epithelial cell proliferation *in vivo* and the inhibition of PI3K-mediated activation by E2 (50) (42). To improve antagonist specificity, G36 was designed with the replacement of an isopropyl moiety for the ethanone group of the original G-1 compound. It was hypothesized that this

modification increased steric inhibition of G36 with ERα/β, thereby improving the selectivity for GPER (51).

Numerous studies employing G-1 and G-15/G36 describe GPER-specific signaling effects in the absence of ER signaling (52) (54) (50) (55). These studies also corroborate the efficacy of GPER targeting compounds to treat multiple pathologies such as breast or endometrial cancer, diabetes, osteoporosis, cardiac remodeling, hypertension and stroke (56) (57). Section 1.6 will discuss some of the discoveries of GPER signaling in vascular function using these GPER-targeting compounds.

1.5 SERMs and SERDs: GPER and ER Targeting with pharmacologic compounds:

Multiple pharmacologic tools have been developed to study estrogen receptor signaling. Some of these compounds are used in the clinic today to treat pathologies involving E2 signaling such as breast cancer and osteoporosis (58) (59). One of the earliest examples of pharmacologic targeting of ERs was the synthetic estrogen ethinyl estradiol, a long-acting form of E2 approved by the FDA in 1943. This synthetic estrogen is prescribed in several types of birth control pills. Ethinyl estradiol constitutively activates ERα to propagate a negative feedback loop to the hypothalamus/pituitary gland axis, which in turn downregulates the production of follicle stimulating hormone and luteinizing hormone (FSH and LH), thereby preventing ovulation.

Since the implementation of ethinyl estradiol, multiple additional pharmacologic ER-targeting compounds have been described. These compounds are classified according to their ability to agonize or antagonize signaling of the classic ERα/β, but not by their effects on GPER signaling, which can vary. Compounds termed selective estrogen receptor modifiers (SERMS) bind estrogen receptors to induce either activation or inhibition of estrogen receptors in a tissue-specific manner (60) (61) (62) (63) (64), whereas selective estrogen receptor downregulators (SERDs) work either by direct antagonism of an ER, or by long-term downregulation by targeting ERs for proteosomal degradation (65). SERMs and SERDs of clinical relevance include tamoxifen, raloxifene, and ICI 182,780 (fluvestrant), which are utilized in this thesis (**Fig 1.7**).

Tamoxifen is a SERM of particular interest for its contrasting ER/GPER effects, and also because it is used in the clinic as a chemotherapeutic in breast cancer (52, 66) and yet based on its vascular effects could also potentially be utilized as a therapeutic in vascular dysfunction. The metabolically active metabolite of tamoxifen is 4-hydroxytamoxifen (4-OHT), which is produced in the liver. Tamoxifen inhibits ERα activity by competitive antagonism, competing with E2 for the receptor binding site but not activating it. Studies have described a positive correlation of tamoxifen and improved vascular function, such as increased endothelial function (67). Tamoxifen has also been shown to increase cyclin E (a cell cycle regulator) activation in a GPER-dependent manner in breast cancer cell lines (68). Tamoxifen-mediated

activation of GPER also results in PI3K/AKT activation and intracellular calcium mobilization(69, 70) (71). It has been hypothesized that tamoxifen may improve endothelial function by increasing endothelial cell calcium to mediate calcium-dependent eNOS activation and NO production (72). 4-hydroxy tamoxifen (4-OHT) has been demonstrated to increase migration of the breast cancer cell lines MCF-7 (ERα+, GPER+) and SKBr3 (ERα-, GPER+) in a GPER-dependent manner through EGFR activation that induces cleavage of cyclin E (68) (73). Tamoxifen-induced cleavage of cyclin E into its activated was blocked by both GPER inhibition (siRNA), and by EGFR inhibitor AG1478, demonstrating the roles of GPER and EGFR in this pathway. Finally, tamoxifen metabolite (4OHT) has also been shown to induce cell migration through GPER by activation of focal adhesion kinases (FAK) in endometrial cancer cell lines expressing or lacking ERα (Ishikawa and RL95-2, respectively) (74). Pretreatment of Ishikawa and RL95-2 cells with siRNA targeting GPER reduced tamoxifen-induced FAK phosphorylation (Y397), as did pharmacologic inhibition of the GPER signaling intermediates PI3K/AKT (LY294002) and ERK1/2 (PD98059).

Raloxifene is another SERM compound that has been shown to alter both genomic and rapid signaling of ERα/β and GPER (62). Studies have demonstrated that raloxifene has anti-estrogenic effects in reducing breast and endometrial cancers by promoting apoptosis (75), and pro-estrogenic effects in bone health by improving osteoblast proliferation (76) (73) (77) and improved dopamine neuron function by activating GPER for increased AKT function (78).

GPER has been shown to mediate multiple additional effects of raloxifene. For example, raloxifene-mediated increases in MAPK activity and the resulting cell proliferation were blocked by GPER-targeting siRNA (79). Raloxifene treatment of the osteocyte cell line hFOB resulted in increased proliferation, and this proliferation was abrogated in the absence of GPER signaling using GPER inhibitors or GPER-targeting siRNA (79). Raloxifene has also been reported to have pro-estrogenic effects on the vasculature by improving endothelial function by reducing oxidative stress (80), and improving flow-mediated dilation in women (62). Raloxifene has also been shown to increase nitric oxide (NO) levels in aorta in both ovarectomized and intact rats (81) and to induce vasodilation in pressurized arteries (82) and *in vivo* (83).

Other pharmacologic compounds function as selective estrogen receptor downregulators or SERDs. As SERDs were characterized and designed prior to the discovery of GPER, the nomenclature of SERDs applies strictly to their effects on ERα/β regardless of their effects on GPER. SERD compounds, such as ICI 182,780, work either by direct antagonism of ERs, or by long-term downregulation by targeting ERs for proteosomal degradation (65). Most SERDs target only classic ERs for long-term downregulation while having either no effect or even agonistic effects on GPER, which is the case with ICI 182,780. Studies by Meyer *et al.* demonstrated that ICI 182,780 induces vasorelaxation in human coronary arteries, likely through agonism of GPER (54). Such divergent receptor actions lend to complex effects of these SERMS and SERDs, which are still not fully understood (71).

1.6 A focus on estrogen and the vasculature:

Although the majority of biomedical studies involving E2 address cancers of the reproductive system, there have been numerous studies attributing estrogen action to cardiovascular function and dysfunction. Consistent and dynamic maintenance of arterial blood pressure is vital for adequate perfusion of peripheral tissues and organs and for healthy heart function. An increase in the incidence of cardiovascular disease (CVD) is observed in post-menopausal women, and protection against CVD in pre-menopasual women (lower incidence vs. men) is attributed to ovarian hormone signaling. Given these observations, it is vital to understand the contribution of estrogen to these functions. Elucidating the role of estrogenic signaling in cardiovascular function and disease represents a significant progression in the fields of both vascular biology and gender medicine.

Clinical observations have documented a sex-dependent difference in the resistance to cardiovascular disease (CVD), in which pre-menopausal women have decreased incidence of CVD compared to men (where CVD positively correlates with age) until the onset of menopause, when the risk of CVD in women increases and with time approaches that of men (84) (85) (86). These observations suggested a protective effect of female ovarian hormones, specifically E2, against CVD. In recent years, advances in the field of E2 signaling mechanisms in cardiovascular tissues have yielded complex yet intriguing results.

1.6.1 The structure and functions vasculature:

The vascular system is made up of arteries, capillaries, and veins. The arteries shuttle oxygenated blood from the heart to peripheral tissue; the capillaries facilitate the exchange of gases between blood and target peripheral tissues, and the veins bring deoxygenated blood back to the heart for rexoygenation in the lungs. While regulation from the CNS and peripheral nervous system is important, this thesis focuses on the dynamics of endothelial and smooth muscle cross-talk in regulating vasomotor tone in the artery. The artery is made up of an endothelial layer forming the luminal surface, smooth muscle at the medial layer, and a surrounding layer of fibroblast adventitia and perivascular fat.

1.6.2 The endothelium:

The endothelium is a single cell layer forming the luminal surface of the vessel. Early studies described the endothelium as a simple selectively permeable barrier for various hormones, peptides and immune cells carried in the blood. Later on, the endothelium was found to be a dynamic signaling tissue responsible for the production of multiple paracrine signaling molecules such as NO, prostacyclin, coagulation factors and inflammatory signaling. Studies by Nobel laureate Robert Furchgott in the 1980s elegantly demonstrated the existence of an endothelium-derived relaxation factor (EDRF) that acted upon adjacent smooth muscle cells to mediate vasodilation. In these experiments, rabbit aorta segments with an intact endothelium were

able to dilate to acetylcholine, but arteries that had been denuded of the endothelium could not, suggesting the endothelium was necessary for mediating smooth muscle relaxation, possibly through the secretion of a relaxation factor to act on adjacent smooth muscle. Soon after Furchgott's report, EDRF was found to be a diffusible compound from the endothelium to the smooth muscle in a series of experiments by Busse and Bassange (87, 88). Cannulation of arteries in series showed that arteries denuded of endothelium were unable to dilate independently to acetylcholine, but were able to dilate if an endothelium intact artery was cannulated upstream, acting as a "donor vessel" to deliver EDRF to the downstream denuded artery to induce vasorelaxation. This diffusible EDRF was subsequently demonstrated to possess a short half-life of 20 to 30 seconds (88). In these studies, a surrogate endothelial source (microbeads covered with bovine endothelial cells) was stimulated with ATP and the perfusate traveled downstream to a cannulated rabbit femoral artery. In some cases, a delay coil was added to increase transit time from the surrogate endothelium to the downstream artery. These studies demonstrated an inverse relationship of transit time for perfusate and the vasodilation response of the downstream endothelium-denuded artery (i.e., longer transit time resulted in decreased dilation). EDRF was identified as nitric oxide (NO) by a number of groups, although its identity was debated for several years (89, 90).

Another class of endothelium-derived relaxation factors, the prostanoids, was described by De Mey and Vanhoutte in the 1980s (91) (92, 93).

Prostanoids are lipophilic metabolites synthesized from arachidonic acids by cyclooxygenase (COX1/2) enzymes. Prostanoid metabolite specificity depends on the post-COX cleavage enzymes, which generate prostaglandins, thromboxanes, or prostacyclins. Endothelium-derived contraction factors (EDCFs) include thromboxane A2 (TBA2) and multiple types of prostaglandins (PGFs, PGHs). Thromboxanes and prostaglandins act upon smooth muscle TP receptors at the adjacent VSMCs in increase cyclic GMP formation and PKG activity to induce vasoconstriction and vasomotor tone (94) (95). Prostaglandin F2α (PGF2α) is an EDCF that is commonly used in experimental settings to precontract arteries to test E2-mediated vasorelaxation. Prostaclyclin (PGI_2) is an endothelium-derived vasorelaxing factor that acts upon VSMC prostacyclin receptors to induce vasorelaxation through AC activation and cAMP increase (96, 97) (97) .

Taken together, studies from the 1980s onward have demonstrated the diverse functional importance of the endothelium in maintaining vasotone in the balance of EDRFs such as nitric oxide and prostacyclin, and EDCFs such as prostaglandin and thromboxane. Further studies are needed to grasp the complexity of endothelial regulation in normal vascular tone and disease, including the role of estrogen receptor-mediated endothelial signaling, which is a focus of this thesis.

1.6.3 Vascular Smooth Muscle Cells:

The vascular smooth muscle cells (VSMCs) comprise the medial layer of arteries. VSMCs are arranged in a circumferential orientation around vessels. This circumferential arrangement allows their contraction or relaxation to modify the radius of a vessel, which inversely relates to flow resistance (i.e. a contraction will decrease the radius and result in increased resistance to flow, relaxation increases vessel radius and decreases resistance to flow). VSMCs are connected to each other by gap junctions and are also connected to underlying endothelial cells via myoendothelial gap junctions. The smooth muscle also receives signals from circulating vasopeptides, peripheral (sympathetic) nerves, vascular adipose and adventitial tissue to regulate contractility.

There are three primary mechanisms that elicit vascular smooth muscle contraction; electromechanical (membrane depolarization), cellular calcium sensitization, and pharmacomechanical coupling (receptor-operated mechanisms). Each of these distinct mechanisms, however, is intricately linked in the mediation of contraction. Endothelial signals such as prostanoids and nitric oxide travel to the adjacent VSMCs to effect vasorelaxation and/or vasocontraction by a myriad of mechanisms, including myosin light chain phosphatase (MLCP) stimulation or suppression, calcium mobilization, and membrane depolarization (109, 110).

The mechanisms of contraction in VSMCs depend on calcium activation of the contractile apparatus. Increased levels of intracellular calcium activate calmodulin (CaM), which phosphorylates myosin light chain kinase (MLCK), which in turn phosphorylates myosin light chain (MLC). The phosphorylation of MLC allows myosin to bind filamentous actin at the overlapping zone of the contractile apparatus. Hydrolysis of ATP to ADP allows the myosin filament to slide across the actin filaments, contracting the apparatus and effecting a contraction of the smooth muscle cell. MLC can be deactivated by myosin light chain phosphatase (MLCP), which is tightly controlled through multiple receptor signaling pathways. Hence, the balance of MLCK and MLCP determines calcium sensitivity of the contractile apparatus and thus plays a pivotal role in mediating VSM tone. Intracellular calcium increases can occur in several ways. The intracellular calcium concentration in a resting VSMC is about 100 nM, while the extracellular calcium concentration is about 1 mM. Hence there is a natural concentration gradient for calcium to flow into the cell if appropriate ion channels are open. Additionally, the intracellular membrane potential of a resting VSMC is negative, which creates an electrical gradient for positively charged calcium to flow into the cell if voltage-sensitive channels are opened by receptor-operated stimuli or depolarization of the plasma membrane.

In the presence of a depolarizing stimulus for VSMC contraction (such as a secondary kinase messenger, increased flow or high extracellular potassium), activation of voltage or pressure-sensitive ion channels or inhibition of potassium channels results in depolarization of the cell membrane, which

activates voltage-gated calcium channels (VGCC). Influx of calcium through these channels will activate ryanodine receptors at the sarcoplasmic reticulum to activate calcium-induced calcium release (CICR), adding to the rise in intracellular calcium to induce contraction.

1.6.4 Renin-angiotensin system (RAS) & reactive oxygen species (ROS) in vascular smooth muscle:

An example involving pharmacomechanical-coupled VSMC contraction is the vasopeptide angiotensin II (Ang II). Ang II is generated as part of a complex and tightly regulated pathway referred to as the renin angiotensin aldosterone system (RAAS). RAAS can be activated by a decrease in blood pressure or an increase in sympathetic neural activity sensed by the kidney. Initiation of the RAS response begins at the juxtaglomerular complex (JGC) at the renal afferent tubule. Prepro-renin is secreted in response to the detection of decreased flow in the afferent tubule. Prorenin is converted into the active form renin in the macula densa cells of the JGC and released into the circulation. Cleaved, activated renin is a catalytic protein that travels to the liver where angiotensinogen is produced. Renin cleaves angiotensinogen into the precursor peptide Angiotensin I within the liver. Circulating Ang I is cleaved at the lungs by angiotensin converting enzyme II (ACE 2) into the vasoactive peptide Ang II. This multi-step process ensures a tight regulation of Ang II production. In some cases of hypertension there is an imbalance (hyperactivation) of RAAS activity. Successful drugs used in the clinic to treat

hypertension target RAAS components, such as inhibitors of renin and ACE2 activity as well as Ang II receptor (AT1R) blockers.

Ang II activation of AT1R receptors on VSMCs initiates a signaling cascade that activates NADPH oxidase to increase production of superoxide anion ($O_2^{\cdot}$). Superoxide is known as a “kindling radical” that can form additional reactive oxygen species (ROS) in multiple cell types including VSMCs. Superoxide can be converted into other forms of ROS, such as hydrogen peroxide via the enzyme superoxide dismutase (SOD). ROS can mediate VSMC contraction in multiple ways, including activation of IP_3R for calcium increase (111). Other mechanisms such as ROS-mediated activation of PKC and RhoA kinase are areas of active research (112) (113) (114) (115).

The NADPH oxidase (NOX) protein family functions as a catalytic source of superoxide anion. NOX expression is ubiquitous across cells types, with certain tissues expressing greater amounts of specific isoforms, such as NOX2 in endothelial cells, NOX1 in vascular smooth muscle, NOX3 in phagocytic cell types (such as macrophages) and NOX4 in adventitial fibroblasts (116). NOX5 is a specialized, calcium-dependent isoform expressed in multiple cell types that is species-specific to humans or primates (*i.e.* not expressed in rodents). The catalytic NOX complex is made up of several subunits and co factors. The catalytic subunit of NOX exists as several isoforms, including NOX1, 2,3, 4 and 5. With respect to smooth muscle, the catalytic subunit NOX1 is located at the plasma membrane and associated with the scaffolding protein p22phox. There are also cytosolic NOX subunits including the low molecular weight GTPase

Rac1 and several adaptor NOX subunits. These subunits can vary by cell type, but in the vascular smooth muscle NOXO1 and NOXA1 reside with Rac1 in the cytosol. Upon receptor-mediated stimulation, Rac1 ushers the translocation of the cytosolic subunits to the plasma membrane for NOX assembly. When activated, NOX forms the superoxide anion radical by transferring an electron from its substrate NADPH through the FAD intermediate onto molecular oxygen. Increased NOX activity has been shown to result in up-regulation of NOX gene transcription, suggesting a positive feedback of NOX through ROS-sensitive transcription factors (116).

It is known that a dysfunctional or hyperactivated RAAS can result in increased ROS accumulation, contractile sensitization, proliferation and hypertrophy of VSMCs, resulting in increased vascular resistance referred to as hypertension (117-119). Chronic hypertension is one of the most prevalent diseases in developed countries worldwide, and its progression is related to the development of multiple CVD types such as heart failure or stroke. Its etiology is complex and multifactorial including genetics, age, lifestyle and gender. (120-122).

1.7 Estrogen, ER and GPER studies in endothelial cells:

It is well known that ERα activation contributes to multiple aspects of endothelial function, including eNOS-mediated vasodilation (NO production), vascular permeability, cell migration and upregulation of antioxidant-related genes.

In 2002, Chambliss *et al.* demonstrated a signaling mechanism for E2's protective effect in endothelial cells. They reported that E2 was able to phosphorylate eNOS, the enzyme responsible for the production of the vital vasodilator nitric oxide (NO), in a dose-dependent manner through a Gi-coupled mechanism (98) (99). Evidence has shown that ERα associates with eNOS in endothelial caveolae, where acylation of eNOS and palmitoylation of ERα coordinate colocalization of the proteins at this specialized lipid raft. These studies demonstrated that ERα activation could increase endothelial bioavailability of NO through rapid activation of eNOS by ERα at the eNOS activation sites Y81 and S1179. Additional studies in HUVECs show that ERα may mediate complex protein-protein interactions of eNOS with co-factor proteins such as Hsp90 (100). Membrane-associated ERα and its truncated isoform ER46 (17) may activate several kinases upstream of eNOS including ERK1/2, which activates phosphatases to dephosphorylate the inhibitory residues threonine 495 and serine 615,as well as c-Src, which activates tyrosine 81 (101) (102), and also PI3K/AKT, which phosphorylates ser1177 (101). Interestingly, the role of ER signaling in calcium mobilization and subsequent calcium-mediated eNOS activation needs further investigation to establish a mechanistic tie between these two events.

In terms of long-term regulation of eNOS or other antioxidant gene expression, studies have shown that ERα activation with the isoflavone S(-)-equol can activate the master transcription factor Nrf2, which is responsible for the transcriptional regulation of multiple antioxidant enzymes by coordinating

transcription complexes at antioxidant response elements (AREs) in the DNA (103). ERα–mediated activation of Nrf2 depends on PI3K signaling (104). In HUVECs, ERα activation has been linked to increased expression of heme-oxygenase, Nrf2, NOX2 ad VEGF (103). The upregulation of these genes by ERα was dependent on PI3K intermediate signaling.

The activation of eNOS by E2 has not always been considered to be beneficial. Studies by White *et al.* in 2005 and 2010 showed that E2-stimulated eNOS activation in the presence of decoupled eNOS in endothelial cells exacerbates oxidative stress by the scavenging of NO into the reactive oxygen species (ROS) metabolite peroxynitrite (ONOO) (105) (106) . ROS accumulation instigates endothelial dysfunction in two ways, first by reducing NO bioavailability by oxidizing it into ONOO, and secondly by decoupling the eNOS enzyme, switching its catalytic activity to produce superoxide. Hence, stimulation of eNOS by E2 may actually promote endothelial dysfunction if the endothelium is already in a ROS-enriched state, such as in atherosclerosis or hypertension, although such mechanisms have yet to be demonstrated.

Using the GPER-specific agonist G-1, multiple groups have demonstrated GPER-mediated activation of eNOS at the critical residue ser1177 in human, rat and mouse endothelial cells (101, 107) (102). Since studies of GPER signaling in multiple cell types have shown that GPER activates c-Src, ERK1/2 and PI3K/AKT signaling cascades, current research, including this thesis, addresses the roles of c-Src, ERK1/2 and PI3K/AKT as secondary messengers mediating GPER-induced NO production. The differential effects of SERMs

and SERDs on GPER-mediated eNOS production are an intriguing area that needs further study. In long-term genomic regulation of eNOS and/or antioxidant-related enzymes, data have shown that addition of E2 to breast cancer cell line MCF-7 increased expression of the antioxidant enzymes glutathione peroxidase and Mn superoxide dismutase (Mn-SOD/SOD3) (108) in an ERK1/2- and NFkB-dependent manner. The contribution of GPER to this ERK-mediated expression may be implied in endothelial cells, but has not been investigated. The contribution of GPER-mediated AKT activation is well known, and the elucidation of a role (if any) for GPER in these signaling events has yet to be directly demonstrated.

1.8 Estrogen, ER and GPER studies in vascular smooth muscle cells:

The majority of studies concerning E2 action in the vasculature focus on endothelium-mediated NO production, but several studies do outline critical activities of E2 in vascular smooth muscle cells (VSMCs). E2 has been demonstrated to reduce proliferation in VSMCs in vascular ischemia models using mouse carotid arteries (123). Additionally, E2 has been shown to mediate decreases in VSMC proliferation, which can lead to hypertrophy of arterial walls and increase vascular resistance, a critical component of hypertension development. Inhibition of VSMC proliferation by E2 can also combat the progression of atherosclerosis (124). E2 has also been shown has to activate neuronal NOS and inducible NOS in VSMCs (125). While neuronal NOS (nNOS) can be a contributor of NO in VSMC leading to vasodilation in conditions of aging and hypertension (where a ROS-enriched cellular

environment is present), excess NO can be scavenged by ROS into the peroxynitrite radical ($ONOO^-$), which in turn can exacerbate oxidative stress in the VSMC (125). Studies by White *et al*. in 2005 and 2010 reported that E2 treatment of VSMCs in a state of uncoupled nNOS leads to increased superoxide levels and increased coronary artery contraction (105). Thus, the contributions of E2 in maintaining vascular health may be dependent on the relative health of the VSM.

The role of GPER in mediating E2-induced acute VSMC signaling has also been investigated. Studies by Lindsey *et al*. report that GPER can activate smooth muscle adenylyl cyclase (AC) resulting in cAMP increases to induce eNOS-independent vasorelaxation in isolated mesenteric arteries and smooth muscle cells (106) (126). Yu *et al.* expanded on these studies using human and porcine coronary artery smooth muscle cells to show that GPER-mediated activation of AC leads to increased cAMP and activation of PKA to inhibit RhoA/ROCK kinase activity, which promoted the activity of MCLP, resulting in vasorelaxation (127). In terms of genomic effects of GPER in VSMCs, long-term activation of GPER by G-1 has been shown to down regulate AT1R receptor (mRNA) expression (128). G-1 also inhibits VSMC proliferation in a similar manner to that described previously for E2 (129). Additional studies of GPER in the short and long-term regulation of vascular smooth muscle function are needed to provide further insights into additional pathways, and the overall balance of endothelial and VSMC signaling in arterial function.

1.9 Estrogen, ER and GPER studies in isolated arteries:

In terms of acute vasoreactivity, the ability of E2 to induce NO-dependent vasodilation in arteries of human, rat and mouse is well known (130) (131). Kleinert *et al*. have also reported that E2 increased eNOS expression in an ERα-dependent mechanism (132). Multiple studies indicate that both ERα and GPER contribute to the actions of eNOS across different species and vascular beds (54, 133). Our laboratory first reported the ability of the GPER-specific agonist G-1 to directly stimulate vasorelaxation in an eNOS-dependent manner in 2010 (54). Others and we have also shown that G-1 induces a modest, eNOS-dependent vasorelaxation in thoracic aorta, mesenteric and carotid arteries (126, 131, 134) of mice. Other reports have shown that GPER-mediated (G-1) vasorelaxation in rat mesenteric arteries induced a similar vasorelaxation response as E2, suggesting at least a collaborative role of both receptors in mediating total vasorelaxation effects (130) (126). In rat cerebral and pial arteries, the GPER agonist G-1 improved vascular function in an eNOS-dependent manner, which increased protection in an ischemia-reperfusion injury model of stroke (135). Furthermore, reduced vasorelaxation to estradiol in arteries from aged rats (male and female) were found to correlate with the downregulation of GPER expression with age (136).

Our laboratory has utilized the GPER KO mouse in characterizing the actions of GPER in vasoreactivity. Our GPER KO mouse model shows similar levels of eNOS expression compared to WT (137). Our colleagues in Zurich, Switzerland previously reported that carotid arteries from GPER KO mice had

increased contraction to ET-1, and that these enhanced contractions were due to elevated thromboxane production (137). These results suggested that deletion of GPER increases endothelium-derived contraction responses to thromboxane, suggesting GPER also promotes vasorelaxation by decreasing EDCF activity. Finally, our laboratory has demonstrated that arteries isolated from GPER KO mice have reduced basal NO levels in mice fed an atherosclerotic diet, but NO levels in healthy GPER KO mice are not different from WT (138).

Studies by Lindsey *et al.* demonstrated that a small portion of GPER-mediated vasorelaxation occurs in an eNOS-independent mechanism by activation of smooth muscle adenylyl cyclase activation and cAMP increase in mesenteric smooth muscle cells. They confirmed their observations from cultured primary cell systems in arterial function by showing vasorelaxation of mesenteric arteries through GPER activation through an adenylyl cyclase and cAMP mechanism (126) in endothelium denuded human coronary artery vasocontraction (127). Interestingly, in human coronary arteries Yu *et al.* demonstrated a larger G-1-mediated vasorelaxation response in endothelium-disrupted human coronary arteries. Finally, studies from 2010 demonstrated a vasocontraction response to E2 in coronary arteries with pharmacologically uncoupled NOS, which serves as a pharmacologic model of vascular disease (105). Taken together, the effects of GPER in vasorelaxation may vary substantially between vessel bed and disease state.

1.10 Estrogen, ER and GPER studies in whole animal and humans:

In contrast to the multiple mechanistic reports of E2 and GPER in vascular cell types and whole vessels, studies of E2 replacement in both rodents and humans have yielded complex results. Chambliss *et al.* report that non-nuclear pools of ERα are responsible for mediating protective cardiovascular effects (such as reduced hypertrophy and maintenance of NO bioavailability) in the absence of breast epithelial cell or uterine proliferation (139). It has also been demonstrated that acute E2 infusion directly at the tissue of rat hearts reduced coronary vascular resistance and increased coronary blood flow (140). In terms of chronic E2 infusion, there are mixed results of vascular reactivity and protection. Studies of E2 replacement in male or ovarectomized female ApoE-deficient mice have shown positive effects, such as protection against atherosclerotic plaque development and reduced oxidative stress in the vascular wall in a high-cholesterol diet (72, 141). Our laboratory has also demonstrated that infusion of GPER-specific agonist G-1 provides the same level of protection against atherosclerotic plaque formation and reduces inflammation in arteries of mice on an atherogenic diet (107). Chronic infusion of G-1 has also been shown to decrease blood pressure in ovarectomized females, but not male rats, and to reduce cardiac remodeling in rat models of hypertension (128, 142).

Human studies of E2 replacement provide less clear results of E2 *in vivo*. While acute administration of E2 in patients improved vasodilator responses and reduced cardiac ischemia (143), chronic infusion (directly into circulation)

of E2 in post-menopausal hormone replacement therapy (HRT) trials have yielded mixed results. Such results were dependent on the source and dose of exogenous estrogen, the age of women treated (relating to the length of time they had been post-menopausal) and their state of preexisting CVD (144). An early study of estrogen replacement, the Nurses' Health Study in the 1970s, indicated E2 replacement reduced CVD risk by 35-50% *vs.* placebo (145). However, later studies such as the Women's Health Initiative (WHI) in the 1990s and the Heart and Estrogen Replacement Study (HERS) in 2002 showed no vascular protection and even reported aberrant cardiovascular dysfunction (143, 146, 147). The WHI examined the effects of exogenous E2 replacement in menopausal women using conjugated equine estrogens (CEEs). Although the WHI was originally designed to examine the effects of estrogen supplementation from CEE to treat post-menopausal symptoms, the study was suspended due to an increased incidence of stroke and myocardial infarcts (148).

Despite these trials, it has been considered that the activation of specific post- estrogen receptor signaling pathways is key to maintaining vascular protection while circumventing deleterious side effects (143). Pharmacologic targeting of GPER-specific pathways using the GPER-specific agonist G-1 has shown promising results in animal models for the activation of favorable estrogenic pathways in vascular tissues. Chronic infusion of G-1 in rats resulted in decreased blood pressure (128), but neither the ERα KO nor GPER KO mouse models are intrinsically hypertensive (149) (150). In some cases,

E2 or G-1 protected from certain forms of hypertensive challenge in rodents (130), but have failed to protect against increased blood pressure in other rodent models (151). Both E2 and G1 infusion have been shown to protect against atherosclerosis in mice (107, 141). However, in some cases, G-1 infusion in rats protected females, but not males (151) from salt- sensitive hypertension.

1.11 Rationale, Hypothesis and Specific Aims:

The mixed results of the literature demonstrate the complex biology of estrogen and its receptors, which suggest that there are additional pathways not yet identified that could be contributing to the often unclear effects of estrogen replacement and vascular health. There are suggested vascular benefits of estrogen, but the role of GPER in mediating these benefits is still unclear.

The goal of this dissertation project was to address gaps of knowledge in GPER signaling in endothelial and vascular smooth muscle, and to implement these findings into a functional context of vessel reactivity and whole animal models. These cell types engage in dynamic cross talk signaling for maintenance of stable peripheral resistance and proper blood pressure control. Maintenance of blood pressure is vital for perfusion of peripheral tissues and organ function. The direct signaling pathways of GPER, as well as the role of GPER in SERM/SERD-mediated effects in endothelial and smooth muscle cells and whole vessel function remain unclear. Elucidation of the specific

signaling pathways of GPER will aim to resolve conflicting results in animal and clinical studies concerning estrogen in vascular function, and is significant for progression in the fields of gender medicine and vascular biology. To address these gaps in knowledge, two specific aims were proposed for examining the signaling pathway(s) of GPER in endothelium and vascular smooth muscle (**Fig 1.8**):

Specific Aim 1: Determine the signaling pathway and contribution of GPER to estrogen-mediated eNOS activation and NO production. We **hypothesize** that GPER regulates a portion of the estrogen-stimulated production of NO through stimulation of eNOS in an AKT/PI3K pathway to activate eNOS residue ser1177, and that genomic deletion or pharmacologic inhibition of GPER reduces the vasorelaxation response to estrogen.

Subaim 1.1: Determine the signaling pathway and contribution of GPER to the activation of eNOS in an ERα-independent manner in endothelial cells.

Subaim 1.2: Assess the effects of GPER deletion on vasorelaxation to E2 using GPER KO mice.

Subaim 1.3: Determine the role of GPER in mediating the effects of SERMS in endothelial NO production and vasorelaxation to SERMs tamoxifen and raloxifene and the SERD ICI 182,780.

Specific Aim 2: Determine and characterize the effects of GPER signaling in Ang II-mediated contraction of vascular smooth muscle. Based on preliminary data showing a role for GPER in endothelial NO generation, we

hypothesize that GPER KO arteries will have an increased vasocontraction response to Ang II due to reduced NO levels, which may result in a hypertensive phenotype in rodents due to a loss of NO-dependent vasodilation.

Subaim 2.1: Identify and characterize a role of GPER in Ang II-mediated smooth muscle contraction and vasotone by utilizing the GPER KO mouse arteries and primary VSMCs.

Subaim 2.2: Elucidate the acute and/or genomic cellular mechanism(s) of GPER in Ang II contraction in genomic deletion (GPR KO) and pharmacologic conditions (short or long-term employment of G36 to inhibit GPER).

Subaim 2.3: Examine the effects of GPER deletion (KO) and pharmacologic inhibition (G36) in a mouse model of RAS-mediated (Ang II-induced) hypertension.

Fig 1.1

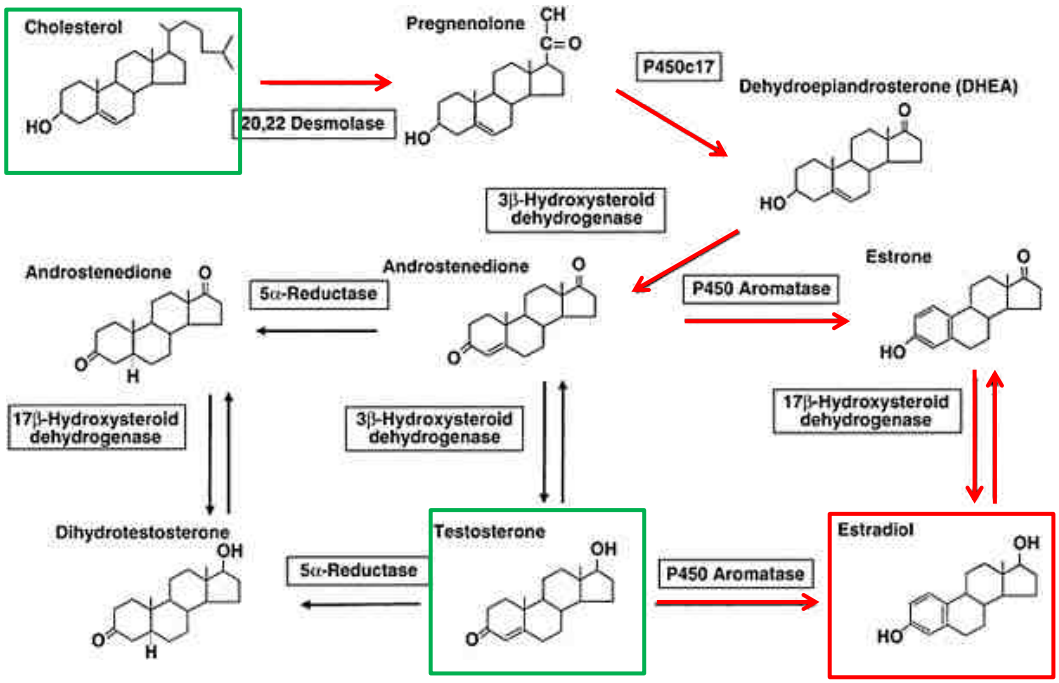


Fig 1.1 Estrogen synthesis is a multi-step process: Cholesterol is converted to pregenenolone, DHEA , and finally into the androgen androstenedione in the thecal cells of the ovary. Androstenedione can be converted into testosterone by the enzyme 3βHSD. Both androstenedione and testosterone are converted into estrogens by the P450 enzyme aromatase. Androstenedione is first converted to estrone by aromatase and then into estradiol by the enzyme 17β HSD. Testosterone can be directly converted to estradiol by aromatase. Aromatase is expressed at the granulosa cells (but not thecal cells) in ovary and at some peripheral tissues (endothelial cells adipose and liver) for estrogen synthesis. Modified from Ray & Gupta, Drugs Fut 2006, 31(1): 65 ISSN 0377-8282 Copyright 2006 Prous Science CCC: 0377-8282 DOI: 10.1358/dof.2006.031.01.959122

Fig 1.2

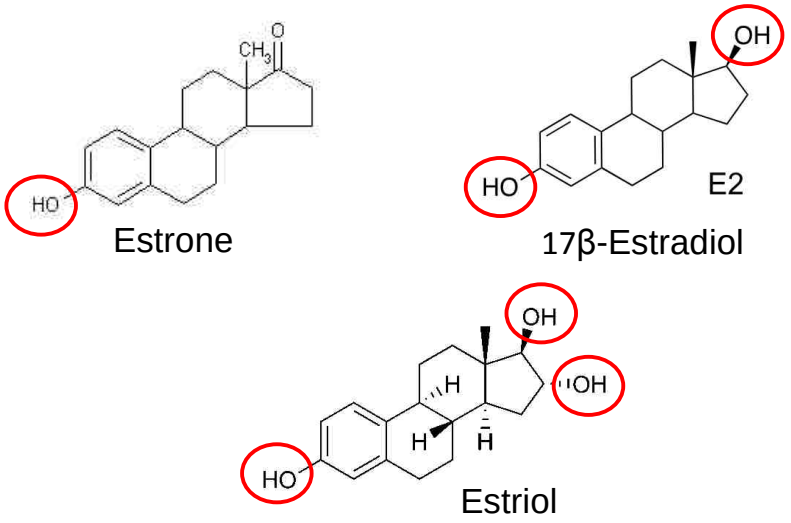


Fig 1.2 There are three variants of endogenous estrogens: Estrogens are named relative to the number of hydroxyl groups they have. Estradiol is the predominantly active form in premenopausal women, while estrone is the predominant form in post-menopausal women. Modified from Ahokas, R, McKinney, E, *Glob. libr. women's med*., *(ISSN: 1756-2228)* 2008; DOI 10.3843/GLOWM.10101

Fig 1.3

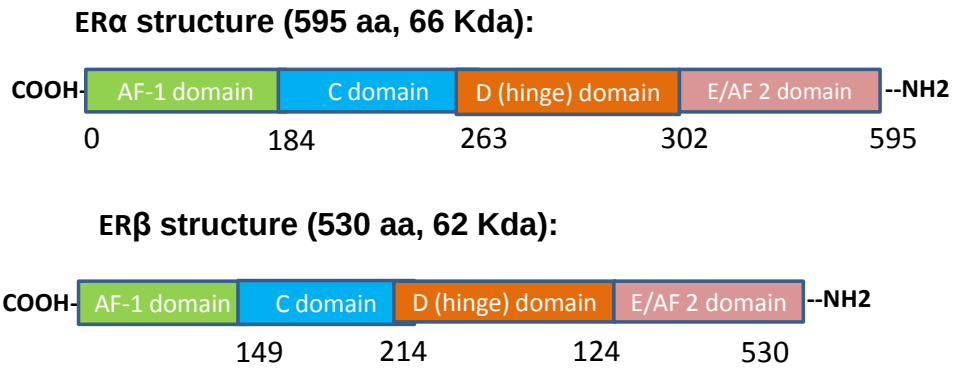


Fig 1.3 classical estrogen receptor and structure : The classical estrogen receptors alpha and beta share an 96% homology at the C and D domain and approximately 30% and 60% homology at the AF-1 and AF-2 domains, respectively (ER β relative to ER α). At the C terminus there is an AF-1 binding domain for co-factors. The C domain is responsible for DNA binding. The D domain is a "hinge" domain where the receptor changes conformation upon ligand binding at the E/AF-2 domain at the amino terminus. Additional co-factors can bind the AF-2 domain. Despite receptor homology, ERβ is largely considered to have opposing effects to ERα in most tissues.

Fig 1.4 A

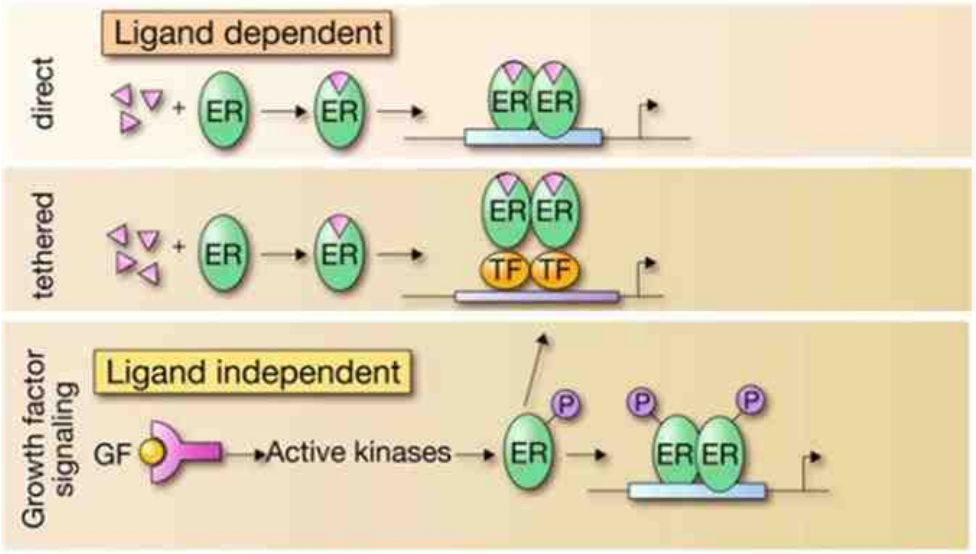


Fig 1.4 Estrogen receptors signal by genomic and rapid pathways: **A)** In genomic or "classical" signaling, activated ERs form homo or hetero dimers, and act as transcription factors for gene regulation by binding at estrogen response elements (EREs) at promoter regions. There are three forms of classic signaling, direct signaling is where ERs bind directly at the DNA, tethered or indirect signaling where dimerized ERs form a complex with multiple transcription factors and/or co-factors which then bind the DNA as a complex, and ligand independent signaling, where secondary kinases such as ERK 1/ 2 activate ERs by phosphorylation of the receptors to mediate downstream transcription factors. Modified from N. Heldring *et.al*. Physiological Reviews Published 1 July 2007 Vol. 87 no. 3, 905-931 DOI: 10.1152/physrev.00026.2006

Fig 1.4 B

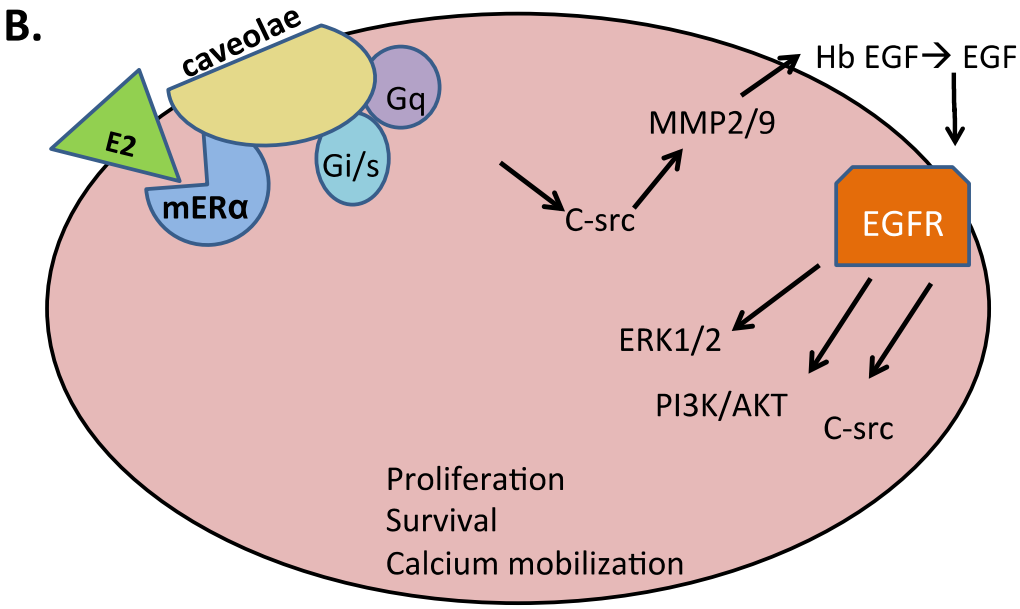


Fig 1.4 B) Estrogen receptors signal by genomic and rapid pathways (con't): In rapid or non-genomic signaling, non-nuclear ERs associated with the plasma membrane or cytosol initiate downstream activation of secondary messengers such as c-Src and ERK1/2, and/or transactivation of the epidermal growth factor receptor tyrosine kinase (EGFR) which initiates any number of downstream kinase activations such as PI3K /AKT, effecting physiologic changes on the order of seconds to minutes, such as Calcium increase and eNOS phosphorylation. These effects can also lead to genomic modifications, which complicates the separation of rapid and genomic signaling.

Fig 1.5

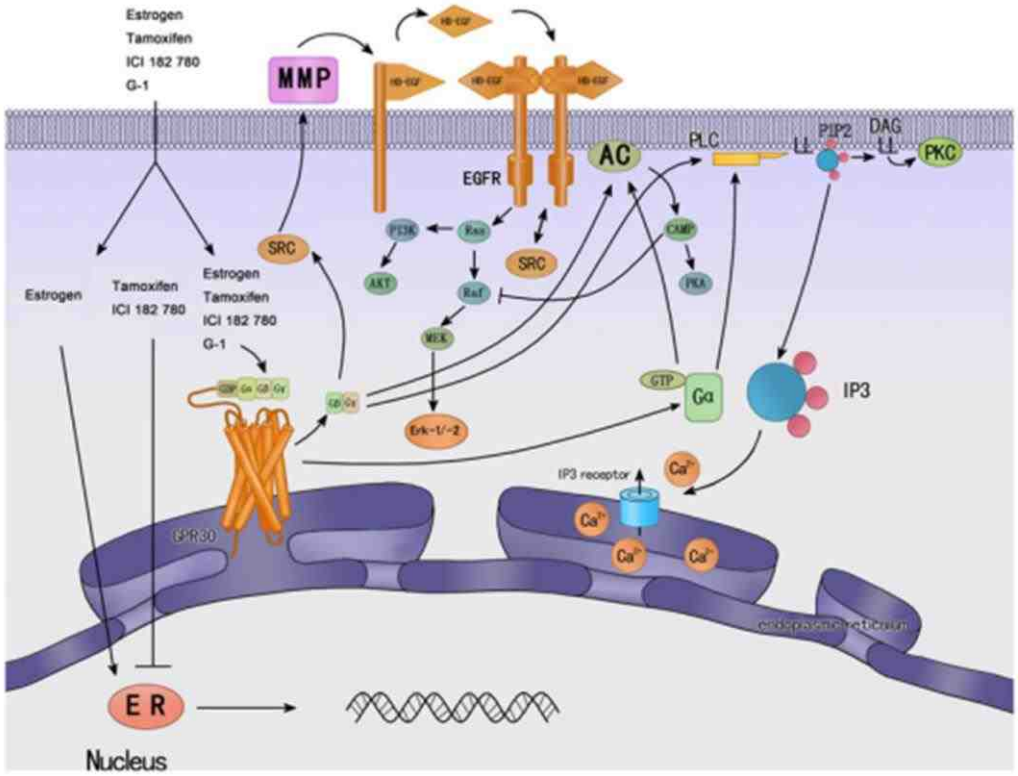
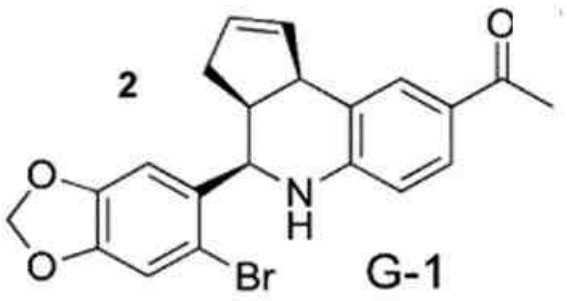
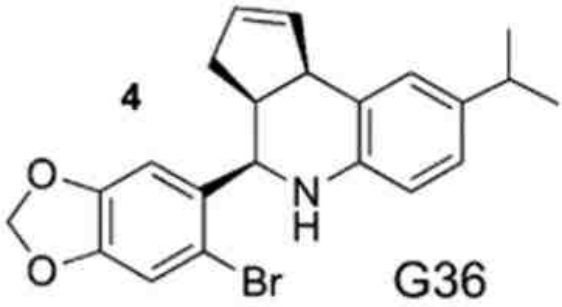
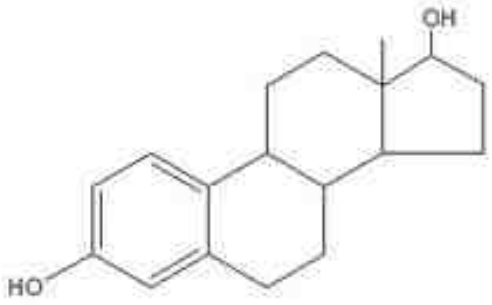


Fig 1.5 signaling pathways of the G protein-coupled estrogen receptor: Following GPER binding to ligand E2, c src is phosphorylatedin through the alpha protein Gs. SRC activate matrix metalloprpteases (MMPs) which cleave heparin bound EGF into its active form to bind EGFR. Activated EGFR can also activate src, ERK1/2 and PI3K/AKT kinase signaling. GPER can also activate adenylyl cyclase via Gs coupled alpha protein. AC can activate PKA signaling and RhoA kinase for multiple downstream kinase effects. GPER activation of A Gq alpha protein can activate Phospholipase C (PLC) which will cleave PIP2 into DAG and IP3. IP3 activate IP3 receptors to increase intracellular calcium. DAG can activate PKC which is also an important secondary messenger of multiple targets. Modified from Gigoux & Fourmey, Front. Endocrinol., 29 April 2013 | doi: 10.3389/fendo.2013.00050

Fig 1.6



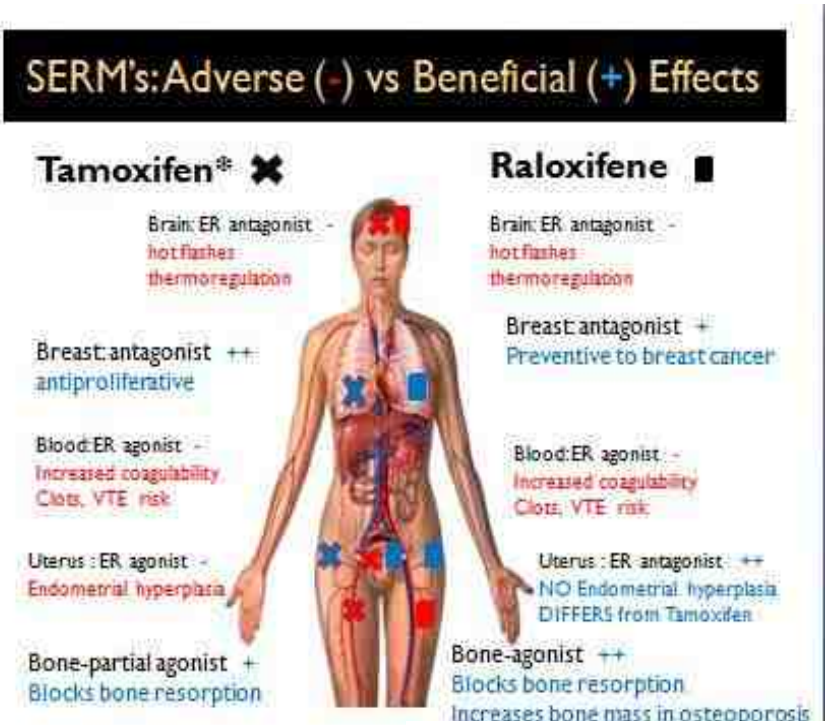


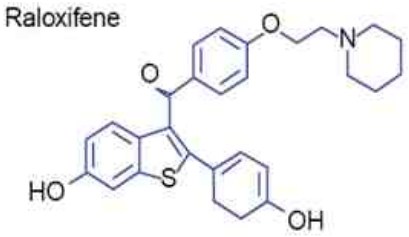


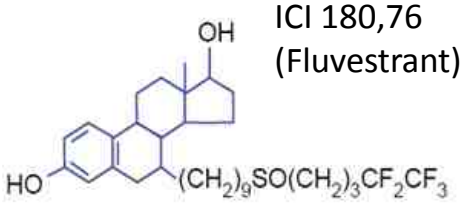
17β-ethinyl estradiol

Fig 1.6 GPER specific targeting compounds: G-1, the GPER specific agonist has a ethanone moiety to increase steric hindrance to prevent ERα/β binding. The GPER-specific antagonist G36 replaces the ethanone moiety with a methyl group to prevent binding with ERα/β. Modified from Dennis & Prossnitz *et.al*. 2003.

Fig 1.7







ICI 180,76
(Fluvestrant)

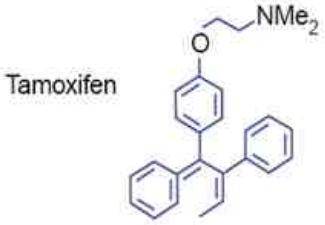


Fig 1.7 SERMs and SERDs have differential tissue dependent effects: Raloxifene and Tamoxifene are SERMs which alter ER signaling in a tissue specific manner. ICI182,76 (fluvestrant) is a SERD that targets ERs for proteosomal degradation. Adapted from Howell et al Best Pract. Res Clin Cndocrinol Metab 2004 Mar;18(1):47-66cam

Fig 1.8

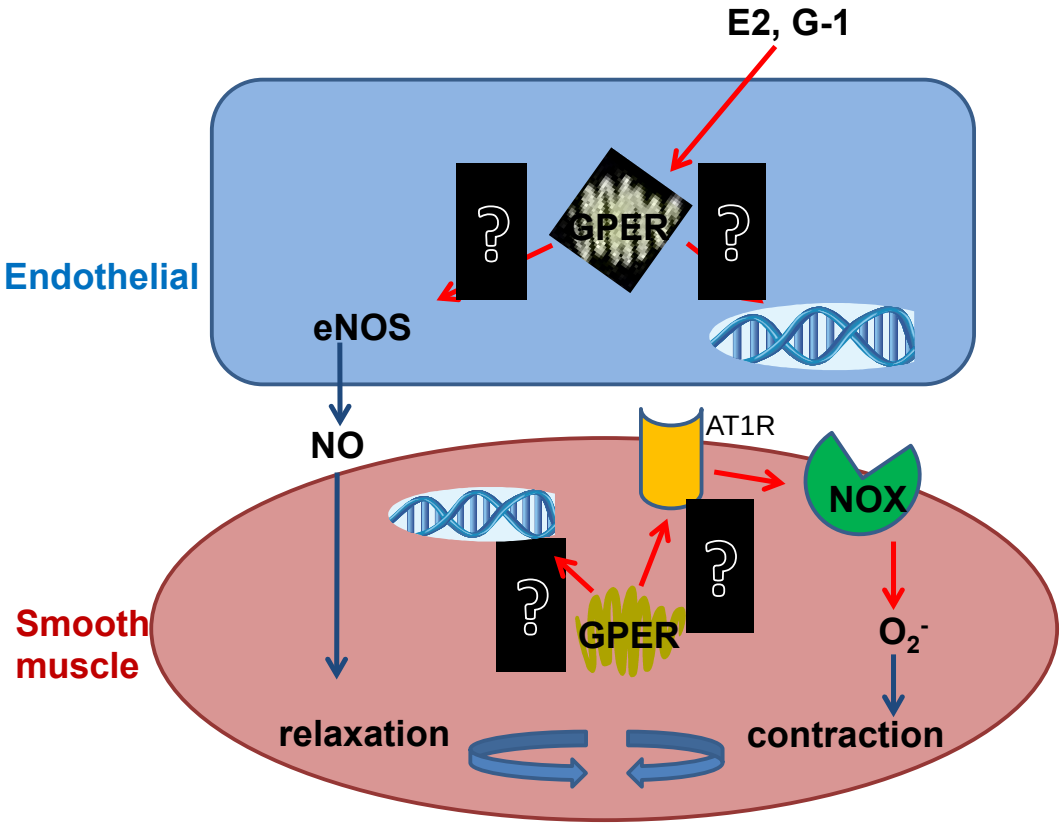


Fig 1.8 Investigative schematic of GPER in endothelial and smooth muscle signaling in vascular regulation: This thesis aims to investigate mechanism by which GPER signaling may activate endothelia NOS (eNOS) with by rapid signaling and/or genomic regulation to promote NO production. At the smooth muscle, we aim to elucidate the role of GPER in mediating Ang II vasoconstriction and NOX induced superoxide production in both rapid signaling and genomic functions.

CHAPTER 2

G PROTEIN-COUPLED ESTROGEN RECEPTOR CONTRIBUTES TO ESTROGEN-MEDIATED ACTIVATION OF ENDOTHELIAL NITRIC OXIDE SYNTHASE AND VASORELAXATION

2.1 ABSTRACT

Although the ability of 17β-ethinyl estradiol (E2) to induce rapid activation of endothelial nitric oxide synthase (eNOS) was reported over a decade ago, the pathways and relative contributions of ERα and the more recently described G protein-coupled estrogen receptor (GPER) are unresolved. Given that rapid signaling of GPER acts through cross-activation of EGFR and PI3K/AKT, and that AKT is responsible for eNOS activation at ser1179, we hypothesized that GPER activates eNOS at ser1179 in an EGFR and AKT-dependent mechanism. We report that GPER-specific agonist G-1 induced phosphorylation of eNOS at the activating residue ser1179 in endothelial cells with a maximal activation at 100 nM. We found that pharmacologic inhibition of GPER with G36 reduced E2-mediated NO production by ~60%. Inhibition of nitric oxide synthase enzymes with LNAME blocked G-1-mediated NO production, indicating NO production by GPER is an eNOS-specific event. Inhibition of EGFR (AG1478) and PI3K/AKT (LY291004) reduced NO production to GPER agonist G-1 by 85% and 76%, respectively. Inhibition of the kinase intermediate c-Src (PP2) inhibited NO production to G-1 by ~70%, and ERK1/2 inhibition (PD98059) blunted NO production to G-1 by ~80%, suggesting that GPER activates eNOS through multiple pathways and regulatory residues. Genomic deletion of GPER in mice (GPER KO) resulted in a 45% reduction in vasorelaxation to E2 in thoracic aorta. Finally, we report that inhibition of GPER in endothelial cells reduces NO production to the selective estrogen receptor modifier (SERM) compound tamoxifen (4-OHT) and to the selective estrogen receptor downregulator (SERD)

ICI 182,780. As the effects of clinical estrogen replacement therapy (ERT) have had unclear results for protecting vascular health in post-menopausal women, the implication of GPER agonism (G-1) as a possible therapeutic for increased NO production is a promising alternative.

2.2 INTRODUCTION:

Once thought of as a simple barrier layer, the vascular endothelium is now known as a diverse regulatory tissue of vascular reactivity, blood pressure, leukocyte extravasation, and serves as a source of growth, clotting, and other paracrine factors (152, 153). In the 1980s, Nobel laureate studies by Furchgott *et al.* identified the endothelium as the source of a vascular relaxation factor, which they termed endothelial-derived relaxation factor (EDRF) (154). These studies instigated a landslide of research that went on to characterize the signaling cascades and functions of endothelium in secreting EDRF, which was later identified as nitric oxide (NO) (155), in mediating arterial relaxation (156).

The enzymatic source of NO for vasodilation is endothelial nitric oxide synthase (eNOS), a multi-unit heterologous enzyme. Although several isoforms of nitric oxide synthase (NOS) have been identified such as inducible NOS (iNOS) and neuronal NOS (nNOS), eNOS is the predominantly expressed isoform in endothelial cells and the largest contributor of NO in healthy vascular function. NO formation is catalyzed by eNOS in two steps: first by transferring an electron from NADPH substrate through FAD^{+} onto molecular oxygen at the eNOS heme-domain, and second by cleaving nitrogen from the amine group of

an arginine substrate. The short half-life of NO (20-30 sec) necessitates rapid diffusion from the endothelium to the smooth muscle through the myoendotheilal gap junctions (157). Several groups have characterized eNOS co-localization with cholesterol-rich caveolae domains of the plasma membrane, and have described a post-translational acylation of eNOS that modifies the enzyme for anchoring to the caveolae scaffolding protein caveolin-1 (cav-1) (158, 159) (160). Additionally, post-transcriptional palmitoylation of ERα promotes trafficking to caveolae and association with cav-1/eNOS complexes (28). Physiologic mediators of eNOS activation include shear stress and pressure- sensitive receptor tyrosine kinases, which induce downstream AKT activation and phosphorylation of eNOS at ser1179 (161) (162). The neurotransmitter acetylcholine (Ach) can act on muscarinic type 3 (M3) G protein-coupled receptors on endothelial cells in experimental settings, which will increase intracellular calcium for calcium-dependent eNOS activation as well as PI3K /AKT activation and phosphorylation of eNOS at ser1179.

Clinical studies show that the incidence of CVD in pre-menopausal women is lower compared to men until the onset of menopause, when the rate of CVD in women rapidly equalizes to that of men (163-165). These observations suggest the loss of ovarian hormones coincided with a loss of protection against CVD in women, implicating a protective role of E2 in CV health.

Investigations of the receptors and pathways mediating E2 signaling in the vasculature have focused on ERα and the more recently described G protein-coupled estrogen receptor (GPER, formerly GPR30). Once thought to only exert

chronic, genomic effects through nuclear-localized ERα and β, studies demonstrating rapid signaling responses to E2 through ERα and GPER have been characterized in vascular cells (63, 166). Multiple studies have shown that activation of ERs and/or GPER induces vasorelaxation in an eNOS-dependent manner (54, 98, 130, 136). However, the contributive roles of ERα and GPER and their mechanisms in eNOS activation by E2 and other ER-targeting compounds (SERMs and SERDs) remain unresolved. We hypothesized that GPER co-mediates acute activation of eNOS by E2 and contributes to total NO production to estrogen with ERα. We sought to extrapolate the signal transduction pathway for GPER-mediated eNOS activation, and to determine the contribution of GPER to E2-mediated vasorelaxation by the endothelium. To answer these investigative points, we utilized molecular approaches to determine eNOS phosphorylation and NO production, and conducted vascular reactivity studies in the GPER KO mouse.

2.3 METHODS:

2.3.1 TIVE cell culture: Telomerase-immortalized human umbilical vein endothelial cells (TIVEs) were a generous gift from the Renne laboratory at the University of Florida (Gainesville, FL), and their derivation has been described previously (167). TIVEs were cultured in M199 basal media supplemented with 20% FBS, 1 µg/mL penicillin, streptomycin and gentamycin antibiotics and 100 µg/mL bovine neural derived endothelial growth factor (Sigma). TIVEs were cultured up to passage 12, and Western blotting for GPER expression for endothelial and fibroblast markers was performed every 4 passages.

2.3.2 Western Blotting: TIVE cells were grown to 80-90% confluence in 6 cm dishes, experimentally treated, and lysed on ice with 80 µL of NP-40 lysis buffer supplemented with 1% SDS, 5 µM sodium orthovanadate and 5 µM NaF to preserve phosphorylated proteins. Samples were centrifuged at 10,000 xg and supernatant was collected and stored at -80°C. For each sample, 20 µg of total protein was resolved by 10% SDS PAGE and blotted to PVDF membrane. Blots were blocked for 1 hour at room temperature in TBS-T 0.01% supplemented with 3% newborn calf serum and incubated overnight at 4°C with the following antibodies: 1:500 phospho- eNOS (mouse IgG S1179, BD Science cat #612392), 1:10,000 β- actin (mouse IgG Millipore MAB1501), and 1:10,000 GPER targeting second extracellular loop (clone #9368, rabbit IgG).

2.3.3 Nitric Oxide detection: Nitrate byproduct was assessed as indirect detection of total nitric oxide (NO) using the AbCam Nitric Oxide detection Kit (ab#65328). Following treatment, in HEPES buffered physiologic saline solution (in mmol/L: 134 NaCl, 6 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose 10 HEPES; pH 7.4), TIVEs were lysed at room temperature in sample buffer, and either stored overnight at -80°C or immediately loaded to a 96-well plate and treated with nitrate reductase and nitrate reductase substrate. Samples were incubated at room temp for 90 min to convert nitrates. Griess reagents were used to develop azo purple to measure nitrate levels. Photometric detection of azo purple absorbance at 540 nm was read on a Syngergy H1 plate reader at room temperature. Three readings per plate were taken. Background signal was subtracted and net azo signal was normalized to total protein (determined by

Coomassie blue readings at 533 nm) for each replicate. Samples were done in triplicate and experiments were repeated at least 3-5 times.

2.3.4 DMT isometric tension studies: Thoracic aorta was excised from male mice of BL6 WT or GPER KO littermates aged 3-5 months (25-30 g). Aorta was cleaned of fat in ice-cold PSS (in mmol/L: 129.8 NaCl, 5.4 KCl, 0.43 NaH_2PO_4, 19 $NaHCO_3$, 1.6 $CaCl_2$, 5.5 glucose; pH 7.4), cut into 3 mm rings, and mounted onto pins of a DMT myography chamber, and allowed to recover at 37°C for 30 min. Isometric tension was applied to a resting tension of 1900 mg and two successive contractions to 60 mM KCl were conducted. Viability was assessed as PE contraction (1 μM 5 min) response to 70% of the KCl max reading, and at least at 70% relaxation of the PE response by 1 μM acetylchoine (5 min). Rings were pre-contracted with PGF2α to 30-40% of the KCl max value, and dilation response to 3μM of E2, G-1 or ethanol vehicle were recorded for 60 min. Meclofenamate (1 μM 30min) was added 30 min prior to pre-contraction with PGF2α and subsequent estrogen dilation to E2 or G-1.

2.3.5 Statistical Analysis: N= 3-10 separate experiments for all studies. Unpaired T test was used to determine differences in mean between treatment groups, and error bars represent SEM. A p-value less than 0.05 was considered experimentally significant.

2.3.6 Animals care and use: All animals were housed according to IACUC protocols and animal procedures were approved by the University of New Mexico care and compliance of animal welfare guidelines. Mice were given *ad libitum*

access to water and standard chow. Animals were anesthetized with 1 mg/kg sodium pentobarbital, and response to pain was assessed by toe web pinch prior to opening the peritoneal cavity. The diaphragm was exposed and cut to ensure animal death.

2.4 RESULTS:

2.4.1 GPER activation contributes to estrogen mediated NO production by activation of eNOS at ser1179. GPER expression in TIVEs was confirmed by fluorescence microscopy (**Fig 2.1 A**). We first examined the effects of GPER-specific agonist G-1 on activation of eNOS by determining the phosphorylation status of the critical activation residue ser1179 in human TIVE cells. We observed a G-1-mediated, dose-dependent increase in phosphorylation of eNOS at ser1179 with a significant activation at 100 nM compared to vehicle (**Fig 2.1 B**)(53 +/-7 *vs.* 19+/-4 n=4, p=0.035). We next sought to confirm that activation of eNOS by G-1 increased NO production using colorimetric detection of nitrates. Detection of NO demonstrated that Ach (536 +/-4.7), E2 (425+/- 4) and G-1 (308 +/-4.3) increased NO production in TIVEs (vs. veh 94.0 +/-0.5, p= 0.006, n=4-6), and that non-selective ER activation (E2) induces greater NO production than GPER activation alone (**Fig 2.2**). Our results here confirm the observations of others (126) (131) that GPER can mediate eNOS activation and NO production in endothelial cells independently of ERα. The augmentation of NO production to E2 likely arises from the collaborative signaling of both ERα and GPER in mediating non-selective ER-induced eNOS activation.

We next sought to determine to contribution of GPER activity in E2-mediated NO generation in endothelial cells. Using GPER-specific antagonist G36 (100 nM 30min). We found that acute pharmacologic inhibition of GPER (G36) reduced NO production to E2 by ~60% (407 +/-3.0 and 235 +/- 2, p=0.009, n=6), but did not reduce NO production to acetylcholine (**Fig 2.3**) (543+/-12 vs. 533+/-9, p=0.838, n=5-8). These data suggest GPER partially contributes to E2-mediated NO production in endothelial cells, but does not contribute to E2-independent mechanisms of eNOS activation, such as Ach-induced eNOS activation.

2.4.2 GPER induces NO production in endothelial cells through multiple pathways. We next sought to determine the secondary signaling kinases involved in GPER-mediated eNOS activation. To determine the signaling pathway of GPER in eNOS activity, inhibitors targeting multiple constituents previously reported in the GPER pathway (35, 168) (33, 169) were used to assess the pathway of GPER- mediated NO production in endothelial cells. One well-known mechanism of GPER signaling is cross-activation of the epidermal growth factor receptor (EGFR). GPER activates G proteins to activate c-Src kinase, which activates MMPs to induce the shedding of EGFR ligand heparin-bound EGF. EGFR activation mediates a substantial number of downstream secondary messengers such as PI3K/AKT, which lies directly upstream of eNOS phosphorylation at ser1177, and ERK1/2, has been shown to phosphorylate both the inhibitory residues threonine 495 and serine 116, as well as the crucial activating residue ser1179 of eNOS. Both GPER and EGFR activate c-Src

kinase, which can directly activate residue tyrosine 81 on eNOS (170). Inhibition of EGFR with AG1478 reduced NO production to G-1 by 85% (210.4 +/-1.4 vs. 42.7 +/-2.8), and reduced NO production to E2 by 86% (211.7 +/-9.4 vs. 51.6 +/-1.1), indicating that both GPER and classic ERs signal through EGFR for eNOS activation (**Fig 2.4 A**).

Next, the PI3K/AKT inhibitor LY294002 (1 μM) was used to inhibit NO production to E2 or G-1 (100 nM) in endothelial cells. PI3K/AKT inhibition significantly abrogated NO production to both G-1 and E2 by ~76% in each case (E2 422.3 +/-2.5 vs. 196.3 +/-1.6) (G-1 337.3 +/- 5.5 vs. 185.7 +/-2.2) (**Fig 2.4 B**), suggesting that non-selective ER (E2) and GPER-specific activation of eNOS occur in a PI3K/AKT-dependent manner. Both non-selective ER-mediated (E2) and GPER-specific (G-1) NO production were eNOS-dependent, as demonstrated by a reduction of 80% (140 +/-5.68 vs. 54.99+/-5.31) and 85% (180.5 +/-52.5 vs. 56.14 +/-4.65) of NO production to G-1 and E2 in TIVE cells pretreated with the NOS inhibitor LNAME (1 μM for 30min) (**Fig 2.5**).

The involvement of ERK1/2 and c-Src signaling in GPER-mediated eNOS activation was determined using ERK inhibitor PD98059 (3 μM) and c-Src inhibitor PP2 (3 μM) prior to G-1 or E2 stimulation of eNOS in TIVE cells. Inhibition of c-Src (PP2) reduced NO production to G-1 by nearly 70% (140+/=5.56 vs. 53.5+/-7.39 p=0.08) (**Fig 2.5**), and reduced NO production to E2 by ~70% (180+/-52.5 vs. 65.01 +/- 4.82) suggesting an additional signaling axis of GPER-mediated eNOS activation through c-Src, likely through activation EGFR for phosphorylation of ser1179 as well as c-Src phosphorylation of residue

tyrosine 81. Inhibition of ERK1/2 with PD98059 reduced NO production to G-1 by ~60% (140+/- 5.56 vs. 59.43 +/-11.04 p=0.002). Inhibition of ERK1/2 also reduced NO production to E2 by ~60% (180 +/- 52.5 vs. 71+/-13.3 p=0.08). As ERK1/2 has been debated as both an activating kinase for ser1179, and also as a mediator of phosphorylation of the inhibitory residue Threonine 495 on eNOS. Given our data here, we suspect that ERK1/2 signaling contributes to activation of eNOS mediated by GPER and ERα through phosphorylation of activating residues rather than through inhibition of eNOS at Thr495, or at least the activation of eNOS overrides any inhibitory effects of ERK1/2 through ER-mediated signaling.

2.4.3 GPER inhibition reduces NO production induced by selective estrogen receptors modulators (SERMs). Selective estrogen receptor modifiers or downregulators (SERMs/SERDs) are used in the clinic for a variety of treatments, both pro-estrogenic and anti-estrogenic, depending on the tissue and cell type (171). SERMs impart pharmacologic effects by activation or inhibition of classic ERs, however SERM classification is specifically defined by their effects on classic ERs, regardless of their modifications to GPER signaling. For example, the SERD ICI 182,780 and SERM tamoxifen (4-OHT) have been demonstrated to act as antagonists towards ERα but conversely act as GPER agonists (56). Since some SERMs and SERDs have been previously reported to have effects on improved endothelial function (56, 171), and yet are shown to have inhibitory effects on ERα in multiple cell types (56), we hypothesized that the SERMs tamoxifen and raloxifene and the SERD ICI 182,780 would induce

NO production through agonism of GPER. TIVEs were pretreated with vehicle (0.01% DMSO) or 100 nM G36 for 30- 45 minutes prior to administration of tamoxifen activated metabolite 4-hydroxy tamoxifen (4-OHT) (1 μM), raloxifene (1 μM), or ICI 182,780 (1 μM) for 10 min prior to cell lysis and NO colorimetric detection. Acute pharmacologic inhibition of GPER (G36) reduced NO production to 4-OHT by ~53% (121 +/-12 vs. 57.65+/-.0625, p=.039). GPER inhibition also reduced NO production to ICI 182,780 by ~27% (106+/-9.77 vs. 77.6+/-6.7 p=0.04). Inhibition of GPER produced a trend in decreased NO production to raloxifene, but was not statistically significant (p=0.086) (**Fig 2.6**). These data indicate that GPER plays a minor role in mediating NO production to SERMs, or that SERMs may have ER-independent effects in mediating endothelial health and function.

2.4.4 GPER genomic deletion decreases vasorelaxation to E2 but not SERMs and does not alter murine systolic blood pressure. Since GPER inhibition reduced NO production in TIVEs, we next tested the physiologic output of GPER-mediated eNOS-dependent vasorelaxation in mouse thoracic aorta response to E2 or G-1 in WT BL-6 or GPER knockout (GPER KO) mice. Thoracic aorta from GPER KO mice had a ~45% reduction in relaxation (-35% +/- 7.42%) to general estradiol mediated dilation (E2 3 μM) compared to E2 dilation in WT aorta (-63%+/- 6.43%) (**Fig 2.7**). The vasorelaxation response of KO aorta to Ach (1 μM) did not differ from WT dilation to Ach (-93% +/- 3.52 vs. 95%+/- 5.68), corroborating earlier results from NO production in TIVE cells that inhibition of GPER had no significant effect on Ach induced NO production.

Interestingly, there was no statistical difference between vasorelaxation responses to E2 (non-selective ER activation) or GPER-specific (G-1) in mouse thoracic aorta (**Fig 2.7**). Our data here are in agreement with previous reports by Lindsay *et al* and Yu *et al* (172) (127) and also agree with our data from NO colorimetric detection in TIVEs that G-1 and E2-mediated NO production did not significantly differ in most aspects, or differed only by a marginal amount (**Fig 2.2**). These results suggest that GPER is sufficient for mediating total ER-induced vasorelaxation responses, but that ERα may not be. It is possible that non-selective ER activation by E2 may be involved in counteractive pathways against eNOS activation (such as ERK2-mediated phosphorylation of inhibitory residue threonine 495 on eNOS) as data from the TIVEs suggest. Given that COX activity was inhibited with meclofenamate prior to observing vasorelaxation responses to G-1 or E2, it is unlikely that E2-induced EDCF production would counteract an E2-induced vasorelaxation response obesrved in our myography experiments.

Vasorelaxation to the SERD ICI 182,780, and the SERM (activated) metabolite 4-hydroxy tamoxifen (4-OHT) were tested in WT thoracic aorta pretreated with vehicle (.01% DMSO) or G36 (1 μM) to assess the role of GPER in mediating the vasorelaxation effects of these compounds (**Fig 2.8**). Data on SERM myography indicated that GPER inhibition did not significantly blunt vasorelaxation to ICI 182,780 or 4-OHT. These results suggest that the SERM and SERD compounds have alternative signaling pathways, including possibly ER-independent pathways.

Since GPER is shown to contribute to estrogen-mediated NO production and vasorelaxation, we sought to assess if GPER genomic deletion had effects on global blood pressure in male or female mice compared to WT littermates. We employed conscious tailcuff readings of systolic pressure, whereby mice were trained to sit quietly under light restraint for 90 seconds to obtain 6 readings of systolic pressure daily for 7 to 10 days (see section 2.3 for detailed methods). Using conscious tailcuff blood pressure measurements, we found that systolic blood pressure in the GPER KO mice was no different from WT in young (3 months) and aged (16 month) male or female mice (**Fig 2.9 A**). Heart rate from these animals were no different between WT and GPER KO mice, suggesting that there was not a compensatory change in heart rate to regulate systolic blood pressure (**Fig 2.9 B**).

2.5 SUMMARY & DISCUSSION:

In summary, we show that GPER contributes to E2-mediated acute eNOS activation by showing that G-1 induced eNOS phosphorylation at ser1179, promoted NO production, and by showing that inhibition of GPER (G36) results in decreased NO production to E2. NO detection revealed that non-selective ER activation and GPER-specific activation induces eNOS-dependent NO production through multiple pathways including EGFR, PI3K/AKT, c-Src and ERK1/2. We also demonstrated a 55% reduction of the relaxation response to E2 in thoracic aorta of GPER KO mice. We demonstrated a contributive role of GPER in SERM mediated NO production through eNOS activation in endothelial cells. Finally, we

showed that systolic blood pressure was no different between WT and GPER KO mice.

Previous studies have clearly implicated a role for estrogen in cardiovascular health (71, 130). These vasoactive effects appear to be mediated primarily through an eNOS-dependent mechanism, which can be activated both by non-selective ER agonism and GPER-specific activation. However, recent studies have presented an additional GPER mechanism for vasorelaxation at the smooth muscle level though GPER-mediated activation of smooth muscle Gs and adenylyl cyclase to increase cAMP and activate PKA and RhoA kinase for increase MLCP activity and reduced contraction in VSMCs by calcium desensitization (126, 127). To date, studies have not elucidated the exact contribution of GPER activation in endothelial NO production, nor the exact signaling pathway of GPER in estrogen-mediated eNOS activation and how it compares to signaling pathway of ERα. The data presented here is significant and progressive in several aspects in that it reveals the pathway of GPER signaling in eNOS activation, and demonstrated for the first time that the E2-mediated relaxation response is reduced with genomic deletion of GPER in GPER KO mice.

We build upon the current body of knowledge using molecular approaches and knockout animal studies to resolve the differences between GPER and ERα in eNOS activation in TIVEs. We showed that GPER-mediated eNOS activation likely occurs through the regulation of multiple regulatory residues due to its activation of multiple eNOS-interacting kinases, such as PI3K/AKT, c-Src and

ERK1/2, which lends to the complex effects of estrogen in endothelial function. Additionally, we demonstrate that the complex effects of SERMs and SERDs in mediating endothelial function is only partially dependent on GPER signaling, which implicates that SERMs or SERDs may have additional effects outside of modifying ER signaling, or that they have differential effects in the endothelium (activating rather than antagonizing).

While the predominant body of work studying ER-mediated vasorelaxation derives from investigations of eNOS-dependent mechanisms, several other sources of endothelial derived relaxation factors (EDRF) exist. Prostacyclin (PGI_2) is a vasodilator compound derived from metabolism of arachidonic acid intermediates by endothelial cyclooxygenase (COX) enzymes. Inhibition of COX 1 and 2 in multiple vessel types, including the thoracic aorta rings used in these studies did not diminish vasorelaxation to G-1 nor E2, indicating that COX-derived EDRFs are likely not involved in estrogen-mediated vasorelaxation. Additionally, previous studies by our laboratory investigating increased vasocontractility in obesity models of GPER KO and high fat diet mice showed that GPER deletion had no effect on COX-mediated vasocontraction increases from thromboxane sensitization in adipocytes (173), which further indicate GPER is not involved in COX-mediated signaling for vessel tone or relaxation. Additionally, multiple independent reports show that inhibition of NOS activity using LNAME in endothelial cells abrogates NO production to E2 and G-1 (126) (172) (174) (166). These observations demonstrate that E2-mediated vasorelaxation is likely an eNOS-dependent mechanism.

We must also consider endothelial derived hyperpolarizing factor (EDHF), which is another mechanism of endothelial regulation of tone and vasorelaxation. Some studies show that EHDFs may persist for adjacent hyperpolarization of adjacent VSMC when both eNOS and COX are inhibited, at least in some vascular beds (175) (176) (177) either by possibly acting through endothelial small and intermediate conductance (SK and IK) channel activation, or by diffusible factors from endothelial cells that activate smooth muscle ion channels. It has also been hypothesized that NO can act directly as a hyperpolarizing agent. Additionally, GPER-specific agonist G-1 has been shown to induce hyperpolarization in some vascular beds (possibly in a secondary manner through the actions of NO) (176, 178). Our myography studies did not address mechanisms of ERα or GPER in EDHF responses, and as such we cannot exclude the possibility of a partial contribution of this signaling mechanism in E2 and GPER-mediated vasorelaxation.

Our systolic blood pressure data showed no difference in SBP between GPER KO and WT mice regardless of sex or age. These data, along with our myography studies, agree with previously published observations that GPER agonism does not intrinsically reduce blood pressure in mice (172) (179). However, estrogen receptors are hypothesized to mediate observed gender differences in blood pressure and susceptibility to hypertension (130, 134). Other laboratories have documented a hypotensive phenotype in different strains of GPER KO mice (174) and others have demonstrated that G-1 infusion lowers blood pressure in rats (128). Also, recent data has demonstrated that a single

nucleotide polymorphism resulting in a hypofunctional GPER variant occurs in approximately 30% of the female population and is associated with a subtle increase in resting blood pressure (180). Given these reports, the role of GPER in maintaining blood pressure is unclear. In mouse models, there may be compensatory effects in the genomic deletion of GPER, such as increased ERα signaling to substantiate E2-mediated NO production, or decreased cardiac output to maintain mean arterial blood pressure in response to increased peripheral resistance in our GPER KO animals. It is also possible that we were unable to observe subtle differences in systolic blood pressure due to our method of measurement with tailcuff. Although conscious tailcuff measurement of SBP has been implemented in many instances to show differences in experimental models where shifts in BP can be more dramatic (such an Ang II infusion or high salt diet vs. control normotensive), this method may not be sensitive enough to measure subtle differences in SBP in healthy mice. To this effect, a compensatory change in heart rate may have occurred in these animals, but again our data from the CODA 6 system may not have been sensitive enough to detect such changes.

This study also presents characterization of an endothelial cell line that has not been previously used to study endothelial pathways of estrogen, the Telomerase Immortalized human umbilical Vein Endothelial (TIVE) cell line (167). Our data establish the molecular and functional identity of TIVEs as a useful reagent in conducting endothelial studies for estrogen mechanisms. Myography data in this study also corroborated our results established in the TIVEs, further

implicating their utility in conducting molecular studies on estrogen signaling in endothelial biology. Stable expression of GPER, ERα and endothelial markers and low expression of mesenchymal markers throughout passaging implicate TIVEs as a novel reagent to study endothelial signaling with the convenience of cell line stability derived from a well-known tool for endothelial studies, the HUVEC.

It must be noted that cultured endothelial systems cannot accurately represent a response from an endothelium in vivo. For example, a vital component of endothelial function is the response to shear stress along the luminal surface, which is a classic stimulus for shear induced, NO mediated vasodilation. While our cell culture system cannot take into account the signaling differences that may arise from a system incorporating shear stress stimulation of NO production, we can establish that E2 stimulation of endothelial cells can lead to an increase in NO production, and also result in NO-mediated vasorelaxation at the tissue level, which was independent of shear stress. We hypothesize that a shear stress exposed endothelium may have a stronger response to E2 stimulation because of increased basal NO due to shear stress exposure, which is further augmented with receptor-mediated activation. We additionally recognize here that myography of aortic rings also lacks a flow-response design that more accurately mimics in vivo endothelial responsive conditions for vasodilation. Nevertheless, the acute reactivity of a vasorelaxation response (in the absence of intraluminal pressure, but holding at optimal resting tension) to agonist-induced (G-1 and E2) was readily measured in our system, which

suggests that ER and GPER stimulation of NO and vasorelaxation can occur independently of shear-induced eNOS activation.

Taken together, this study utilized unique tools such as TIVEs and the GPER KO mouse to establish the detailed GPER signaling axis for eNOS activation, and resolved the contribution of GPER to E2-mediated vasorelaxation in thoracic aorta and blood pressure maintenance in the GPER KO mouse. Importantly, the GPER-specific agonist G-1 and GPER-specific antagonist G36 are useful reagents in that present research suggests these compounds lack the deleterious side effects seen with generic estrogen replacement or with other SERMs known to have tissue-specific effects or complications (181). Studies of E2 replacement in mouse studies as well as in hormone replacement therapy (HRT) clinic have presented mixed results of E2 in mediating CVD risk reduction (120, 143, 182). Here, the resolution of the GPER signaling axis and its contribution to E2-mediated dilation further enrich the resourcefulness of G-1 and G36 as compounds that could have broad clinical implications in reducing CVD risk in post-menopausal women by promoting eNOS activity, increasing NO bioavailability and maintaining endothelial function. Given that there are GPER specific agonists (G-1) and antagonists (G36) that avoid deleterious complications of estrogen replacement (143), it is plausible that GPER-specific modulation could aid in promoting or maintaining cardiovascular function in post-menopausal women, given the lack of reproductive side effects GPER-specific compounds have such as uterine weight gain seen with estrogen replacement or supplementation in mice (51) (183) (E2) or ERα-antagonizing SERMs or SERDs

such as tamoxifen or ICI 182,780. Additionally, the efficacy of GPER-mediated NO in the endothelium can also deliver vasoprotective effects from atherosclerosis and inflammation. To this effect we published such results demonstrating GPER agonist G-1 indeed reduces atherosclerotic plaque development and inflammation in mice fed an atherogenic diet (107). We may conclude that a mechanistic implication of this protective effect stems from GPER-mediated NO production by the data given in this thesis. We conclude that GPER may moderate multiple cardiovascular benefits such as vasorelaxation by promoting eNOS activation and NO production, and that this mechanism is essential for mediating the benefits of estrogen signaling in cardiovascular health.

Fig 2.1

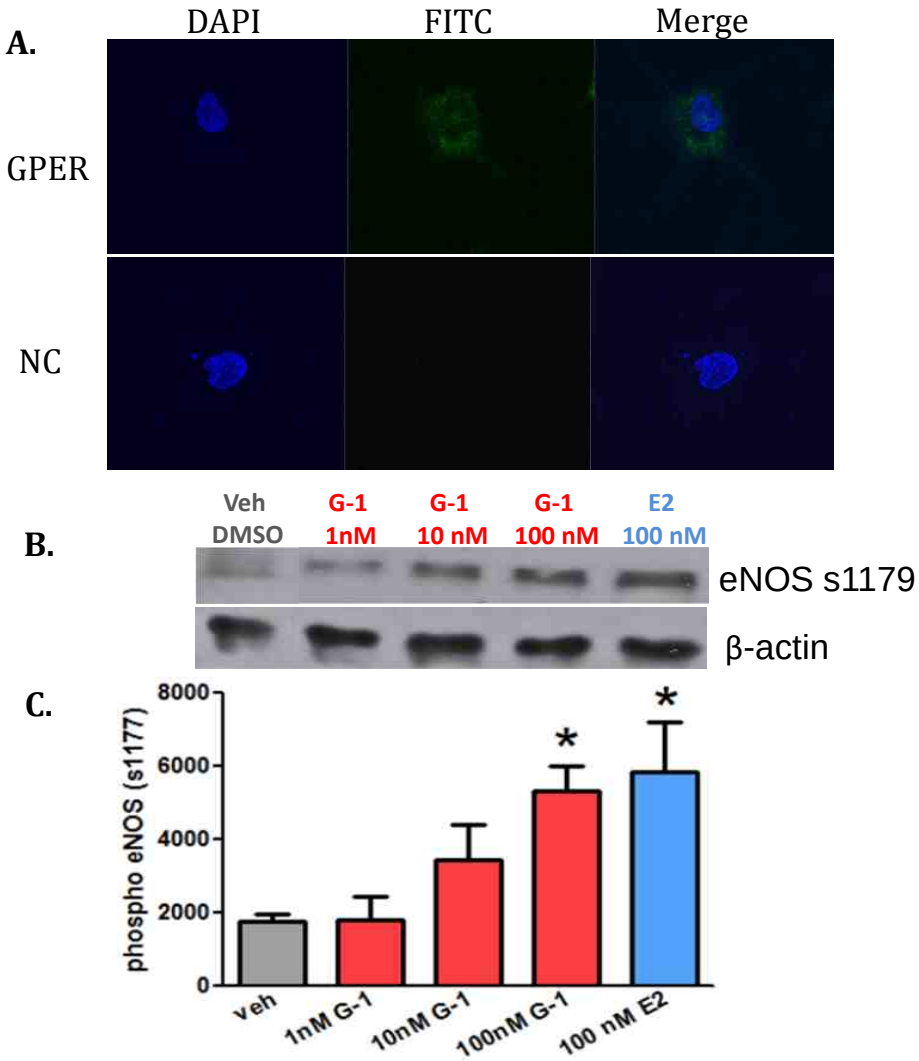


Fig.2 GPER activation stimulates eNOS phosphorylation at ser1179: A) GPER expression in TIVEs was confirmed with fluorescence microscopy. **B)** TIVE cells were treated with increasing doses of GPER-specific agonist G-1 for 20 min, lysed and blotted for phosphorylation of eNOS critical activation residue ser1177. **C)** Densitometry of 2B shows that GPER activation increased eNOS phosphorylation in a dose-dependent manner. *p=0.03 vs. veh

Fig 2.2

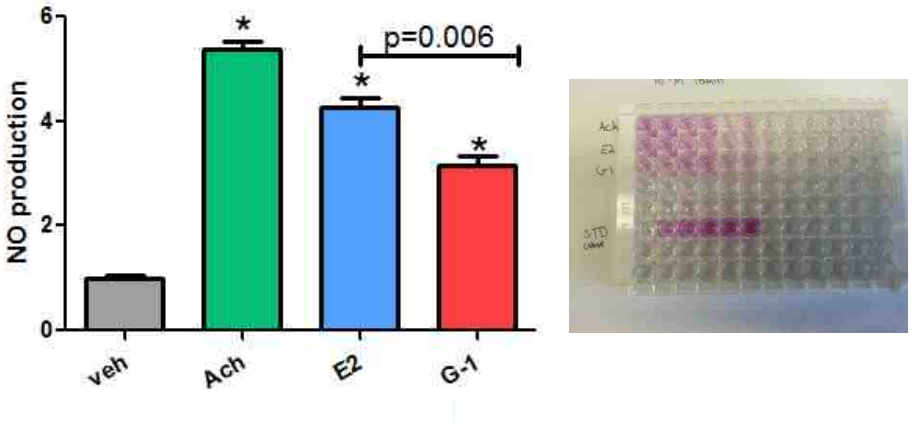
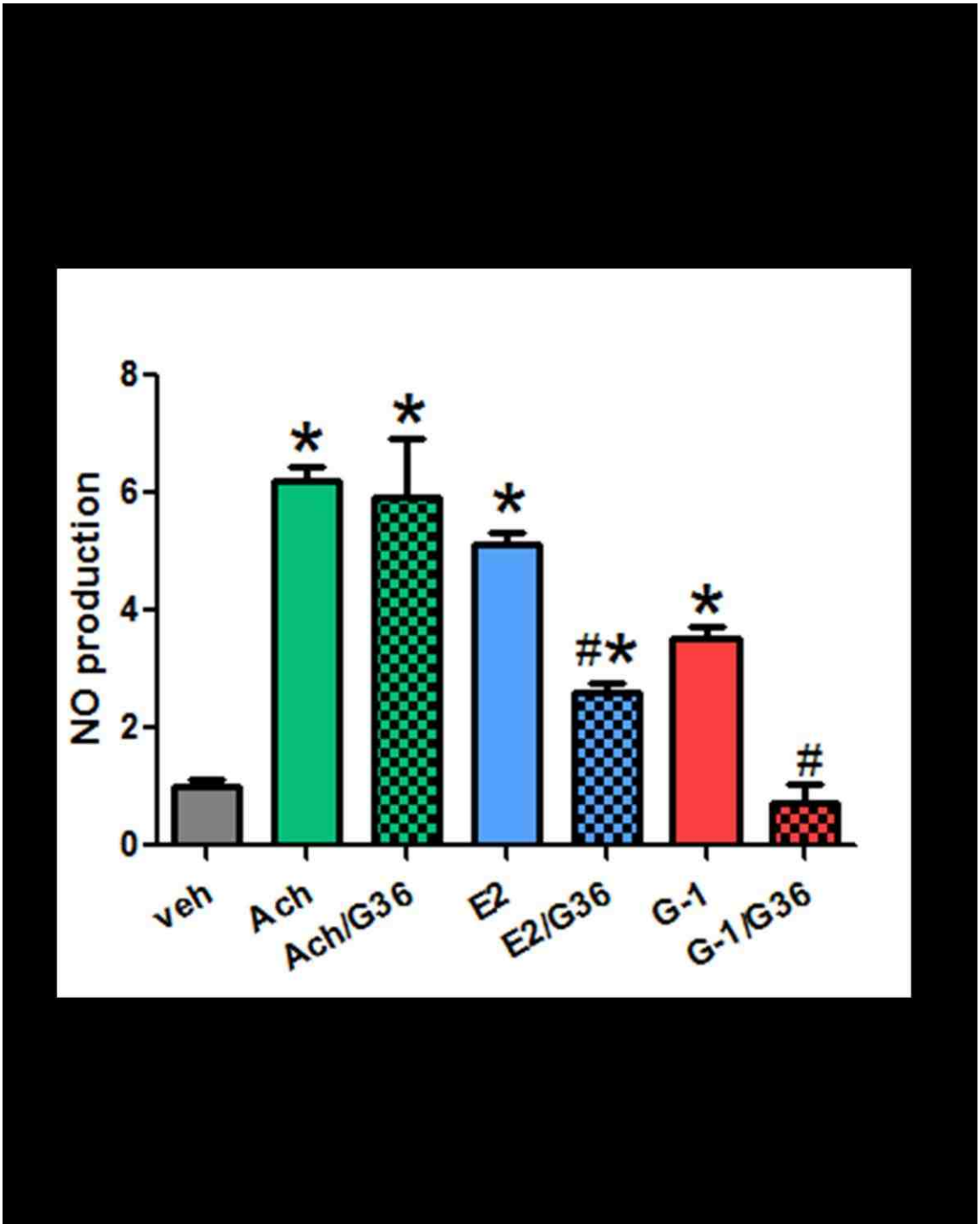


Fig 2.2 GPER activation induces nitric oxide production: TIVE cells were treated with 100 nM of estrogen (E2), GPER agonist G-1, acetylcholine (Ach) or 0.01% DMSO vehicle control (veh) for 10 min and were lysed and subjected to Griess reagent detection of nitrate byproducts as an estimate of NO production. Both non-specific ER activation (E2) and GPER-specific activation (G-1) resulted in NO production. Estrogen mediated NO production was not as robust as Ach.


NO production
8
6
4
2
0
*
*
*
#*
*
#
veh
Ach
Ach/G36
E2
E2/G36
G-1
G-1/G36

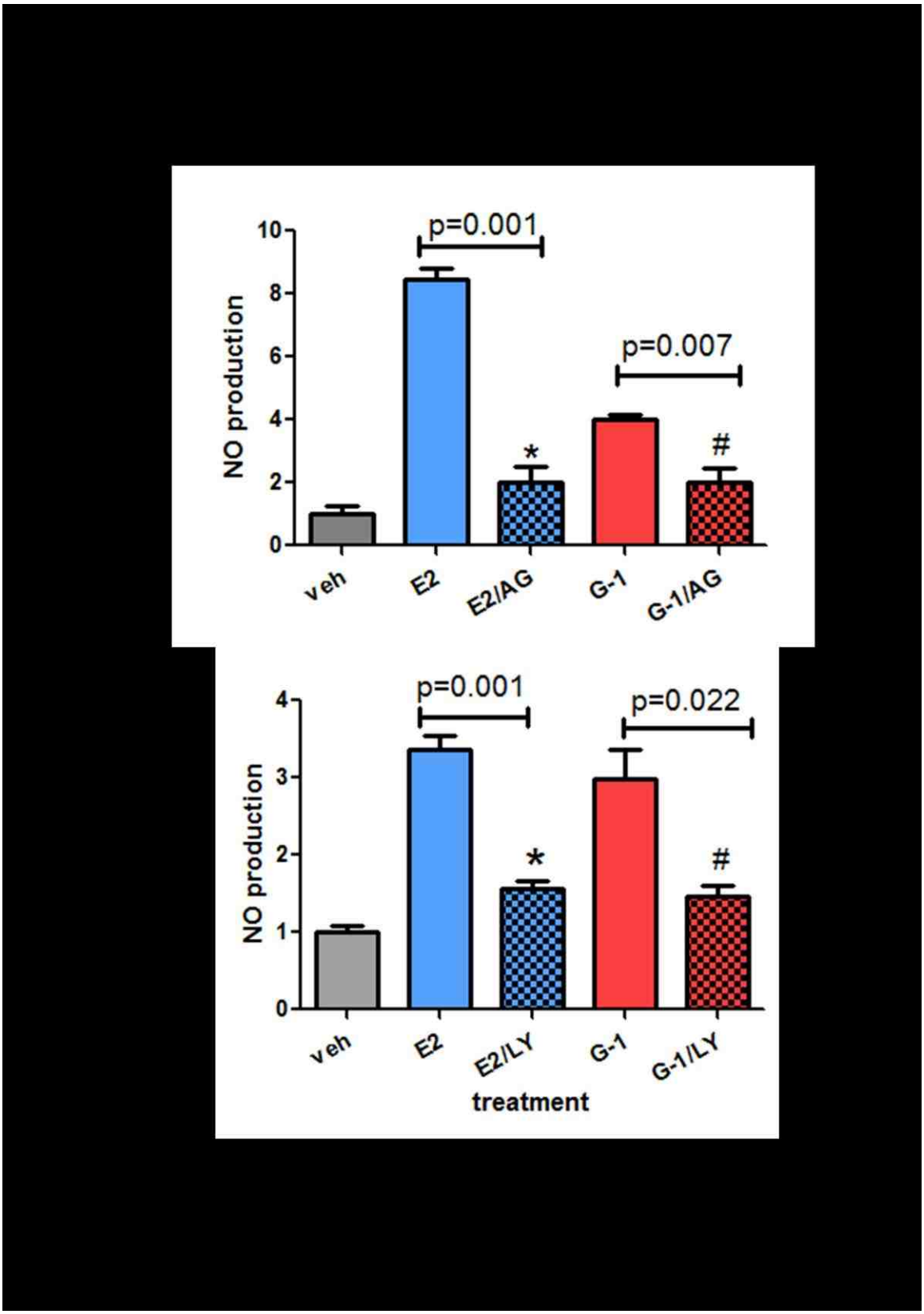

p=0.001
p=0.007
NO production
10
8
6
4
2
0
*
#
veh
E2
E2/AG
G-1
G-1/AG
p=0.001
p=0.022
NO production
4
3
2
1
0
*
#
veh
E2
E2/LY
G-1
G-1/LY
treatment

Fig 2.5

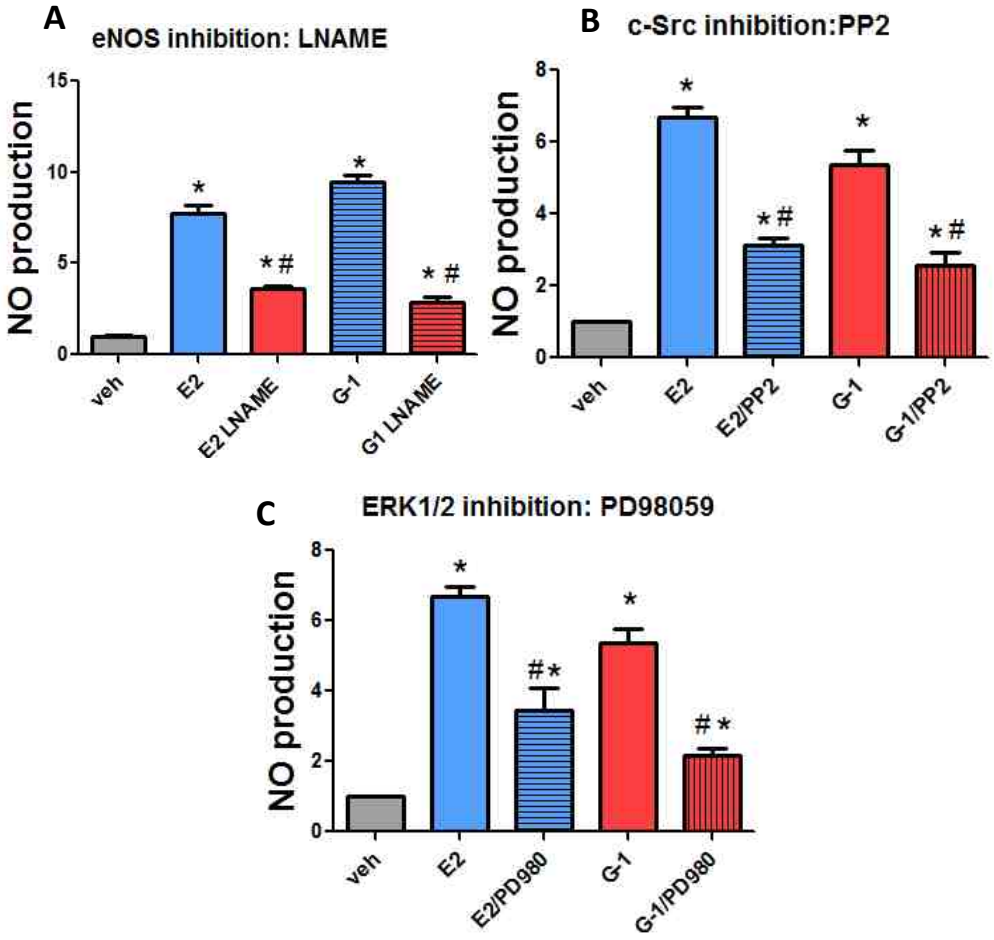


Fig 2.5 E2-mediated NO production requires c-Src and ERK1/2: Endothelial cells were pretreated with 1 μM of NOS inhibitor LNAME (A) c-Src inhibitor PP2 (B), or ERK1/2 inhibitor PD98059 (C). Inhibition of eNOS blocked NO production to estrogen and GPER specific activation. Inhibition of c-Src and ERK1/2 kinases also prevented NO production to E2 and GPER-specific agonist G-1. These data indicate that multiple pathways of activation are required to elicit the E2 and GPER induced production of NO. *P<0.05 vs. E2, #P<0.05 vs. G-1 or E2 without inhibitor

Fig 2.6

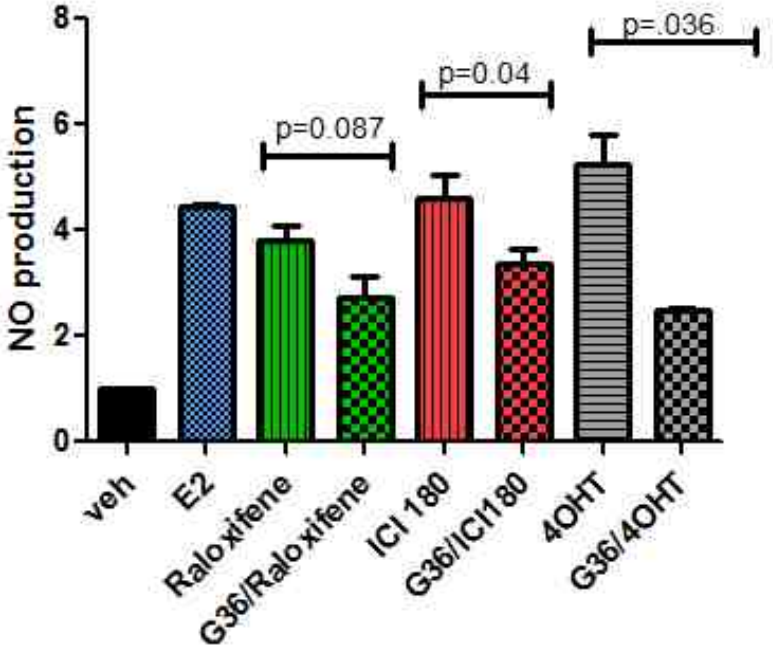


Fig 2.6 GPER inhibition reduced NO production to selective estrogen receptor modifiers and down regulators: Endothelial cells were pretreated with GPER-specific antagonist G36 (1 μM) or 0.01% DMSO vehicle for 30-40 min., and treated with SERMs 4 hydroxy-tamoxifen (4OHT) or raloxifene, or the SERD ICI182,76 for 15 min. Inhibition partially blocked NO production to 4OHT and raloxifene, as well as blocked NO production to the ICI182,76.

Fig 2.7

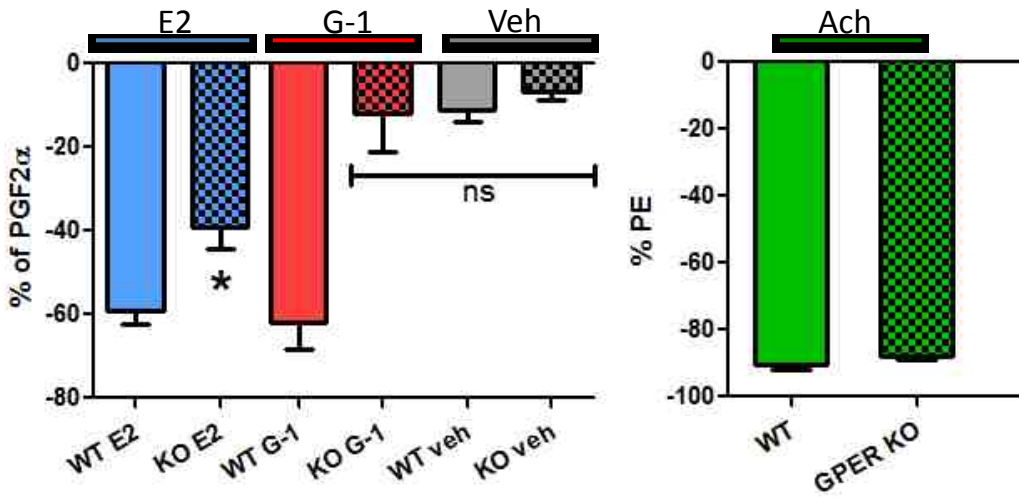


Fig 2.7 Genomic deletion of GPER results in reduced vasorelaxation to E2 in thoracic aorta: Thoracic aorta harvested from GPER KO mice or WT BL-6 littermates were subjected to isometric force myography to measure vasorelaxation to E2 (3 μM)or to acetylcholine (1 μM) .Aortas were precontracted with PGF2α and then treated with a single dose of Ach or E2. Vasorelaxation is expressed as a percent reduction from the PGF2α precontraction value. Compared to WT aorta, GPER KO aorta displayed a 45% reduced vasorelaxation to estrogen (E2). There was no observed difference in vasorelaxation between GPER KO and WT aorta to acetylcholine. N=6-8 animals per treatment, * P< 0.05 vs. WT E2

Fig 2.8

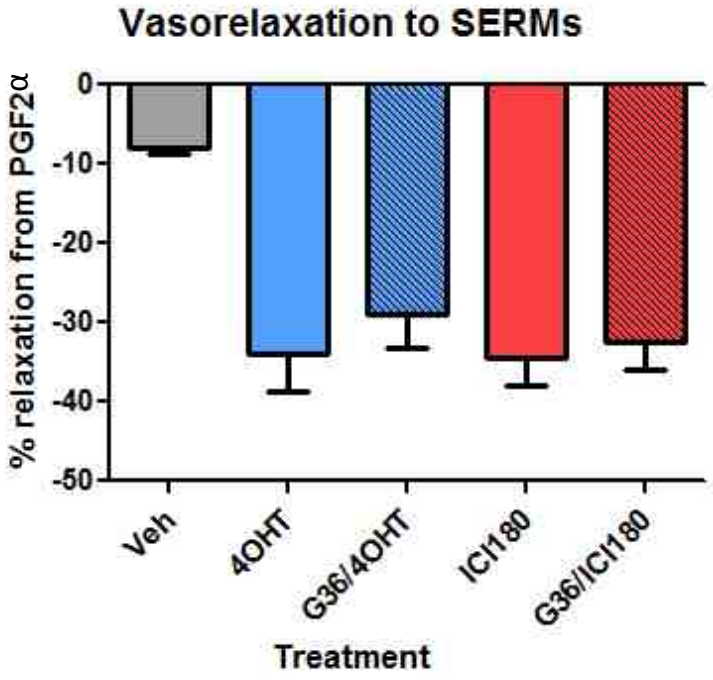


Fig 2.8 GPER inhibition does not reduce vasorelaxation to tamoxifen or ICI 180,76: thoracic aorta form WT mice were pretreated with GPER-specific antagonist G36 (1 μM 30 min) prior to vasorelaxation with the active metabolite of tamoxifen (4-OHT) or ICI180,760. GPER inhibition did not significantly reduce vasorelaxation to either compound.

Fig 2.9

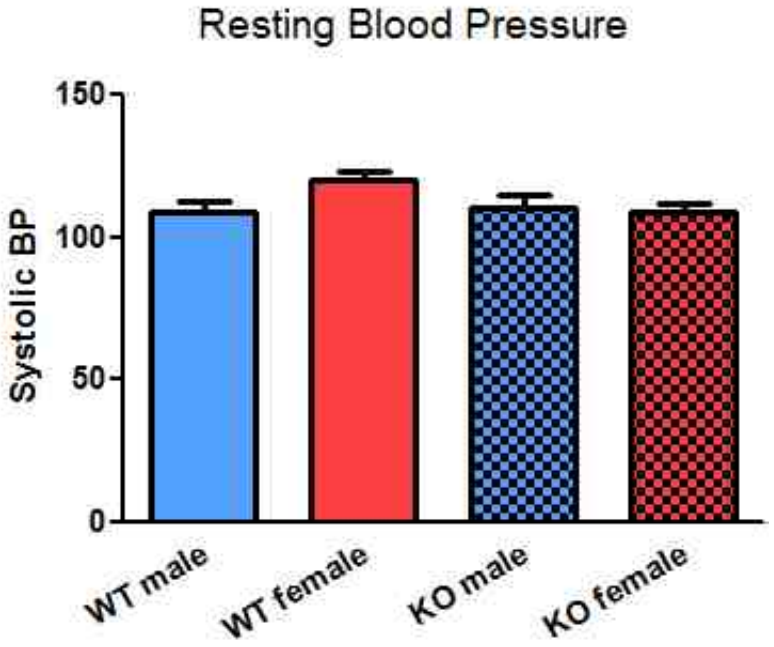


Figure 2.9 GPER KO animals do not exhibit increased systolic blood pressure: Mice that were wildtype (WT) or GPER null (GPER KO) were subjected to conscious talicuff measurement of systolic blood pressure (SBP) at 3,6,12,and 18 months of age. The graph above depicts SBP at 18 months of age. There was no detectable difference in SBP between GPER KO and WT littermates at any age point. N=4-5 per group.

Fig 2.10

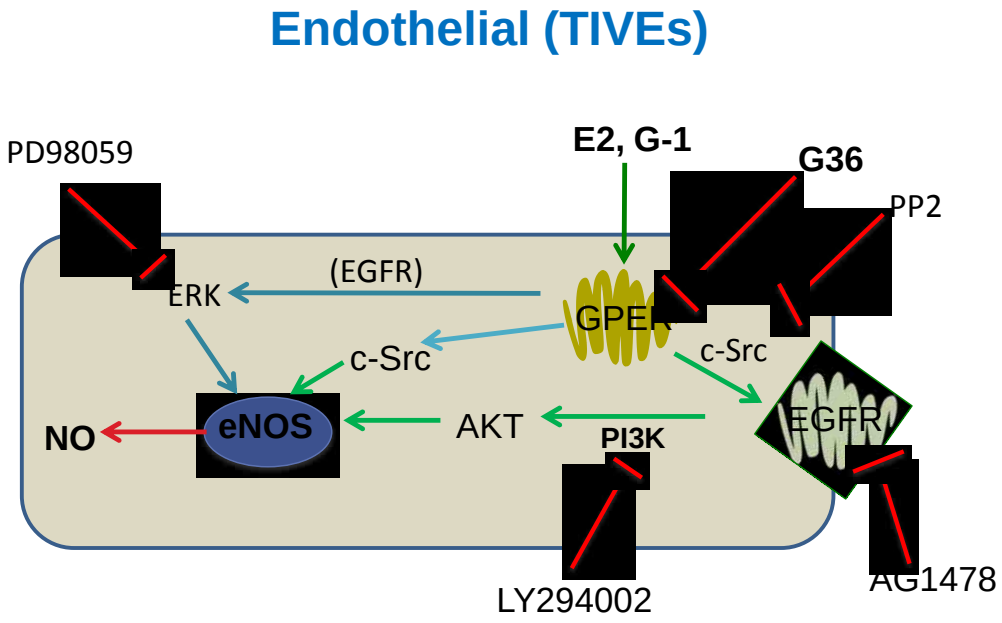


Fig 2.10 Summary of chapter 2 findings: Using a human endothelial cell line, it was determined that GPER activation induces NO production by eNOS through several signaling pathways including EGFR and PI3K/AKT which are upstream of ser1171, and c-Src activation which is upstream of Y87, and ERK1/2 which is upstream of thr495. GPER specific agonist G36 also reduced NO production to the SERMs tamoxifen and raloxifene, as well as reduced NO production to the SERD ICI182,76.

CHAPTER 3

GPER REGULATES NADPH OXIDASE EXPRESSION AND SUBSEQUENT SUPEROXIDE PRODUCTION TO ANGIOTENSIN II IN VASCULAR SMOOTH MUSCLE

3.1 ABSTRACT:

Reactive oxygen species (ROS) are an essential mediator of vascular smooth muscle cell (VSMC) signaling and contractility. A catalytic source of ROS is the family of NADPH oxidase (NOX) proteins. As such, pharmacologic regulation of NOX activation and/or expression could serve as a potential therapeutic to reduce oxidative stress, which is a common component to vascular disease such as hypertension. Myography studies imparted a novel observation that the arteries from mice expressing global knockout for the g protein-coupled estrogen receptor (GPER KO) have reduced contraction to vasopeptide Ang II compared to wild type (WT) arteries. The reduction seen in KO arteries was mimicked in WT by pharmacologic inhibition of NOX using the gp91ds-tat peptide. Lucigenin detection showed that VSMCs from GPER KO animals had a remarkable loss of superoxide production to Ang II and NADPH compared to WT and human VSMCs, as well as a reduced calcium response, coinciding with reduced arterial contraction seen in our GPER KO vessels. Chronic, but not acute inhibition of GPER reduced superoxide production in WT mouse and human aortic SMCs, suggesting a genomic mechanism of regulation of GPER over NOX1. Indeed we found a ~50% reduction in NOX1 expression in GPER KO SMC, as well as a 50% reduction in NOX1 expression in human aortic SMCs treated with G36 ($p = .0227$). To assess the physiologic efficacy of our findings, we tested GPER inhibition or deletion abrogated a hypertensive response to Ang II peptide infusion. GPER KO and G36-pretreated WT animals resisted hypertension onset, and their vessels had reduced superoxide levels compared to hypertensive WT animals. These data implicate that GPER partly mediates

expression of NOX1 in VSMCs, and that inhibition of GPER can subsequently reduce ROS levels to mitigate Ang II-induced hypertension by decreasing NOX1 expression.

3.2 INTRODUCTION:

Reactive oxygen species (ROS) are a vital molecular component of cellular signaling and physiologic function, and has long been implicated in normal vascular function. Disruption of the ROS balance has been demonstrated as a causative factor in atherosclerosis, hypertension, and stroke (184, 185). Although multiple forms of ROS exist, the superoxide anion radical ($O^{\cdot}$) is a special type of ROS, generated in part by a family of enzymes, NADPH oxidases (NOX), expressed in most cells. In the vasculature, NOX1 is commonly expressed in smooth muscle, while NOX2 is expressed in endothelial cells and NOX2 and NOX4 in adventitial fibroblasts (185, 186). Typically, NOX catalytic enzymes function by transferring an electron from the substrate NADPH through FAD+ onto molecular oxygen to generate the superoxide anion radical, although the isoform NOX4 exclusively generates hydrogen peroxide (H_2O_2) (185, 186). Superoxide has been shown to oxidize cysteine residues of ROS-sensitive transcription factors and other downstream signaling targets such as VEGF, hypoxia inducible factor (HIF), and protein kinase C (PKC) (187, 188). Additionally, superoxide and other types of ROS (such as H_2O_2) can activate voltage gated L-type calcium channels (L-VGCC), and IP_3 receptors (IP_3R), which drive cytosolic calcium increase (189). PKC activation and intracellular calcium increase are implicated in myosin light chain kinase (MLCK) activation and smooth muscle contraction. Ang II, a vasoconstrictor peptide binds to AT1R receptors at the smooth muscle, which activates a signaling cascades such as rac1 to promote assembly and activation downstream NOX to produce ROS in vascular

smooth muscle cells, resulting in arterial contraction (190-192). The superoxide anion can also be rapidly reduced to H_2O_2 by the enzyme superoxide dismutase (SOD), or oxidize the nitric oxide radical (NO) into the ROS peroxynitrate ($ONOO^-$), and hence also serves as a "kindling" radical for downstream ROS generation and activation of multiple ROS-sensitive targets (184).

In 2005 and 2010, Experiments by White and colleagues described a novel effect of estrogen (E2) inducing contraction in porcine coronary arteries when nitric oxide synthase (NOS) enzymes were uncoupled with L-NNA, resulting in increased ROS production and ROS-mediated contraction (105) (106). However, the receptor(s) involved and subsequent signaling mechanism(s) were not fully established, including sources of ROS separate from pharmacologic disruption of NOS. Importantly, these studies failed to address signaling differences between estrogen receptors such as alpha, beta or GPER when conducting mechanistic studies on the contractile effects of estrogen in NOS-uncoupled aortas and VSMCs (106). Interestingly, the Women's Health Initiative (WHI) clinical study in 1991 concluded that post-menopausal women suffered a 30% increase in CVD from synthetic estrogen replacement, suggesting that estrogen may play a role in post-menopausal CVD (193). Taken together, these separate reports may implicate a mechanistic effect of E2 to induce ROS production in arterial contraction and vascular dysfunction.

The deregulation of NOXs in hypertension has been described in several animal models (185, 194-197). In 2005, Griendling *et al.* reported that NOX1 overexpression in transgenic rats exacerbates an Ang II-model of hypertension. Conversely, inhibition of NOX using siRNA or gp91ds-tat (NOX peptide inhibitor) abrogated Ang II-mediated

hypertension (198, 199). Several mechanisms of NOX activation have been addressed in the literature, such as a feedback loop of Ang II receptor AT1R to cross activate both epidermal growth factor receptor (EGFR) and NOX1 (200, 201), as well as NOX1-generated superoxide cross-activation of EGFR via c-Src oxidation (202) , and EGFR activation of ERK1/2 to activate transcription factors MEF2B and JunB for increasing NOX1 transcription (203).

Our laboratory has reported that GPER can cross-activate EGFR (40) in several cell types by activating c-Src to activate matrix metalloproteases (MMPs) which cleave and activate heparin-binding EGF (HB-EGF), a ligand for EGFR. This link may be important in SMC for GPER in mediating superoxide production via EGFR crosstalk with NOX in the mechanisms listed above. GPER functions have already been partially characterized in endothelial function and CVD (130, 135, 204); however, more investigation in vascular smooth muscle is needed to fully understand GPER function in the vasculature.

Whereas previous reports implicated an ability of E2 to promote oxidative damage in lieu of uncoupled NOS or diseased arteries, our studies here expand upon this observation to establish new insights regarding E2 in cardiovascular health through the contribution of GPER signaling to the oxidative damage connected to E2 replacement in clinical studies. While we originally hypothesized that GPER KO animals would have an increased Ang II contraction due to reduced eNOS activity from lack of GPER, we report here a novel observation that GPER KO animals have reduced Ang II contraction. This reduced contraction arises from diminished NOX expression

and results in decreased ROS production to the vasopeptide Ang II in VSMCs, as well as in a rodent model of ROS-mediated hypertension.

3.3 METHODS:

3.3.1 Aortic SMC harvest and culture: *Ex vivo* harvest of fresh murine smooth muscle cells from aorta of GPER KO and WT age-matched littermates were used to study the signaling mechanisms of superoxide production. Mice were euthanized with sodium pentobarbital (0.1mg/kg ip). Aortas were dissected from mice and cleaned of fat in ice-cold sterile HPSS (HEPES-buffered saline solution, pH 7.4) supplemented with penicillin, streptomycin and gentamycin. Collagenase I digest (1mg/mL) was performed at 37°C for 20 min to loosen the adventitia. Adventitia was then peeled off the vessel, the vessel was cut open lengthwise, and endothelium rubbed off with a sterile toothpick. The tissue was washed twice with sterile HPSS and allowed to rest for 2 hours in DMEM with 10% FBS in a humidified 37°C incubator. Aortas were incubated in digest buffer containing elastase I and collagenase I at 37°C for 20 min to disperse SMCs. Digested aortas were titurated with a sterile glass pipette, digested another 25 min and titurated again to disperse the smooth muscle cells. Cells were collected and seeded in 2 mL of DMEM with 10% FBS and antibiotics. Primary AoSMC's were split 1:2 to in T-25 flasks coated with 0.01% Poly-L lysine and cultured to passage 4 for experiments. QPCR and for α-actin, GPER, and Vimentin (a fibroblast marker) were used to ascertain the purity of primary VSMC culture.

3.3.2 Lucigenin detection of superoxide production to assess the role of GPER: To determine if there are differences in superoxide generation between direct chronic

inhibition of GPER and the global GPER KO mouse-derived SMCs, we examined the role of the GPER in Ang II and NADPH-induced superoxide production in WT and GPER KO primary aortic smooth muscle cells. Lucigenin is a cell-permeable dye that is specifically reduced by superoxide to emit luminescence at 550 nM, resulting in indirect detection of superoxide in live cells in real time. At 90% confluence cells in p.2 to p.4 were pretreated with GPER antagonist G36 for short term (30 min) and long term (3 days) prior to harvesting for lucigenin analysis. These conditions simulated acute inhibition and genomic loss of GPER, respectively. Cells were trypsinized, washed in warm HPSS, counted twice, and resuspended in 200 µL HPSS prior to lucigenin detection. A final working concentration of 5 µM lucigenin was prepared by adding 50 µL of 25 µM lucigenin per well of a white opaque 96-well plate. After 5 min of dark adaptation at 37°C in a Synergy H1 plate reader, 200 µL of cell suspension is added to the wells, resulting in a final concentration of 5 µM lucigenin. Total luminescence readings were recorded at 37°C every 20 sec for 10 min to obtain a baseline. After taking a 10 min baseline, 100 nM Ang II (final concentration) was added per well and luminescence reads every 20 sec for 20 min. Following Ang II readings, 100 µM NADPH was added and luminescence recorded under the same conditions. In some cases, NOX-specific inhibitor gp91ds-tat (3 µM) will be used to pretreat WT or KO cells for 30 min prior to lucigenin analysis. Luminescence will be calculated as 5-minute interval averages over 20 min of treatment, minus the background reading average at 10 min, and normalized to cell number.

3.3.3 DHE analysis of superoxide levels in live primary VSMCs: DHE was employed as an additional detection method of indirect superoxide detection to confirm

superoxide dynamics between WT and GPER KO SMC and frozen aorta. The dye dihydroethidium, or DHE, is specifically oxidized by superoxide into hydroethidium, which emits red fluorescence. At passage 2 or 3, cells were seeded into an 8-well coverslip chamber coated with 0.01% poly-L lysine. Cells were washed with warm PBS and treated with 5 μM DHE in HPSS for 20 min at 37°C on a rocking platform in the dark. In some cases, cells will be treated with 100 nM Ang II at 10 min into the 20 min DHE incubation. Alternatively, some cells will also be pretreated with 3 μM gp91ds-tat for 30 min prior to DHE or DHE/Ang II incubation. DHE was gently aspirated off and cells were carefully washed twice with warm PBS and viewed within 5-10 min on an epifluorescence scope (Zeiss Axiovert 200M) with a rhodamine filter at 10x.

3.3.4 DHE fluorescence on aortic rings: Following two weeks of Ang II osmotic pump treatment (Alzet Corp. 0.7 mg/kg/day) with placebo or G36 pellet (0.7 mg/kg/day), animals were euthanized (1mg/kg sodium pentobarbital, ip) and the thoracic aorta was excised, cleaned of surrounding fat in ice cold HEPES-PSS (pH 7.4), and embedded in OCT (TissueTek, VWR) over methylbutane prechilled with liquid nitrogen, then stored at -80°C for future use. Tissue samples were cut into 10 μM sections on a cryostat at -30°C, and slides were stored at -80°C for future use. Upon removal from -80°C slides were immediately traced with a PAP pen and immediately incubated with 5μM DHE in HEPES-PSS (pH 7.4) or vehicle (0.01% DMSO in HEPES-PSS) for 15 min at room temperature in the dark. Slides were carefully washed twice and gently wet- mounted with HEPES-PSS, and immediately imaged using epifluorescence at 10X on a Zeiss Axiovert microscope with a rhodamine filter. At least 3-4 aortic slices per animal were

used, and at least 3 images of each slice (i.e, 9-12 images per animal) were used to assess an average representative image.

3.3.5 RNA isolation from cells and tissues: At 80% to 90% confluence in p.2 to 3, primary AoSMCs were scrapped in a T-25 flask with 500 µL of TriZol reagent (Sigma) on ice, and stored at -80°C for future use. RNA was extracted with chloroform (200µL per 1 mL trizol used) and precipitated with 70% ethanol. Samples were subjected to DNAse I digest, and cleaned on RNEasy columns according to manufactures instructions (Qiagen). RNA was eluted into 30 µL RNAse free water, quantified with a NanoDrop and stored at -80°C. Approximately 300 ng of RNA was used as input for reverse transcription synthesis of cDNA.

3.3.6 QPCR to assess GPER genomic effects on NOX expression: To investigate a genomic contribution of GPER to NOX-related gene expression, we resolved differences in NOX transcript expression between WT, KO and GPER-inhibited cells. Untreated WT, GPER KO SMCs and, WT SMC treated with 100 nM GPER specific antagonist G36 or 0.01% DMSO for 3 days were used. Media was replaced with fresh G36 or vehicle every 24 hours. RNA was extracted as described above and quantified by averaging 3 readings on a NanoDrop spectrometer. Approximately 300 ng of RNA per sample was reverse-transcribed (Applied Biosystems RT kit), and diluted at 1:9 in RNAse free ultra-pure water. Subsequent QPCR performed with SYBR green detection, and relative quantification determined using GAPDH as a housekeeping gene. Mouse primers were used for the following targets: NOX1, NOX 2, NOX 4, p22, p47phox, p67phox/NOXA1, AT1R, AT2R, NOXO1, and Rac1. Raw cT values were normalized to GAPDH expression, and comparison of relative transcript expression to

untreated WT SMC as a positive control was calculated and graphed as arbitrary units or percent expression of WT, as indicated.

3.3.7 Western blotting to determine NOX1 levels in GPER KO and WT SMC:

Primary cells derived from WT (BL-6) or GPER KO aorta were lysed in sub-confluent (70-80%) T25 flasks at p.3 on ice using NP-40 lysis buffer supplemented with 1 µg/mL protease inhibitor (sigma), 10% SDS, 0.5% NaF and 0.5% sodium orthovanadate by volume. Lysates were centrifuged at 13,000xg for 10 min at 4°C and soluble supernatant was aliquoted and stored at -80°C. For sample preparation for Western Blot, 20 µg of lysates (determined by Coomassie colorimetric assay) were supplemented with 6X Lamelli buffer and heated to 95°C for ten minutes to denature proteins. In the case of GPER blotting, 30 µg of lysate was used.

Lysates were resolved on a 10% SDS PAGE and blotted onto PVDF membrane for 90 min at 75 volts on ice. Blots were blocked in 3% newborn calf serum (NCS) TBS-T (0.01%Tween-20) at room temperature for one hour. Blots were then carefully cut and blotted for NOX1 (10 µg/mL, polyclonal rabbit IgG, Sigma #4200097), beta actin (millipore, mouse IgG 1:10,000), alpha actin (Abcam mouse IgG 1:1,000) or GPER antibody targeting the second extracellular loop (clone 9368, rabbit IgG 1:5,000) in 3% NCS TBS-T overnight at 4°C with gentle rocking. Blots were washed three times for 5 min each with TBS-T at room temperature, incubated with appropriate HRP-conjugated secondary antibody at 1:5,000 TBS-T for one hour, and washed three times in PBS-T. Blots were developed with Super Signal West Pico Chemiluminescent substrate (ThermoSci cat#34087) and imaged on X-ray film. Quantification of band intensity of NOX1 was ratiometrically determined by taking the densitometry ratio of target band to

corresponding beta actin band in Image-J. When needed, blots were stripped using Restore stripping buffer (Thermoscientific cat#21059) for 20-30 min at room temperature and washed three times in TBS-T. Blot were briefly imaged to ensure no bands remained, and re-blocked for 1 h in 3% NCS TBT-T before reprobing with primary antibodies overnight at 4°C.

3.3.8 Immunofluorescence of NOX1 expression in VSMC: At passage 2, WT or GPER KO SMCs were seeded into a 24-well plate on coverslips with Poly-L lysine. In some cases WT cells will be pretreated 3 μM gp91ds-tat for 30 min as a negative control. Cells will be treated with 100 μM NADPH for 2-20 or 30 min. Cells will be washed twice with PBS, fixed in 4% PFA on ice for 15 min, and blocked in phosphate buffered saline with 0.01% Tween-20 (PBS-T) for 1 hour at 25°C. Cells will be incubated in primary antibody against NOX1 (rabbit IgG Sigma SAB4200097) at a dilution of 0.5 μg/mL in 3% BSA PBS-T overnight at 4°C in a humidified chamber. The next morning, cells were washed three times for 5 minutes in PBS-T, and incubated with FITC conjugated anti-rabbit secondary antibody at 1:500 at 25°C for 1 hour in the dark. Cells were washed three times in PBS-T, washed briefly with nano-pure H_2O, and mounted with vectasheild mounting medium and type 00 coverslips. Slides were imaged with a Leica confocal microscope using 480 nm excitation.

3.3.9 Human Aortic smooth muscle cell culture: Primary Aortic human smooth muscle cells (18 yo male no history of CVD cat#CC2571 cryovial of 500,000) were purchased and shipped from Lonza (Bethesda MD). Cells were seeded at 3,350 cells/cm^2 using the SmGm2 bullet kit of basal growth media with supplements and antibiotics (Lonza cat#CC3182). Cell were passaged at 70-80% confluence and split at

1:3. To ensure quiescence prior to experiments, cells were incubated in serum free media overnight prior to lucigenin experiments or collection of lysate or RNA.

3.3.10 Chronic and acute pharmacologic GPER inhibition G36 in huSMC:

A final concentration of 100 nM G36 was prepared in 4 mL full serum SmGm2 media and human VSMCs cultured in T-25 flasks (to 70% confluence) were washed twice with 1X PBS and replaced with G36 infused media. Fresh G36 was thawed and used to prepare a 100 nM concentration in media to replace old G36 media every 24 hours**.** Prior to experiments, cells were washed and replaced with 100 nM G36 in serum free media overnight. Some flasks were treated with media containing 0.01% DMSO as a vehicle control. For acute G36 doses, cells were washed with warm PBS and treated with 100 nM G36 in serum free DMEM 30 min prior to experiments.

3.3.11 siRNA (siGPER) transfection in human SMC: Small inhibiting RNA targeting GPER mRNA for degradation was used as an additional method to confirm the effects of chronic inhibition of GPER on NOX1 expression in GPER KO and human aortic VMSCs. For each transfection, 200 picomoles of siRNA was combined with 5 µL of lipofectamine 2000 reagent at ambient temperature for 20 min in 1 mL total volume of low serum DMEM, (0.1% FBS) and then added to a T-25 flask containing 2 mL for a final volume of 3 mL of low serum DMEM overnight. This recipe was multiplied for as many T-25 flasks as needed. The next day, flasks were washed and replaced with full media (10% FBS). Human smooth muscle cells were used from passages 3-5 at 80% confluence. After 72 hours post transfection, cells were either subjected to lucigenin

analysis, or harvested for protein using 400 µL of NP40 base buffer. GPER, NOX1 and NOX 2 expression inhibition were assessed by Western blot.

3.3.12 NOX1 adenoviral transduction of KO VSMC: Adenovrial constructs of GFPlaboratoryeled NOX1 and control GFP adenoviral vector were graciously provided by Dr. Bernard Lesengue at Vanderbilt University. Cells were transduced with a viral titer of 300 vpm in 3 mL total volume of low serum (0.1% FBS) DMEM basal media overnight. The next day virus media was washed off and replaced with full DMEM (10% FBS) media. A positive infection was considered to be at least 70% expression at 72 hours, as confirmed by GFP positive cells versus total cells in bright field microscopy images taken every 24 hours.

3.3.13 Animal surgery for Ang II osmotic pump and subcutaneous pellets: Ang II is the principal effector of the renin-angiotensin system and plays a major role in the initiation and progression of hypertension. To test if the absence of GPER abrogates NOX activity, which is a primary mediator of the Ang II effects in the vascular wall, we tested whether GPER deficiency or inhibition prevents hypertensive response to Ang II by reducing the superoxide response. Ang II was delivered by mini-osmotic pumps (Alzet model 2002, Durect Corp), a safe, well-documented/published device for controlled release of research agents. The mini-pump was implanted in the intrascapular region to permit subcutaneous delivery of Ang II (0.7 mg/kg/d) or vehicle (PBS) for 14 days in adult male WT and GPER KO mice. Some WT animals will be simultaneously treated with the GPER-selective antagonist G36 or agonist G-1. A 21-day time-release pellet of GPER-specific antagonist will be inserted 3 days prior to Ang II pump insertion to deliver G36 at 0.7mg/kg/day throughout the study.

3.3.14 Conscious tailcuff systolic blood pressure measurement: To assess a role for GPER in maintaining superoxide and NO in hypertension, it is vital to examine readouts of vascular pathology, such as increased systolic blood pressure (SBP), in an animal model to test the involvement of GPER in mediating the development of hypertension. Tail cuff measurement of BP is beneficial in that heart rate and pressure are not compromised by anesthesia, and readings can be taken over several time points during the hypertensive development. Conscious tail cuff measurement of blood pressure will be assessed using the CODA 6 tail cuff machine (Kent Scientific), a well-published method of BP measurement (205-207). Mice were trained for 3 days prior to beginning the study. Three sessions of five readings per animal (15 sec per reading) were taken daily to calculate average SBP per animal per group preceding to (for baseline) and throughout the two week study. Animals were given one day to recover from each surgery before resuming readings. Daily averages from each animal were compiled to calculate an average daily BP for each treatment group of 4-5 animals. Group averages were graphed at 3-day intervals along the x-axis.

3.4 RESULTS:

Given that majority of previous studies demonstrated a role of GPER in eNOS mediated vasodilation and NO generation (126) (208) (107) (54, 71), we had hypothesized that genomic deletion of GPER (GPER KO) would result in an increased contraction response to Ang II due to reduced NO levels. The experiments characterizing contraction to Ang II in WT and GPER aorta (figure 3.1 of this dissertation) were performed by post-doctoral fellow Dr. Matthias M. Meyer, MD. In stark contrast, myography of thoracic aorta from GPER KO animals displayed a 58%

reduction in contraction to Ang II (**Fig 3.1 A**). These data suggested that GPER KO animals had a reduced or inactive component of Ang II-mediated contraction. To assess the integrity of the Ang II signaling pathway in smooth muscle contraction in GPER KO vessels, several inhibitors relating to the Ang II pathway were used in WT arteries in an effort to mimic the reduced contraction seen in GPER KO arteries. Interestingly, the peptide inhibitor gp91dstat, an inhibitor of the NADPH Oxidase (NOX) catalytic enzyme of the reactive oxygen species superoxide, reduced WT contraction to Ang II to similar levels seen in GPER KO arteries (**Fig 3.1 B**). Furthermore, gp91ds-tat inhibition of NOX had no additive effect on contractile reduction in the GPER KO arteries, implicating a role for GPER in mediating NOX-generated superoxide in Ang II-mediated contraction.

3.4.1 GPER deletion reduces superoxide production to Ang II and NADPH in primary aortic VSMCs. To further characterize superoxide formation in WT and GPER KO vascular smooth muscle cells (VSMCs), we derived primary aortic VSMCs from our GPER KO mice and their WT littermates. Confocal microscopy and western blot confirmed the absence of GPER expression in our KO VSMCs (**Fig 3.2 A,B**), and demonstrated that GPER localized to cytosolic membranes and neither the plasma membrane nor nucleus in WT VSMCs (**Fig 3.2 C**), which coincides with our original report (40). Using these isolated primary VSMCs, we assessed superoxide production to Ang II, which initiates a VSMC signaling cascade to induce NOX catalytic activity, and NAPDH, a direct ligand of NOX catalytic activity. We found that compared to WT, aortic VMSCs from GPER KO animals displayed a near total abrogation of superoxide formation to Ang II and NADPH stimulation, whereas pretreatment of WT VMSCs with

NOX-inhibiting peptide gp91dstat reduced superoxide production to Ang II and NADPH to levels of KO SMCS (**Fig 3.3 A,B**). To further corroborate these data, we employed an additional measure of indirect superoxide detection using dihydroethidium (DHE). Red fluorescence of DHE oxidized by superoxide was similarly diminished in GPER KO VMSC compared to WT VSMCs when challenged with Ang II. Pretreatment of WT VSMC with gp91dstat to inhibit NOX reduced DHE signal to levels seen in GPER KO VSMCs (**Fig 3.4 C)**. In either method of detection, we saw no differences in resting ROS levels between WT and GPER KO VSMCs prior to stimulation with Ang II or NADPH.

3.4.2 GPER deletion subsequently blunts calcium increase to Ang II in VSMCs.

A vital downstream intermediate of ROS-induced contraction is the increase of intracellular calcium. Given that contraction in GPER KO aorta is reduced, and ROS response to Ang II in GPER KO VMSCs is also reduced, we sought to confirm that intracellular calcium was also reduced due to a decrease in ROS production in GPER KO VSMCs. Calcium fluctuations were detected in live cells in real time by the calcium-sensitive dye Indo 1 AM using a PTI spectrofluorimeter to measure the ratio of calcium-bound to unbound Indo1 AM. Upon stimulation with Ang II (100 nM) the ratio of calcium-bound to unbound Indo1 AM increased in WT, but this response was significantly abrogated GPER KO VSMCs, indicating there was a lack of intracellular calcium increase to Ang II, likely as a result of reduced ROS production to Ang II (**Fig 3. 4**). The loss of calcium response in WT cells was reduced by the NOX-inhibiting peptide gp91ds-tat, indicating that a loss of ROS production was capable of reducing the calcium response. The calcium response to purinergic receptor activation with ATP

(an alternative, Ang II-independent pathway), was robust in both WT and GPER KO VSMCs, indicating the loss of calcium increase in GPER KO VSMCs may be specific to the Ang II and NOX signaling pathway(s). These data indicate that the loss of calcium response in GPER KO VSMCs is likely in part due to a loss of ROS response to Ang II, which also coincides with the reduced contraction response to Ang II seen in GPER KO aorta.

3.4.3 Chronic, but not acute GPER inhibition reduces superoxide production to Ang II in VSMCs. To elucidate a mechanism of GPER in regulating NOX-mediated superoxide production, we designed an experiment to determine if the role of GPER was in rapid activation and/or assembly of NOX subunits, or in genomic expression of NOX subunits. To this end, we employed pharmacologic inhibition with GPER-specific antagonist G36 at 100 nM for three days or 30 min in primary mouse WT VSMCs prior to lucigenin detection of superoxide production to Ang II and NADPH stimulus. We found that chronic, but not acute inhibition of GPER reduced superoxide production in WT VSMCs to GPER KO levels (**Fig 3.5**).

3.4.4 GPER deletion results in decreased NOX1 and p22phox expression in VSMCs. Given that GPER activity exerted long-term, genomic related changes in NOX signaling, we assessed NOX subunit expression in WT and GPER KO VMSCs by QPCR and Western blot. QPCR showed approximately 50% reduction in expression of NOX1, the catalytic subunit and p22phox, the plasma membrane anchoring unit of NOX1 in KO VSMCs compared to WT VSMCs. QPCR showed that there was a loss of approximately half the expression of NOX1 and p22phox in GPER KO VSMC compared to WT, and that RNA levels of other components of NOX and Ang II signaling were

unaffected (**Fig 3.6**). We followed up RNA data by examining protein expression levels by Western blotting and found that indeed, there was a reduction of approximately 55% of NOX1 protein in GPER KO VSMCs compared to WT (n=4 per genotype, p=0.027)(**Fig 3.7**).

3.4.5 GPER inhibition results in decreased superoxide production and NOX1 expression in human VSMCs. To confirm that our observations were not an anomaly of the GPER KO mouse model, we sought to corroborate these data in VSMCs of different species. In the immortalized rat aortic smooth muscle cell line A7r5 we found that chronic (3 day), but not acute (30 min) inhibition of GPER also resulted in reduced superoxide generation to Ang II and NADPH challenges via lucigenin detection (data not shown). Critically, we established that in primary human aortic smooth muscle cells, chronic (3 day) but not acute (30 min) inhibition of GPER using both pharmacologic (G36) and genomic (siRNA) approaches resulted in a reduced superoxide response to Ang II and NADPH (**Fig 3.8 A,B**). Furthermore, chronic inhibition of GPER in human aortic VSMCs also resulted in decreased NOX1 protein expression (**Fig 3.9**). These data infer that GPER signaling regulates NOX1 expression in human VSMCs, thereby contributing to regulation of VSMC contraction to Ang II via NOX1 expression.

3.4.6 GPER deletion results in reduced expression of JunB, a vital mediator of NOX1 expression. To hypothesize a mechanism of GPER regulation in NOX1 expression, we utilized previous reports demonstrating that the transcription factors JunB and MEF2B were involved in upregulating NOX1 transcription in an AP-1 dependent manner (209) (210) (211, 212). Both expression and activation of JunB and MEF2B in multiple cell types is mediated by ERK1/2 (213-215), which is a known

secondary kinase messenger of GPER signaling via cross activation of EGFR. We hypothesized that GPER- mediated ERK1/2 activation leads to increased JunB and MEF2B expression, which in turn increases NOX1 transcription. QPCR of WT and GPER KO VSMC revealed that JunB expression was decreased in KO VSMCs compared to WT VSMCs (n=4 $p<0.05$) (**Fig 3.10**). Taken together, these data implicate that GPER deletion results in decreased JunB expression, which may reduce NOX1 transcription and reduce ROS-mediated Ang II contractions in VSMC.

3.4.7 reintroduction of NOX1 restores superoxide response to Ang II in VSMCs of GPER KO mice. We sought next to determine if reintroduction of NOX1 could restore the superoxide response in GPER KO VSMCs. Constructs of Adenoviral NOX1 with GFP or empty adenoviral vector with GFP were transfected into GPER KO VSMC, and superoxide response to Ang II and NADPH we measured three days later. Indeed we found that restoration of NOX1 by adenoviral transduction restored superoxide production to Ang II in GPER KO VSMCs (**Fig 3.11**). There was no restoration of superoxide signal by lucigenin in KO VSMCs transfected with GFP alone.

3.4.8 GPER inhibition or deletion reduces or blocks the hypertensive response to Ang II in mice. To test the physiologic efficacy of our cellular studies, we employed a rodent model of hypertensive challenge (**Fig 3.12**). We hypothesized that pharmacologic inhibition (G36 infusion) or genomic deletion (GPER KO) of GPER would abrogate hypertensive onset in response to Ang II challenge by reducing the NOX mediated superoxide response and ROS accumulation resulting from RAS-mediated hypertension. WT mice were fitted with a subcutaneous placebo pellet or G36-releasing pellet (0.7mg/kg/day) for three days prior to insertion of a subcutaneous

osmotic pump releasing Ang II peptide (0.7mg/kg/day) or PBS. GPER KO mice also received a placebo pellet and Ang II or PBS osmotic pump. Systolic blood pressure (SBP) was monitored by conscious tailcuff readings from three days prior to pellet infusion up to two weeks post-insertion of osmotic Ang II pump. Mice were euthanized after two weeks of Ang II treatment and their tissues were taken for molecular and superoxide analysis, and vasoreactivity. In WT mice receiving placebo pellet and Ang II pumps, SBP increased by 30 mm Hg, indicating a hypertensive response to Ang II. In WT mice receiving G36 prior to Ang II pump infusion, this hypertensive onset was partially abrogated by ~30 mm Hg. In GPER KO animals receiving Ang II osmotic pumps, there was no hypertensive onset (**Fig 3.13**). Taken together, these data indicate that GPER is significant in mediating the hypertensive onset to Ang II in a mouse model.

3.4.9 GPER inhibition reduces superoxide production to Ang II in aortic rings of mice. To determine if this diminished hypertensive response was due to a lack of superoxide production to Ang II, superoxide levels in aortic rings from each treatment group were measured by lucigenin and DHE. Both methods of ROS measurement showed reduced superoxide levels in aortic rings from GPER KO animals and WT animals receiving pharmacologic inhibition of GPER prior to Ang II hypertensive challenge (**Fig 3.14**). We made the critical observation that there was no significant difference in ROS levels (as detected by DHE) between GPER KO and WT animals receiving PBS pumps, implicating that GPER deletion protects from Ang II hypertension by abating the ROS response.

3.4.10 GPER inhibition or deletion reduces NOX1 expression in aortic rings of mice. Levels of NOX1 protein were measure by immunofluorescence in mouse aortic

rings from different treatment groups. Confocal microscopy of FITC-labeled NOX1 in aortic rings indicated elevated NOX1 protein expression in rings from WT animals receiving placebo pellet and Ang II osmotic pump. Conversely, we saw reduced NOX1 expression in aortic rings from GPER KO animals and WT animals infused with GPER specific antagonist G36 prior to Ang II challenge (**Fig 3.15**). The data from this animal study demonstrate a mechanism whereby inhibition of GPER prevents hypertensive onset by Ang II by mitigating superoxide production through genomic inhibition of NOX1.

3.5 CONCLUSIONS AND DISCUSSION:

Our data demonstrate that GPER regulates NOX1 expression (but not acute activation) in vascular smooth muscle, thereby indirectly regulating superoxide production to Ang II. This genomic regulation may be mediated by GPER activation of ERK1/2 to mediate JunB expression, which in turn promotes NOX1 expression in vascular smooth muscle cells of multiple species (**Fig 3.16**).

A critical conclusion of this report is that GPER activity was required for the physiologic hypertension response to Ang II challenge in mice. Hypertension onset due to increased activity of the renin-angiotensin aldosterone system (RAAS) is commonly observed in the clinic. To this end, multiple successful drugs employed to counter hypertension in the clinic are inhibitors of the RAS, such as ACE 2 and renin blockers (blocking the generation of Ang II peptide) or angiotensin receptor blockers (blocking the signal transduction of Ang II in cells) (216-219). Our data suggest that another feasible route of hypertension treatment may be the reduction of NOX1

expression via pharmacologic inhibition of GPER, which will to reduce the NOX superoxide response to Ang II, and abrogate hypertension.

The NOX blocking peptide gp91dstat was originally reported by the Pagano laboratory in 2011 (University of Pittsburgh) as effective in blocking NOX formation and catalytic activity to prevent superoxide formation (198). We utilized this peptide in to mimic the loss of contraction and superoxide accumulation in WT aorta and VSMCs seen in GPER KO smooth muscle, and to demonstrate that loss of GPER recapitulates the loss of superoxide seen when NOX is inhibited. The gp91ds -tat peptide prevents NOX activation by blocking the interaction of the scaffolding p22phox subunit with the B loop of the NOX catalytic subunit at the plasma membrane. This peptide was originally tested for preventing superoxide accumulation in cell-free systems and HEK 293 cells transduced to overexpress different NOX isoforms (NOX1,2,4). Although gp91dstat has been described as NOX 2-specific (198), the conservation of the B-loop docking site between NOX isoforms (220) brings into question the specificity of gp91ds-tat targeting, thus the specificity of the peptide is debated in the literature (221). Our data here indicate that the peptide blocks superoxide response in SMCs, of which NOX1 is the primary isoform. Given that our studies were performed in isolated smooth muscle cultures, and that the pathway we tested in ROS development (Ang II) is an endothelium- independent, we suspect that the gp91dstat peptide inhibited NOX1 activity in our experiments.

We demonstrate here that GPER contributes to NOX-mediated superoxide production in response to Ang II and NADPH, and it followed that inhibition of GPER abrogated increases in systolic pressure associated with Ang II infusion by reducing

superoxide production in response to Ang II peptide. We and others have reported that GPER-specific agonist G-1 promotes vasorelaxation through eNOS activation and reduction of oxidative damage (54, 71, 107, 126, 208). However, there are several conflicting reports where G-1 infusion did not prevent hypertension (130). Given the reported findings of White *et al*. (105), we hypothesized that overproduction of ROS by the Ang II infusion model may promote NO scavenging into peroxynitrate radical ($ONOO^-$). This ROS-enriched environment can decouple tetrhydrobiopterin and eNOS, and lead to even greater augmentation of ROS that will be further stimulated by constitutive activation of uncoupled eNOS by G-1. We therefore reasoned that GPER agonist (G-1) infusion may have only moderate or early effects in abrogating hypertension, and that these effects would be lost at later time points due to ROS saturation from Ang II infusion and eventual eNOS uncoupling, which will magnify ROS accumulation. Studies by Lindsey *et al.* employing G-1 to prevent a salt induced hypertension in mRen2 rats showed reduced oxidative stress and proteinuria in the kidney, but no protection from hypertension (151). While these studies indicate that G-1 likely decreased ROS at the kidney by increasing eNOS activity, we propose that the persistence of hypertension may stem from GPER-mediated NOX1 expression increases resulting in ROS accumulation.

In perspective, this study is significant and progressive in several aspects. First, it implicated a novel mechanism by which estrogen contributes to ROS production in healthy vascular function. This finding has implications on the divergent effects estrogen replacement therapy has had on cardiovascular function and stroke, as evidenced by the deleterious outcome of estrogen replacement in the Women's Health

Initiative in the 1990s (143). We propose here a mechanism by which estrogen replacement in post-menopausal women may conversely increase ROS production. This scenario is further supported by the notion that post menopausal endothelium is dysfunctional and has lower basal NO levels and uncoupled eNOS, thereby compounding ROS accumulation. ROS accumulation can occur both through hyperactivation of NOX activity and by increasing NOX expression to sustain ROS production in the smooth muscle. The data we report here may suggest that estrogen replacement therapy could be supplemented with GPER-specific antagonist G36 to reduce vascular oxidative stress in E2 replacement as a possible therapeutic.

Fig 3.1

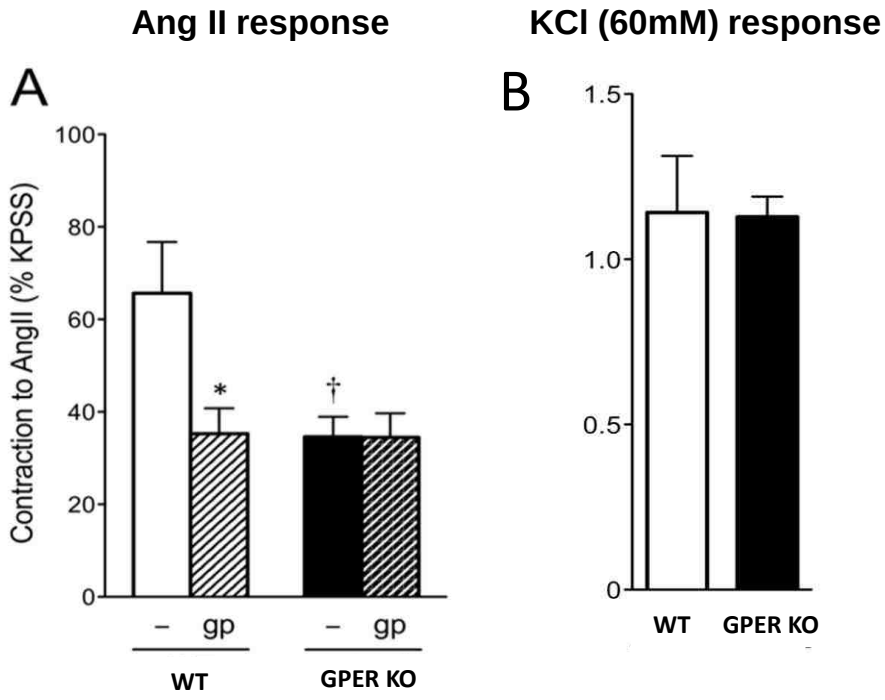
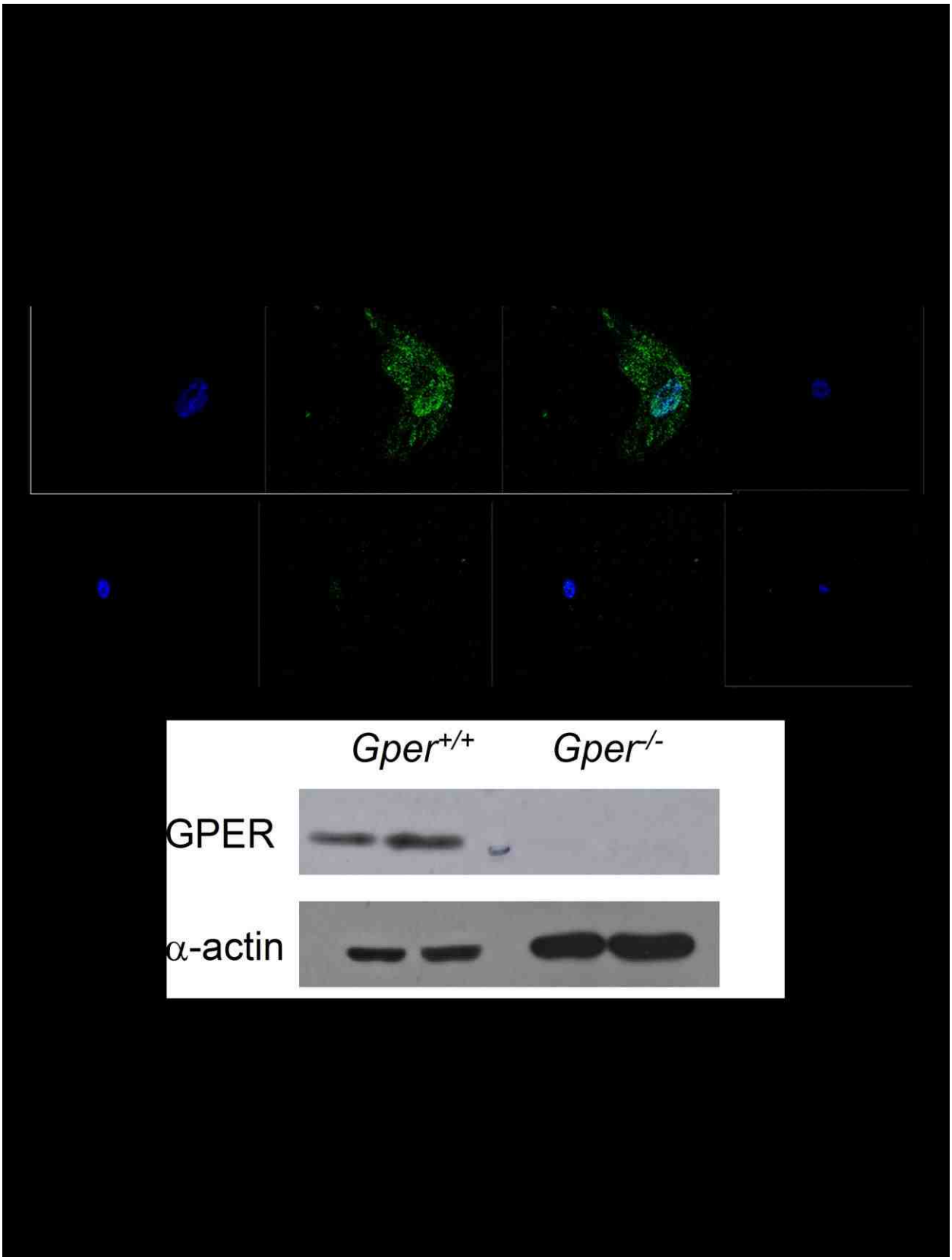


Fig3.1 GPER is required for angiotensin II-induced, Nox-mediated contraction. Contractile responses to Ang II are reduced in GPER-deficient thoracic aorta. The NADPH oxidase inhibitor gp91ds-tat (gp) acutely decreases contraction to Ang II only in WT arteries (A). Contraction in response to KPSS is unaltered by GPER deficiency (B). *n*=4-5/group, **p*<0.05 *vs.* untreated, †*p*<0.05 *vs.* WT. This data was borrowed with permission from Matthias Meyer, MD as preliminary data to this dissertation.


Gper+/+
Gper-/-
GPER
α-actin

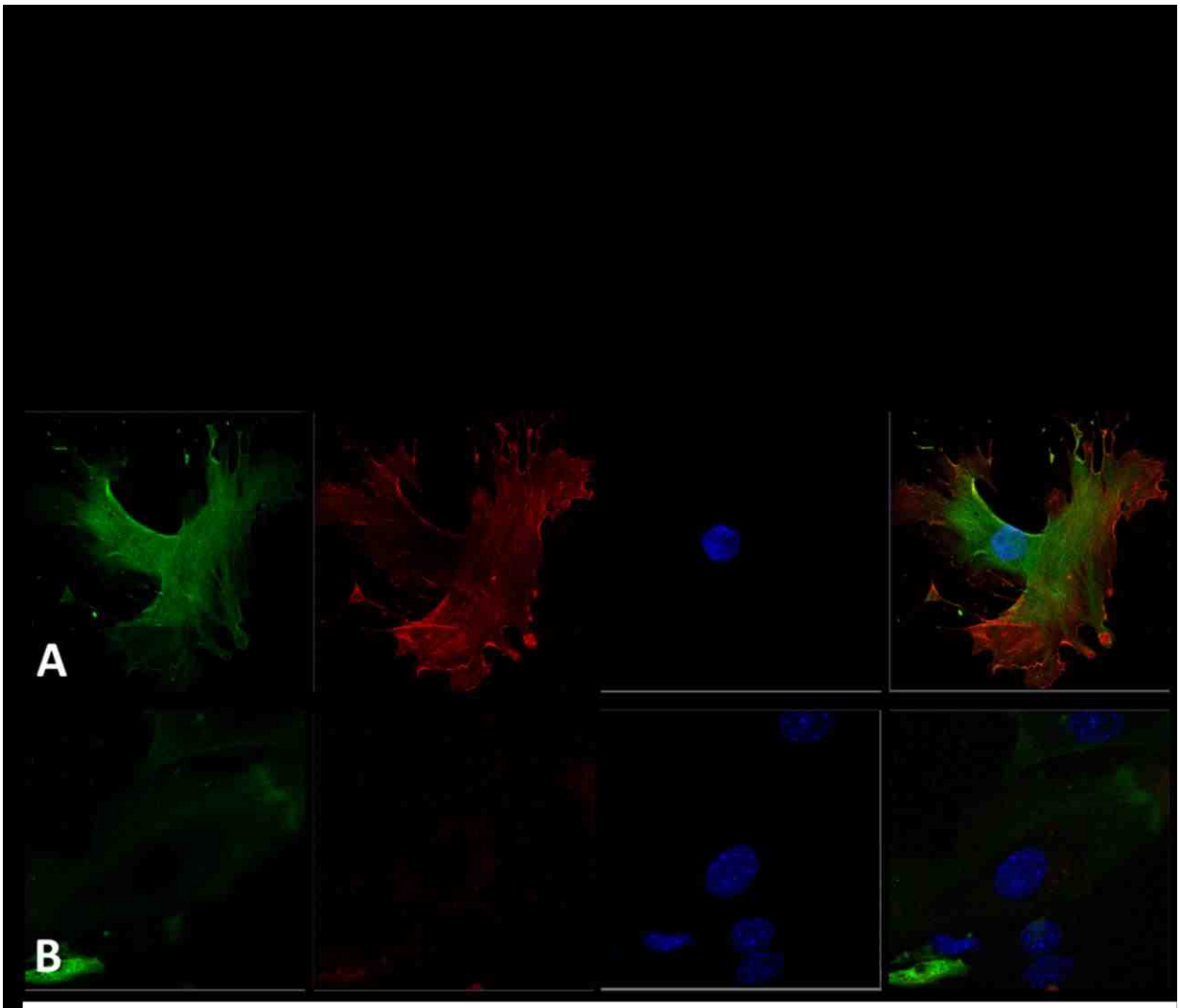
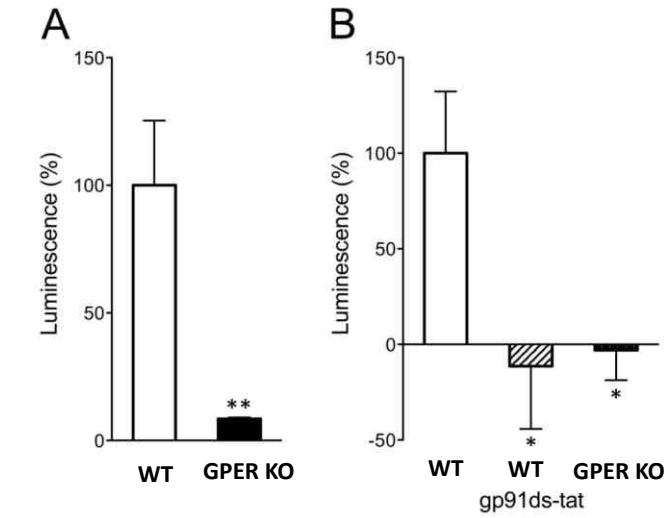


Fig 3.2 C GPER is expressed at intracellular membranes: Primary aortic smooth muscle cells from WT mice in p.2 were stained with GPER antibody targeting the second extracellular loop under permeabilizing (supplemented with 0.01% tritonX-100) (**A**) or non-permeabilizing (PBS with 3% normal goat serum) conditons (**B**). Alpha-actin was used as a positive control for intracellular marker. GPER-targeting antibody only stained cells under permeabilizing conditions, suggesting that GPER is predominantly localized to cytosolic membranes and not the plasma membrane

Fig 3.3



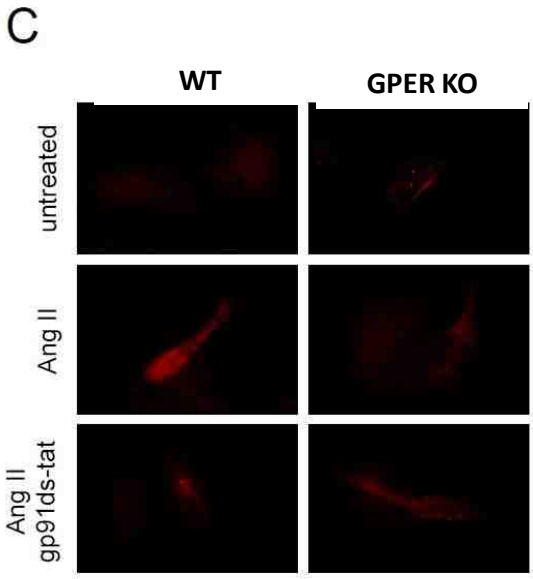


Fig. 3.3 GPER deficiency markedly reduces Nox activity and abolishes angiotensin II-induced, Nox-dependent superoxide formation: NADPH-stimulated superoxide production was markedly reduced in GPER-deficient VSMC (A). Ang II (in the absence of exogenous NADPH) produced a small amount of superoxide in WT cells, which is abolished by the Nox-inhibitor gp91ds-tat and deletion of GPER (B). Luminescence units were normalized to VSMC count. *n*=3-5/group, **p*<0.05, ***p*<0.01 *vs*.WT. C) Detection of superoxide with DHE also indicated a significant reduction in superoxide response to Ang II in GPER Ko VSMCs compared to WT. NOX inhibition reduced Ang II induced superoxide production in WT VSMCs to levels of GPER KO.

Fig 3.4

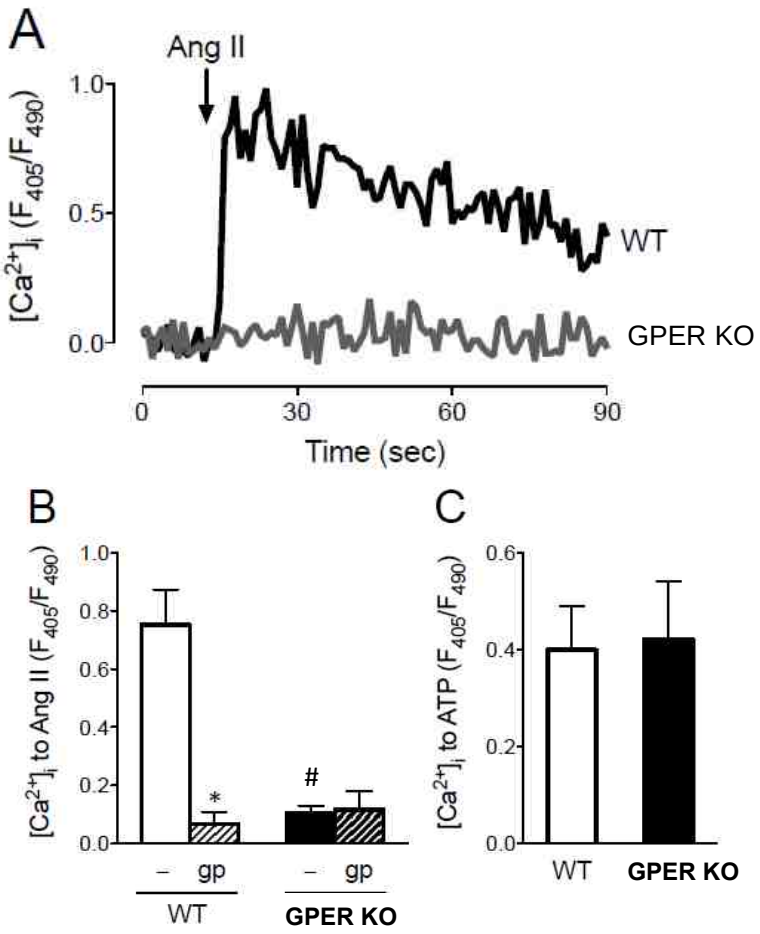


Fig 3.4 GPER KO VSMCs exhibit decreased intracellular calcium response to Ang II: Superoxide increase results in calcium increases in VSMCs. Indo1 AM detection of free intracellular calcium revealed that GPER KO VSMCs had decreased intracellular calcium response to Ang II compared to WT, and that inhibition of NOX using gp91dstat in WT VSMCs reduced intracellular calcium responses to levels observed in GPER KO VSMCs. ATP induced intracellular calcium increases in both WT and GPER KO VSMCs, indicating the abrogated calcium response to Ang II is likely pathway specific. A) Raw trace of bound to unbound Indo1AM ratios in WT and GPER KO VSMCs. B) quantitative summary of raw traces. C) Quantitative summary of ATP-induced intracellular calcium increase. #p<0.05 vs. WT control,*P<0.05 vs. w/o gp

Fig 3.5

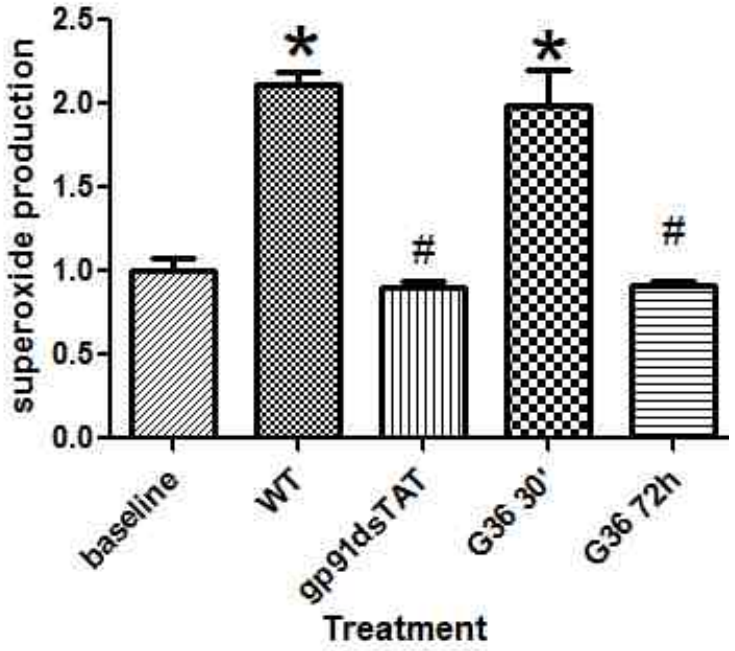


Fig 3.5 Chronic but not acute GPER inhibition reduces the superoxide response to Ang II in WT VSMCs: WT VSMCs in p.3-4 were cultured for 3 days (72h) in 100 nM G3 or vehicle (0.01%DMSO) and treated with G36 (100 nM) 30 min prior to harvest for lucigenin detection of superoxide response to Ang II. As a negative control, some cells were prereated with 3 uM gp91dstat to inhibit NOX signaling for 30 min prior to lucigenin experiments. 72 hour, but not 30 min of G36 pretreatment reduced superoxide response To vehicle levels. To compare, 30 min of NOX inhibiting peptide also fully reduced the superoxide response to Ang II in WT VSMCs. *P<0.05 vs. WT, # P<0.05 vs. veh, n=4-6 per treatment

Fig 3.6

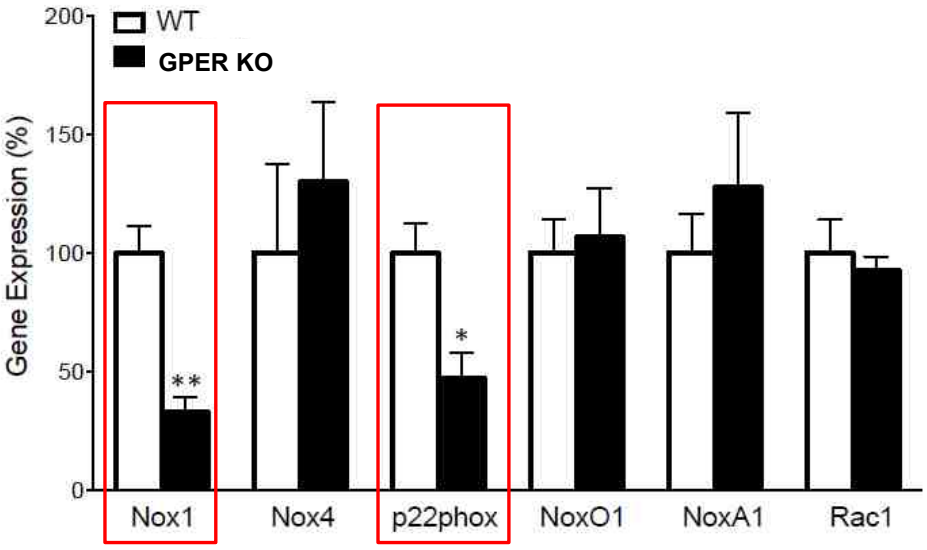


Fig 3.6 GPER regulates Nox1 and p22phox gene expression in VSMC: Deletion of the GPER gene markedly reduces Nox1 and p22phox gene expression, which may explain the lack of superoxide production and arterial contraction. n=6/group, ***p*<0.01 *vs.* WT.

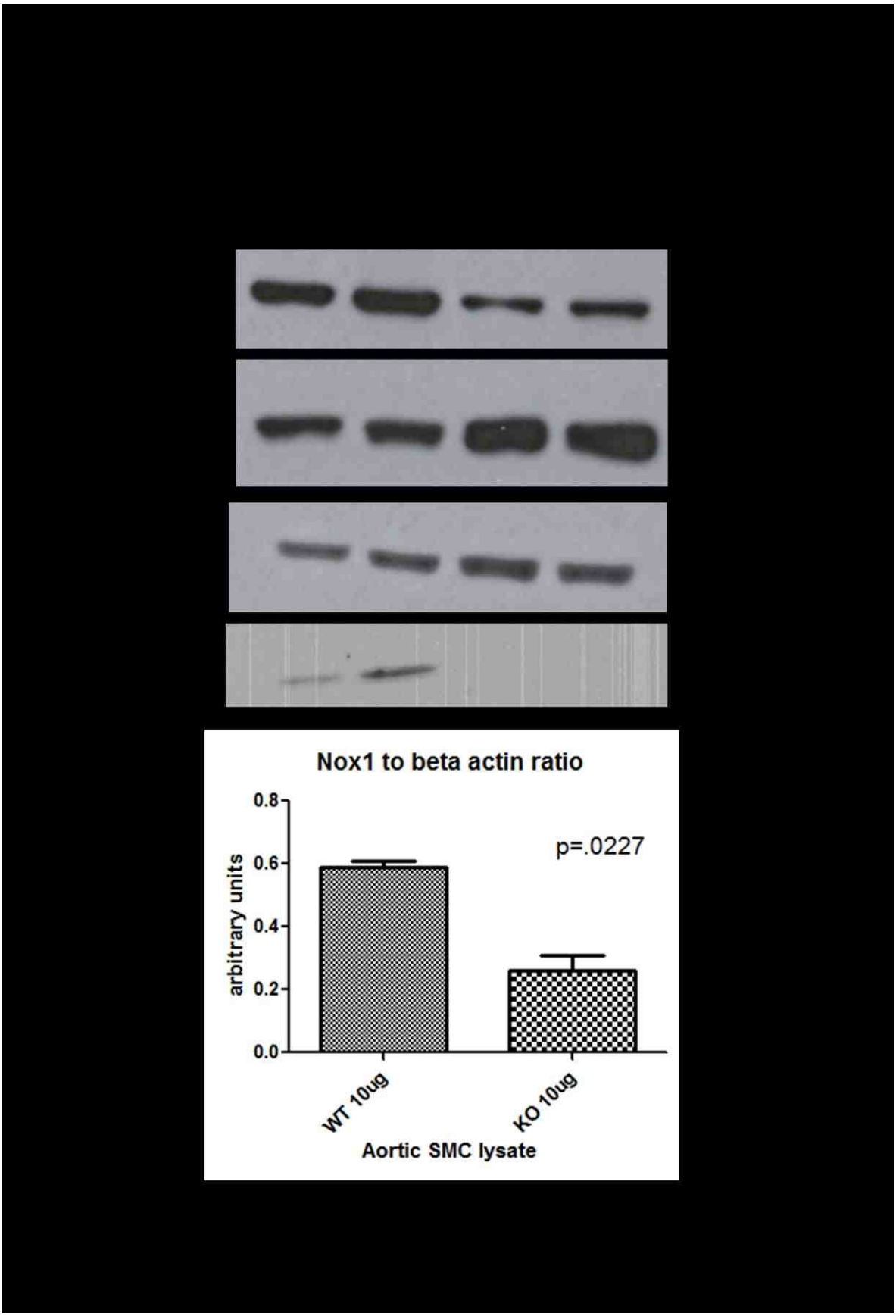

Nox1 to beta actin ratio
p=.0227
arbitrary units
0.8
0.6
0.4
0.2
0.0
WT 10ug
KO 10ug
Aortic SMC lysate

Fig 3.8

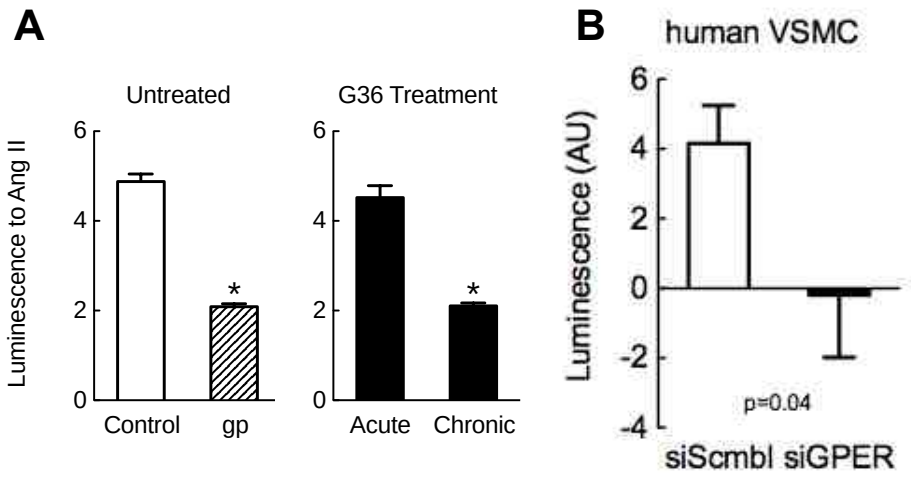


Fig 3.8 Chronic but not acute inhibition of GPER results in reduced ROS production to Ang II: A) Aortic smooth muscle cells from rat A7r5, and primary human aortic smooth muscle cells were treated for three days or 30 min with 100 nM G36 prior to harvest for lucigenin detection of ROS production to Ang II. In some instances cells were treated with NOX inhibitor gp91dstat as a control for ROS inhibition. Chronic inhibition of GPER with 3 days (changed every 24 h) but not 30 min reduced superoxide response to Ang II in aortic SMCs from all species. **B**) genomic inhibition of GPER with siRNA also reduced ROS response to Ang II.

*p<0.01 vs. WT

Data shown above for human aortic SMC only

Fig 3.9

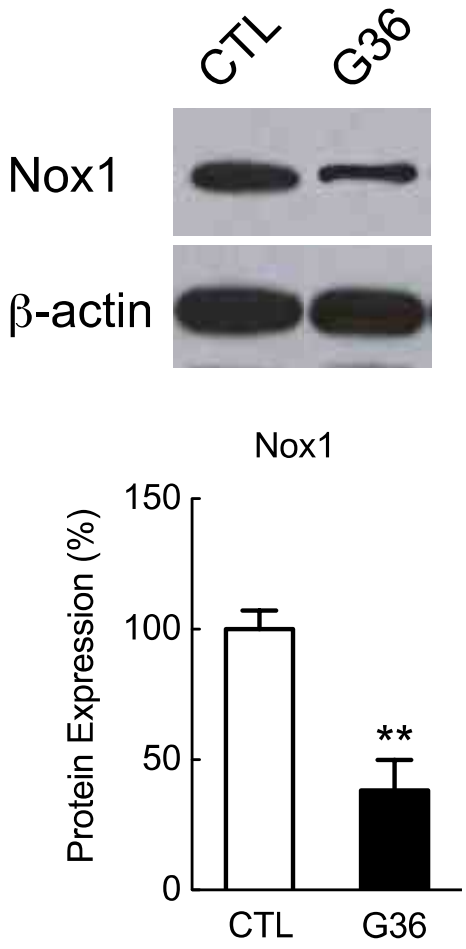


Fig 3.9 Chronic GPER inhibition reduces NOX 1 protein in human VSMCs: Human aortic VSMCs were treated with GPER-specific antagonist G36 (100 nM) or vehicle (0.01% DMSO) for three days and lysed for total protein and blotted for total NOX 1 protein expression. VSMCs treated with G36 had a reduction in NOX 1 protein levels of approximately 40%.

Fig 3.10

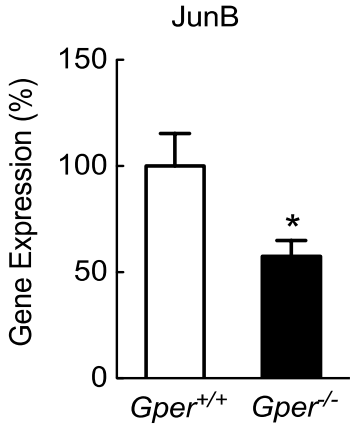


Fig 3.10 JunB expression is decreased in VSMC of GPER KO mice: QPCR detected a 40% reduction in JunB expression in primary VSMCs harvested from GPER KO animals compared to WT VSMCs. RNA was isolated at p.3, n=4 *P<0.05

Fig 3.11

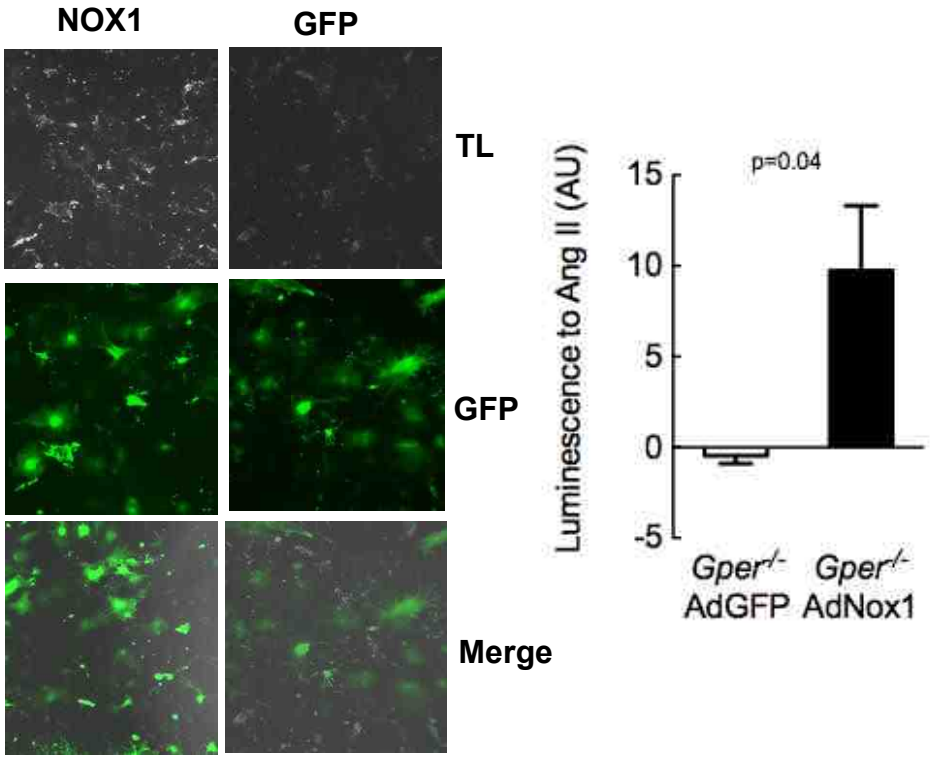


Fig 3.11 reintroduction of NOX 1 restores ROS response to Ang II in GPER KO VSMCs: GPER KO VSMCs were transduced with Adenoviral constructs of GFP-labeled NOX 1 or empty GFP vector. Viral expression of NOX 1 GFP reestablished the superoxide Response to Ang II in GPER KO VSMCs, but adenoviral GFP alone did not. TL= transmitted light

Fig 3.12

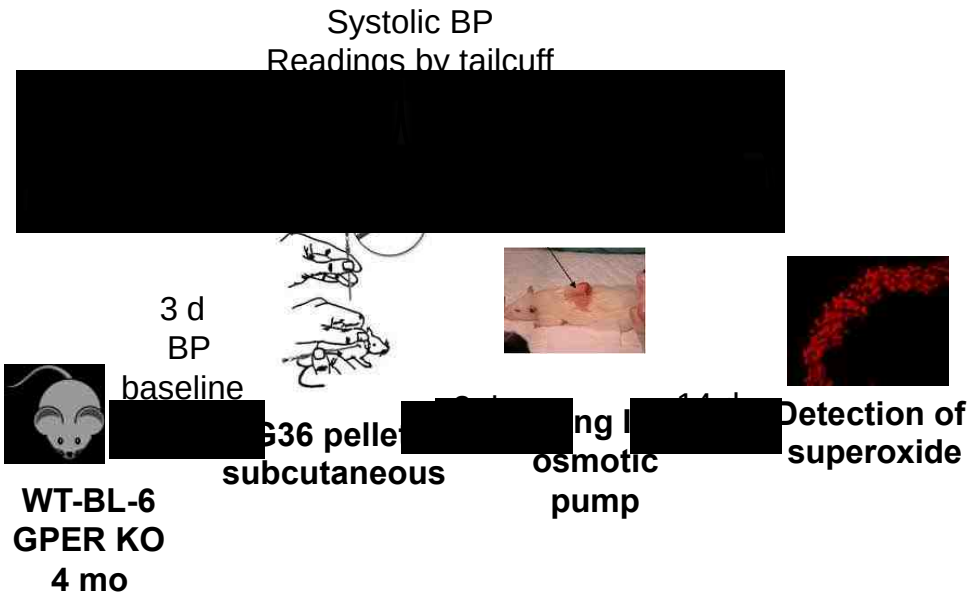


Fig 3.12 Experimental design of mouse hypertension: Mice were trained for one week to sit calmly for conscious tailcuff readings of systolic blood pressure (SBP), and then a baseline SBP reading was taken for three days. After baseline SBP was established, mice were given a subcutaneous pellet releasing G36 or placebo pellet at the left hind leg, and were allowed to recover for three days while still receiving SBP readings. Mice were then fitted with an osmotic pump releasing Ang II or PBS at the intrascapular region. SPB readings were taken for two weeks following osmotic pump insertions. At two weeks, animals were euthanized and their tissues were harvested to assess ROS accumulation and for additional molecular analysis.

Fig 3.13

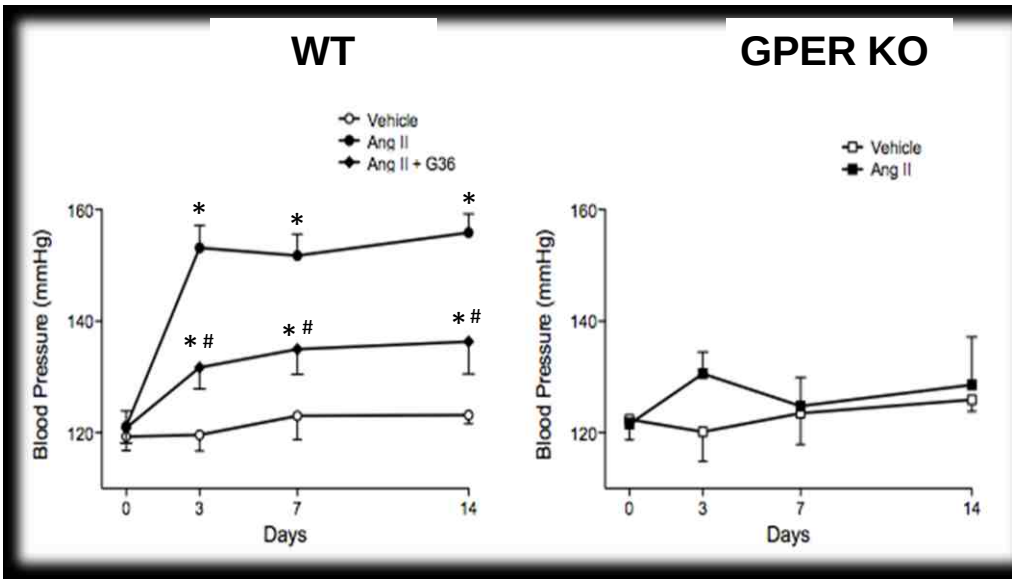


Fig 3.13 GPER deletion and inhibition reduces hypertension response to Ang II infusion in mice: A) WT mice receiving subcutaneous pellets releasing GPER-specific inhibitor G36 (diamonds) had an abrogated hypertension response to Ang II infusion by osmotic pump compared to mice with placebo pellet (circles). B) GPER KO mice did not exhibit hypertensive onset to Ang II infusion (black squares).
*p < 0.05 vs. veh, #p< 0.05 vs. WT AngII

Fig 3.14

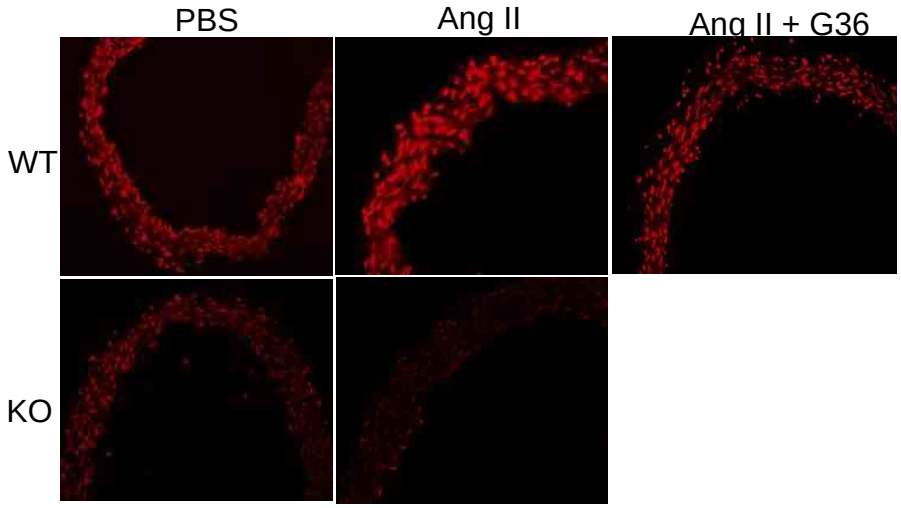


Fig 3.14 Loss of GPER reduces superoxide deposition in aorta of mice receiving Ang II hypertensive challenge: Thoracic aorta slices fro mice treated with G36 or placebo or Ang II or PBS vehicle osmotic pumps were treated with DHE to detect superoxide levels. Thoracic aorta from mice treated with GPER specific antagonist G36 had reduced superoxide compared to thoracic aorta from WT animals that received Ang II infusion and became hypertensive. Thoracic aorta from GPER KO animals receiving Ang II did not show an increase in superoxide. Compared to negative controls (PBS pump). N=4-5animals per group, 5 aorta slices per animal.

Fig 3.15

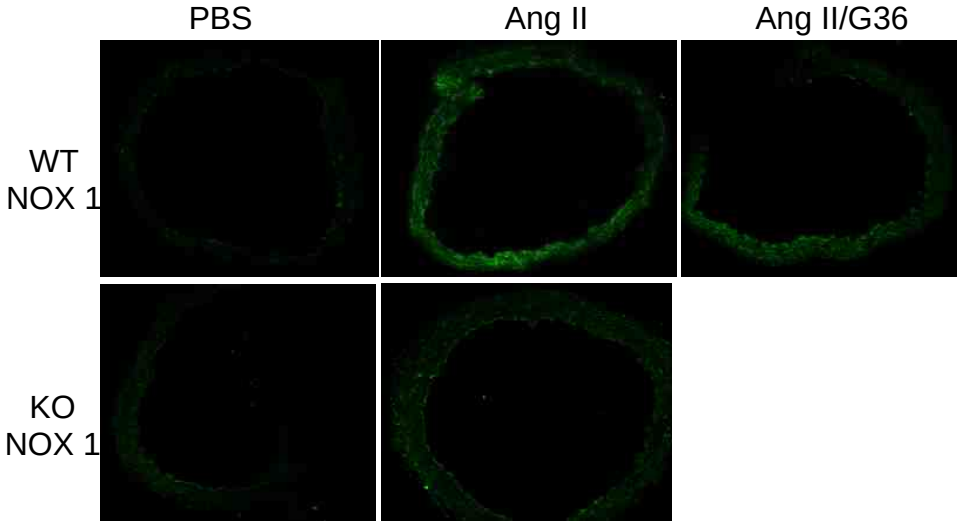


Fig 3.15 GPER inhibition reduces NOX 1 levels in a model of Ang II hypertension: WT,GPER KO mice and WT mice receiving GPER antagonist G36 were challenged for two weeks with Ang II osmotic pump and their aortas were harvested and stained for NOX 1 protein expression. WT animals treated with Ang II had increased NOX 1 expression, while WT animals receiving both GPER antagonist G36 and Ang II had a reduction in NOX 1 expression compared to WT placebo. Thoracic aorta from GPER KO mice receiving Ang II did not exhibit an increase in NOX 1 protein. NOX 1 basal expression was no different between WT and GPER KO animals receiving vehicle (PBS) infusion. N=4 animals per group, 3-4 slices per animal.

Fig 3.16

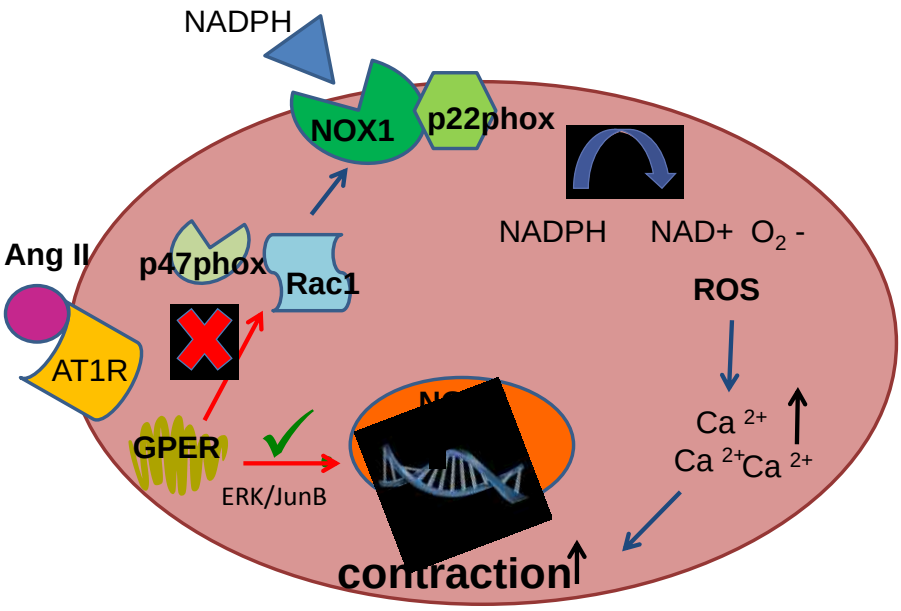


Fig 3.16 Summary: GPER mediates chronic NOX 1 expression, but not acute activation. GPER may mediate NOX 1 expression through ERK1/2 activation to induce JunB expression and activation, which regulates transcriptional activation of NOX 1.

CHAPTER FOUR:

CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Overall Discussion and Future Directions:

The complex role of estrogen in vascular function is not yet fully understood. While E2 has been shown to acutely increase vascular dilation through eNOS activation and increase eNOS expression (98, 222), studies of chronic E2 treatment both in research animals and in the clinic yield mixed results regarding blood pressure effects (223) (224) (143) (225) (128). It has been shown that the G protein-coupled estrogen receptor, GPER, also mediates the vasoprotective effects of estrogen (128, 130), and is likely needed in collaboration with signaling from ERα for eNOS activation and NO production. Furthermore, a role of GPER in mediating vasoprotective effects of SERMS and SERDs has not yet been characterized in the vasculature. Furthermore, the effects GPER may have in vascular smooth muscle regulation of vascular reactivity are unclear. To address these gaps in knowledge, we sought to elucidate the contribution and mechanism(s) of GPER in regulating eNOS activation in endothelial cells, and to characterize the role of GPER signaling in vascular smooth muscle cell contraction.

Utilizing a telomerase immortalized endothelial cell line (TIVE), we demonstrated that the GPER-specific agonist G-1 stimulates eNOS activation through downstream phosphorylation of ser1179, resulting in nitric oxide (NO) production. When GPER was inhibited using the GPER-selective antagonist G36 and siRNA targeting GPER, there was a decrease in NO production in response to E2, but not to acetylcholine, suggesting that GPER is necessary to mediate the full effects of E2 on eNOS activation, but is not necessary for eNOS activation in response to other (non-estrogenic) vasodilators. The contributing role of GPER in E2-mediated vasorelaxation was confirmed with myography experiments on mouse thoracic aorta. Vasorelaxation to E2

in thoracic aorta from GPER KO mice was reduced by ~50% compared to wild type. Importantly, there was no reduction in acetylcholine-mediated vasorelaxation in GPER KO aorta compared to wild type, indicating that GPER is involved specifically in the E2-mediated mechanism of eNOS-dependent vasorelaxation, but is not involved in downstream eNOS-mediated vasorelaxation. Inhibition of EGFR and PI3K/AKT reduced NO production to both E2 and G-1, implicating these intermediate signaling kinases in E2-mediated eNOS activation. Additionally, inhibition of other known signaling kinases, including c-Src and ERK1/2 also reduced NO production in response to E2 and G-1, indicating roles for these kinases in ERα- and GPER-mediated vasodilation. It is likely that ERα and GPER are capable of regulating eNOS at multiple residues in addition to ser1179, such as c-Src-mediated phosphorylation of the activating residue Y81 and ERK1/2-mediated dephosphorylation of the inhibitory residues Thr 495/617 (226, 227) (228).

Given that the SERMs tamoxifen and raloxifene as well as the SERD ICI182,780 have been shown to signal through GPER in multiple cell types (68, 74), we postulated that GPER may be responsible for mediating vasorelaxation in response to these SERMs through eNOS activation. Indeed, when we measured NO production in response to these “anti-estrogens” in TIVE cells, the generation of NO in response to ICI182,780 and tamoxifen was reduced by approximately 35% in TIVEs pretreated with GPER antagonist G36. Although significant, these results leave open the possibility that additional (non-GPER-mediated) mechanisms are involved in the NO response to these compounds.

From the data presented in Aim 1 (Chapter 2), we conclude that GPER indeed contributes to estrogenic activation of eNOS for NO production, and that inhibition of GPER reduces NO production to E2 as well as to SERMs and SERDs. As inhibition of GPER by pharmacologic antagonism (G36) or global deletion (GPER KO) reduces NO production and vasorelaxation to E2 and SERMs/SERDs, it follows that GPER signaling is responsible for partially contributing to the entirety of the E2 response of eNOS. We also conclude that GPER mediates eNOS activity acutely but does not modify long-term eNOS expression as ERα does. We demonstrated that the signaling pathway(s) involved in GPER-mediated eNOS activation are dependent on transactivation of the EGFR and PI3K/AKT signaling pathway, as well as through parallel pathways of ERK1/2 and c-Src activation, which modify additional regulatory residues on eNOS. Therefore, GPER plays a pivotal role in mediating the vasoprotective effects of estrogen by contributing to eNOS activation.

Future directions for Aim 1 include additional characterization of GPER signaling pathways for the different eNOS regulatory residues such as the activating residue Tyr81 and the inhibitory residues Thr495 and Ser617 and 116. Inhibition of c-Src and ERK1/2 showed decreased NO production to both E2 and G-1, which suggests that estrogen receptor mediated eNOS activation may occur through multiple pathways in addition to EGFR and PI3K/AKT activation, which is a novel finding of this study meriting further investigation.

We also presented the interesting observation that GPER inhibition is unable to fully block NO production to SERMs and SERDs. As the SERMs tamoxifen and the SERD ICI 182,780 are known to have antagonistic actions against classic ERs, our data

implicate that these compound may have ER-independent signaling pathways, at least in endothelial cells, that result in increased NO production. This characterization of SERMs in endothelial function here postulates the critical consideration that SERMs and SERDs, while usually only thought of in the narrow aspect of ER signaling modifications, must be considered more broadly for their cross-activity effects in cell signaling independent of ER signaling. Such insights open the discussion for considering ER-independent effects in future SERM/SERD studies both in basic research and in the clinic.

Given that the effects of GPER on vascular smooth muscle contraction are less known, we sought to characterize the contributions of GPER to vascular smooth muscle function by examining contraction to Ang II in arteries from GPER KO mice. Since it is known that GPER contributes to NO production, we hypothesized that arteries from GPER KO animals would exhibit greater contraction to Ang II due to a partial loss of vascular eNOS activity in GPER KO animals. Surprisingly, we observed that the arteries of GPER KO mice exhibited reduced contraction in response to Ang II compared to wild type arteries. We were able to mimic this loss of contraction (in KO arteries) in WT arteries using the NOX peptide inhibitor gp91dstat. Importantly, there was no additive effect of NOX inhibition in GPER KO arteries, suggesting that the decreased contraction to Ang II in GPER KO arteries was moderated through a decrease in (i.e. loss of) NOX activity. VSMCs isolated from GPER KO mice exhibited a dramatic decrease in superoxide production in response to Ang II and NADPH compared to WT. Chronic (3 day, employing 100 nM G36 or siRNA), but not acute (30 min treatment with 100 nM G36) inhibition of GPER in (WT) mouse, rat, and human

aortic SMCs recapitulated the abrogated superoxide response observed in GPER KO VSMCs. Furthermore, GPER KO SMCs as well as WT mouse, rat and human AoSMCs treated for 3 days with the GPER antagonist G36 exhibited a reduction in NOX1 RNA and protein expression that coincided with reduced superoxide responses to Ang II and NADPH. These data suggest that GPER regulates superoxide production by controlling expression of NOX1 in VSMCs.

We next sought to resolve if pharmacologic targeting of GPER using the GPER antagonist G36 could abrogate a hypertensive challenge of Ang II infusion in mice by reducing the superoxide-mediated component of hypertension. Mice were fitted with G36-releasing pellets (0.7 mg/day) or placebo 3 days prior to insertion of an Ang II-containing osmotic pump (0.7mg/day), and systolic blood pressure was measured by conscious tailcuff for two weeks. In agreement with our cellular and ex vivo results, mice pretreated with the GPER-selective antagonist G36 exhibited reduced hypertensive response to Ang II infusion compared to mice pretreated on placebo. Surprisingly, GPER KO animals receiving Ang II infusion were completely resistant to hypertensive effects of Ang II.

Taken together, data from specific aim 2 (Chapter 3) present a novel paradigm of GPER signaling where superoxide production is modulated by GPER-mediated regulation of NOX1 expression. These data present a surprising and conflicting role of GPER in the vasculature, since it had already been established that GPER contributes to endothelial NO production involved in vasodilation, and we now present here that GPER is also responsible for a ROS-mediated mechanism of contraction in vascular smooth muscle. However, we postulate that GPER inhibition may be more

advantageous to treat hypertension onset by blocking ROS accumulation, as current studies of GPER activation with GPER agonist G-1 show mixed results (depending on the estrogen levels in female mice) in preventing hypertension. We suspect that the varied results of G-1 treatment in animal models of hypertension may have been complicated due to increased GPER activity in the smooth muscle, which may have lead to increased NOX1 expression, possibly resulting in increasing ROS production that may have been counteracting the GPER activation and NO production at the endothelium.

Future directions of specific aim 2 will address the characterization of this novel finding in other cardiovascular tissues such as the heart and kidney. Deleterious effects of ROS in the kidney and heart contribute to major aspects of CVD, such as decreased kidney function, cardiac fibrosis, ventricular remodeling and hypertrophy. Evaluating the role of GPER in regulating NOX expression and subsequent ROS production in these tissues may identify a robust mechanism of ROS regulation. These findings implicate GPER as a possible therapeutic target for treating ROS-related CVD in multiple cardiovascular tissues.

Further investigation of the specific mechanisms responsible for GPER regulation of NOX1 expression is needed to fully understand the role of GPER in smooth muscle ROS. Two transcription factors known to mediate NOX1 expression are JunB and MEF2B, which activate the transcription factor AP-2 for binding at the NOX1 promoter site (210). MEF2B and JunB gene expression is regulated by ERK1/2, which is a known secondary messenger of GPER. QPCR of GPER KO and WT VSMCs show that GPER KO VSMC have reduced JunB expression compared to WT. This data suggests

an indirect mechanism of NOX1 downregulation by GPER, possibly through ERK1/2 activation of JunB transcription. It has also been shown that the NOX1 promoter region contains estrogen response elements, suggesting a regulatory role for ERα, however, additional NOX1 promoter elements that may be responsive to GPER activation are unknown.

Accumulation of ROS in vascular tissues occurs naturally during the aging process. Reports show that ROS production in tissues of the cardiovascular system also contributes to CVD such as hypertension, inflammation, atherosclerosis and cardiac fibrosis (229). Characterization of ROS production in the cardiovascular tissues of aged GPER KO and WT animals may implicate a role for GPER in long-term ROS regulation in aging cardiovascular tissue.

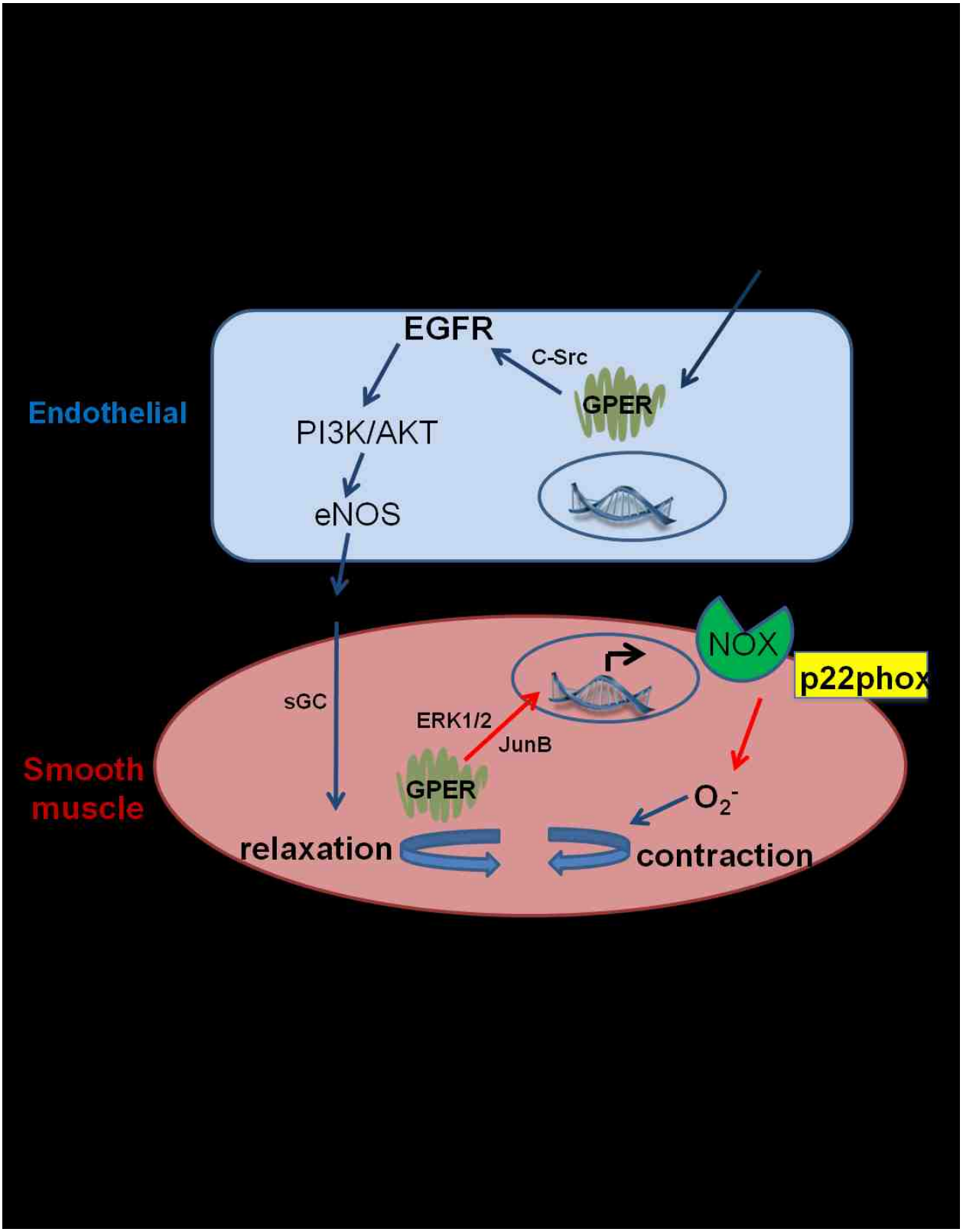
Conclusions from specific aim 2 (chapter 3) implicate the novel prospective that an estrogen receptor (GPER) could be playing a larger role in vascular ROS, and encourages investigations into additional cardiovascular tissues to determine if GPER-mediated ROS plays a larger role in cardiovascular dysfunction.

4.2 Overall Conclusions and Perspectives:

This thesis has outlined several seminal findings in the GPER-mediated regulation of endothelial and smooth muscle signaling in the vasculature. Our data present a counterintuitive paradigm in which GPER is necessary for mediating the estrogenic activation of eNOS in NO production, yet in contrast also contributes to mediating superoxide production through genomic control of NOX1 in vascular smooth muscle. Such a paradigm has been demonstrated in physiology before, such as the

classical example of the effects of vasopeptide endothelin-1 (ET-1) which induced vasorelaxation through calcium increase in endothelial cells (promoting calcium-dependent eNOS activation) and contraction smooth muscle cells (promoting calcium-mediated activation of the contractile apparatus). Given such an interesting juxtaposition of the GPER mechanism, the balance of an individual's cardiovascular health (especially whether an individual has a healthy endothelium), age, and genetics play a vital role in the global effects that pharmacologic alteration of GPER signaling with have in their cardiovascular function. For example, while GPER activation (G-1) may have positive effects in healthy, pre-menopausal women with a functioning endothelium by contributing to NO production, an individual with endothelial dysfunction due to aging or underlying CVD may actually benefit from GPER inhibition (G36) to reduce oxidative stress in lieu of reduced NO benefits GPER may be able to provide in such conditions.

These data provide crucial insights into the etiology of estrogen in hypertension. The development of effective hormone replacement therapy (which commonly utilize E2 supplementation) to combat CVD in post-menopausal women is a significant issue in the clinic. Given the findings of this thesis, an interesting approach could be to supplement E2 replacement with the GPER-specific antagonist G36 to reduce the extent of ROS production that may occur with E2 replacement through GPER activation. Such clinical implications support further study of GPER in the cardiovascular system.


Endothelial
EGFR
C-Src
GPER
PI3K/AKT
eNOS
NOX
p22phox
sGC
ERK1/2
JunB
Smooth muscle
GPER
O_2^-
relaxation
contraction

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