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Estrogen receptor beta: Influence on urokinase-type plasminogen activator in vascular smooth muscle cells

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Submitted to the School of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Biochemistry, Microbiology and Immunology,

Faculty of Medicine, University of Ottawa

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Abstract

It has been proposed that the presence or administration of 17-β estradiol can be protective against heart disease, the primary cause of death for both men and women. Acute events in the progression of atherosclerosis are thought to be primarily the result of plaque rupture, a mechanical event dependent on the structural integrity of a lesion. The mechanism by which 17-β estradiol confers vascular benefits may be related to expression or activation of structural proteins and degradative enzymes influencing plaque composition and stability. Ligand signaling by 17-β estradiol is thought to occur via specific estrogen receptors (ERs) expressed in the cell membrane of vascular cells.

The aim of this thesis was to investigate the influence of ERs on the activity of uPA, a protease that is capable of degrading structural proteins integral to atherosclerotic plaque stability. Vascular smooth muscle cell (VSMC) uPA activity was observed after treatment with 17-β estradiol, 4-hydroxytamoxifen, faslodex (ICI 182,780) and antisense to ERβ, the most recently discovered ER. The addition of ER ligands 4-hydroxytamoxifen and faslodex resulted in a substantial increase in the activity of uPA. This effect was determined to be dependent on transcription, translation and N-glycosylated secretion. However, these effects were independent of transcriptional events involving the estrogen response element. Therefore, the ERs may be acting via AP-1 or Sp1 enhancer regions controlling transcription. Alternatively a signal for the release of pro-uPA from secretory granules may be transmitted via cell surface ERs.

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I would like to thank my supervisor, Dr. E.R.O'Brien, for granting me the opportunity to pursue graduate studies in his laboratory, and for his support and guidance. I would also like to thank the members of the lab for their help and advice – Dr. Xiaoli Ma, Dr. Yong-Xiang Chen, Steve Olsen, and especially Harvey Miller for his technical expertise, patience and encouragement.

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Introduction

Overview

The role of estrogen receptors (ERs) in the vascular system was explored due to the body of evidence suggesting gender differences in cardiovascular disease, with women experiencing clinical events at a later age, presumably due to hormonal benefits derived before menopause (1). The question of how estrogens may mediate these effects has not yet been clearly answered. Urokinase-type plasminogen activator (uPA) is an enzyme that has been implicated in atherogenesis and has an important influence on the stability of the atherosclerotic lesion. The general goal of this thesis is to determine if 17-β estradiol modulates uPA in vascular smooth muscle cells (VSMCs) in a manner that is predictive of a reduced incidence of plaque rupture.

Two specific aims are proposed:

1- Determine the expression pattern of ERα and ERβ in human aortic VSMCs (VSMCs) *in-vitro*. Antisense and ER ligands will be used to modulate the expression and activity of ERs in primary VSMCs.

2- Determine if and how the modulation of ER expression alters uPA activity.

17-β estradiol and heart disease

Epidemiological studies have shown that premenopausal women have a lower incidence of coronary artery disease (CAD) relative to men (2). After menopause, there is a sharp increase in the incidence of heart disease in women (3,4). This gender difference in CAD has been attributed in part to the drop in

serum 17-β estradiol levels that accompanies the onset of menopause (1). As a result, physicians have prescribed hormone replacement therapy (HRT) for women with the intention, to both alleviate menopausal symptoms (e.g. hot flashes) and prevent CAD. Consequently, Premarin (conjugated estrogens and medroxyprogesterone acetate) was the third-most prescribed drug in the United States in 2001 (5). However, recent clinical trials have questioned the efficacy and safety of HRT when prescribed to prevent CAD. The American Heart Association issued an official advisory recommending against the use of HRT as a means of secondary prevention for cardiovascular disease (6). Two large trials (the Heart and Estrogen/Progestin Replacement Study (HERS) and the Women's Estrogen for Stroke Trial (WEST)) indicated that HRT conferred no preventative benefits (7,8). The recent results of the Women's Health Initiative (WHI) HRT component, showed that the use of combined progesterone and estrogen as a primary means of prevention of cardiovascular disease, is hazardous due in part, to increases in thrombosis and coagulation (9,10). Although the double blind randomized clinical studies are very helpful in determining appropriate indications for HRT, they provide little mechanistic information regarding the effects of estrogens on the artery wall, nor explain the discordance with population studies that clearly show a difference in CAD incidence between premenopausal women and men of similar age. Moreover, the formulation, dose and route of administration of the hormones used in these trials are arbitrary and possibly inadequate physiological replacement therapies. The age and cardiovascular health of the women enrolled may also be an important consideration. The WHI

trial enrolled women 50-79 years of age, with a mean age of 63 years at the time of enrolment, instead of limiting treatment groups to women in their late 40's and early 50's when the maximal cardiovascular benefit of HRT might be expected. Hence the questions surrounding the role of estrogens and their receptors in vascular biology remain incompletely understood, and if anything, require more detailed assessment of ligand-receptor biology in vascular cells.

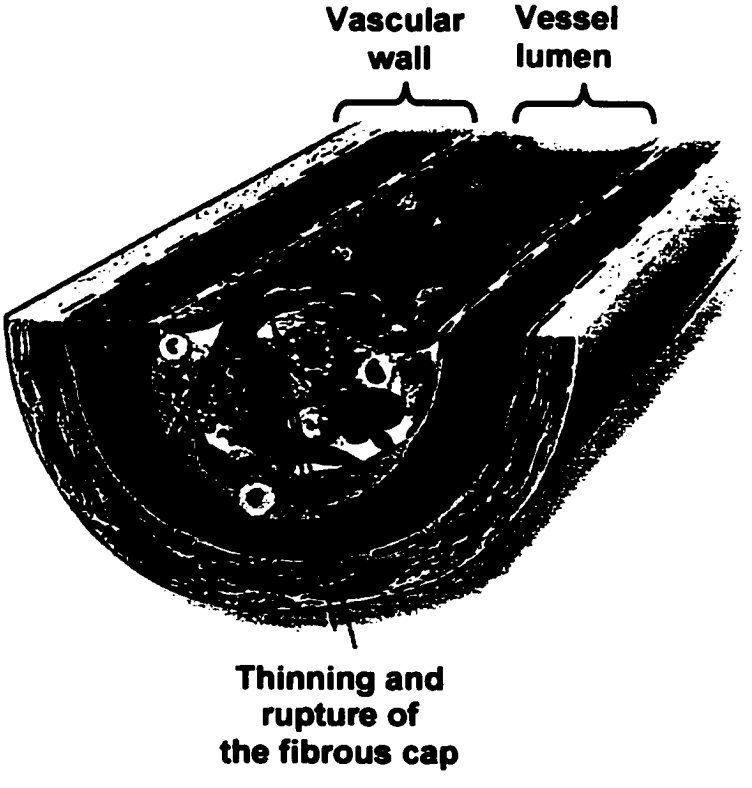
It is believed that 17-β estradiol can be vasoprotective due to its influence on risk factors for heart disease and antagonism to essential steps of atherosclerotic lesion development. Generated primarily in the ovaries, 17-β estradiol can be synthesized by the enzyme aromatase in the vascular wall (11). Two risk factors for heart disease are responsive to HRT. 17-β estradiol has been shown to lower total cholesterol, increase the proportion of high-density lipoprotein "good" cholesterol, and lower low-density lipoprotein "bad" cholesterol, by modulating apoprotein expression from the liver (2,12). 17-β estradiol also affects blood pressure by increasing vasodilation and reducing vascular tone acutely (13,14,15). With regards to specific steps in atherogenesis, 17-β estradiol has been shown to inhibit apoptosis of vascular cells, to promote angiogenesis, and to inhibit proliferation of endothelial cells (2,12). In ovariectomized monkeys, rabbits and rats, administration of 17-β estradiol has been shown to retard atherogenic intimal thickening (1,2,16). In these animal studies, high-cholesterol diets and/or arterial injury are used to induce vascular lesions.

Atherosclerotic plaque rupture

Atherosclerosis in an inflammatory disease of the blood vessel walls (17), leading to the formation of lesions which can ultimately result in death (18). By early adult life, most individuals in developed countries will have some advanced lesions (18). The fibrous cap of an advanced lesion overlies an atherosclerotic core that consists of inflammatory cells and microvessels penetrating the lesion and necrotic debris (19). The cap covers the lesion, but can ultimately rupture, exposing core contents to the blood stream (18). This usually results in the formation of a thrombus, due to interaction of the platelets with certain proteins in the lesion (Figure 1)(18). The thrombus can occlude the vessel and result in an acute ischemic event, such as myocardial infarct or stroke (20).

A plaque becomes vulnerable to rupture due to its composition, and not its size (21). Although a plaque may occlude the lumen such that ischemic effects are produced, the vast majority of lesions that rupture and result in acute myocardial events occlude the lumen by less than 40% (22). Since plaque rupture is a mechanical event, its likelihood depends on the structural integrity of the lesion (23). It is believed that structural proteins such as collagen provide tensile strength and thus, increase stability (24,25). Post-mortem pathology studies have established that the small, soft, and fat-rich plaques, with low collagen content, are more prone to disruption and thrombosis (26,27). Treatment of patients with members of the "statin" family of

Figure 1. Rupture of an atherosclerotic lesion. This figure depicts the rupture of an atherosclerotic lesion. The lumen of the blood vessel and the vascular wall are indicated with parentheses. Macrophages are present in the lumen of the blood vessel, as well as within the lesion in the form of foam cells. Vascular smooth muscle cells are present in the vascular wall, and also in the fibrous cap region and within the necrotic core of the lesion. Note that the rupture of the fibrous cap has permitted platelets from the blood stream to enter the lesion. This figure was adapted from Figure 4 of reference 17.


Vascular wall
Vessel lumen
Thinning and rupture of the fibrous cap

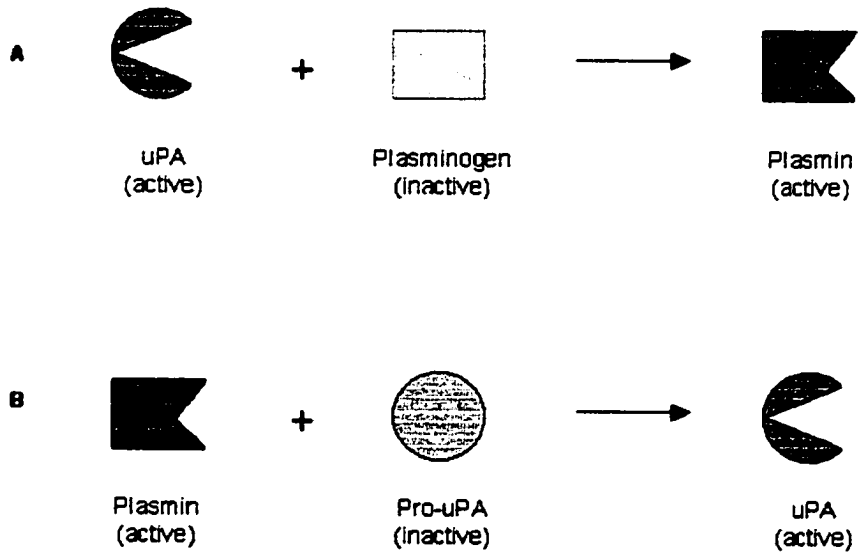
lipid-lowering medications, increases collagen content and decreases lipid content, inflammation, proteolytic enzymes, and cell death in human carotid plaques and experimental lesions produced in rabbits (28,29,30).

uPA

A host of enzymes expressed by vascular cells elaborate and degrade structural proteins found in the fibrous cap. uPA, a key enzyme in this process, is a serine protease secreted from the cell as a zymogen (pro-uPA) (31,32,33). Immediately after secretion, the pro-form binds the uPA receptor (uPAR), at the cell surface (34). Pro-uPA is activated by plasmin, which is normally in close proximity to uPAR (35). Vitronectin is also in close proximity to uPAR, and can bind the primary inhibitor of uPA: plasminogen activator inhibitor 1 (PAI-1) (36,37). PAI-1 is a serine protease inhibitor secreted by many cell types, and it inhibits uPA by means of classical competition (35). Excessive amounts of PAI-1 are secreted, ensuring strict control of pericellular proteolysis (35). Further control occurs at the level of the uPA gene, with the uPA promoter being regulated via AP-1 and SP1 enhancers (38,39). The substrate of uPA is the zymogen plasminogen, which yields the active protease plasmin (35) (Figure 2).

The generation of plasmin by uPA affects the degradation of the extracellular matrix because plasmin is capable of activating members of a family of enzymes known as matrix metalloproteinases (MMPs) (40). The MMPs are endopeptidases whose activity is increased in injured arteries (41,42,43,44,45), and can degrade a variety of structural proteins (46). By facilitating

Figure 2. The interaction of uPA and plasmin. Panel A shows the cleavage of the zymogen plasminogen by uPA to generate plasmin. Panel B shows the generation of active uPA from pro-uPA by plasmin.


A
uPA
(active)
Plasminogen
(inactive)
Plasmin
(active)
B
Plasmin
(active)
Pro-uPA
(inactive)
uPA
(active)

the activation of MMPs, uPA helps shape an enzymatic environment outside the cell, which favours degradation of the fibrous cap. A loss of uPA, as evidenced in the uPA knockout mouse, leads to impaired neointimal formation after arterial injury (47). Substantial upregulation of uPA and uPAR has been shown in VSMCs after injury to the blood vessel wall (48) (Figure 3), and upregulation of uPA expression in the carotid arteries of cholesterol-fed rabbits, led to a 70% greater neointimal area after a four week period (49). Hence, uPA has a pivotal role in atherosclerotic lesion development, as well as the genesis of rupture prone plaques.

Estrogen Receptors

There are two known ERs, ERα and ERβ, both of which are nuclear transcription factors (50). Unlike most steroid hormone receptors, ERβ and ERα are not alternate transcripts of a single gene, but are encoded on separate genes and located on different chromosomes (51). Overall, the ERs have a high degree of primary structure homology, especially in the DNA-binding domain (52). Figure 4 shows the percent homology of the two receptors for specific domains, based on their primary structure. Some tissues express only one of the receptors, while others express both or neither (52). Figure 5 shows a generalized scheme for the cellular signaling mechanisms of estrogens via their receptors. Prior to ligand binding, the estrogen receptors are bound by heat shock proteins/chaperone proteins such as Hsp90. Conformational changes in the protein, which occur on ligand binding, result in the release of the chaperone

Figure 3. In-situ hybridization for uPA in a vein bypass graft. The arrow indicates an area of uPA mRNA hybridization probe binding. The bright dots reveal expression of uPA mRNA, with the brightest areas indicating areas of high concentration of uPA mRNA. This work was performed in the Vascular biology laboratory of the University of Ottawa Heart Institute.

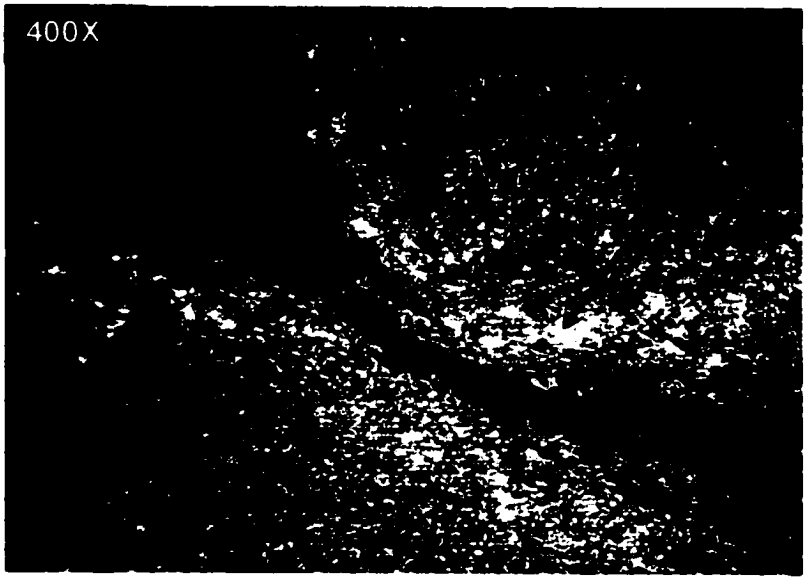

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Figure 4. Primary structure homology comparison of ERα and ERβ. The dark blue bars represent ERα, while the pale blue bars represent ERβ. NHD refers to the N-terminal Non-hormone binding domain, DBD refers to the DNA-binding domain, Hinge refers to the central hinge domain, LBD refers to the ligand-binding domain, and F refers to the C-terminal domain. This figure was adapted from reference 52.

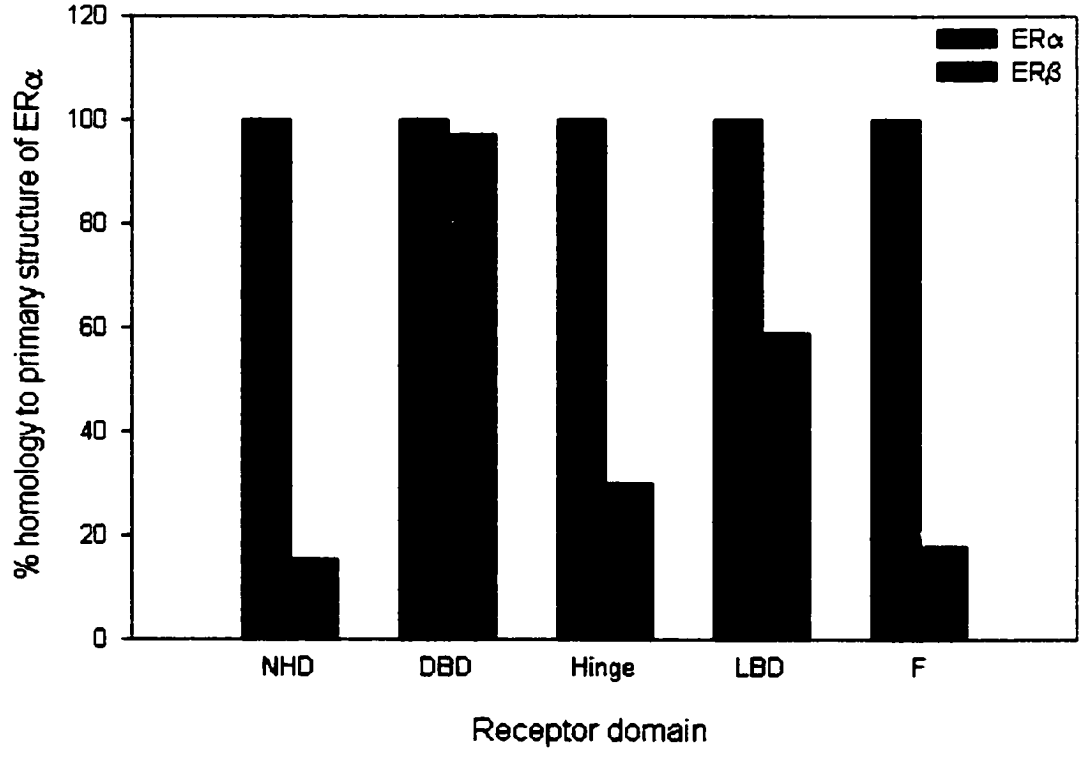
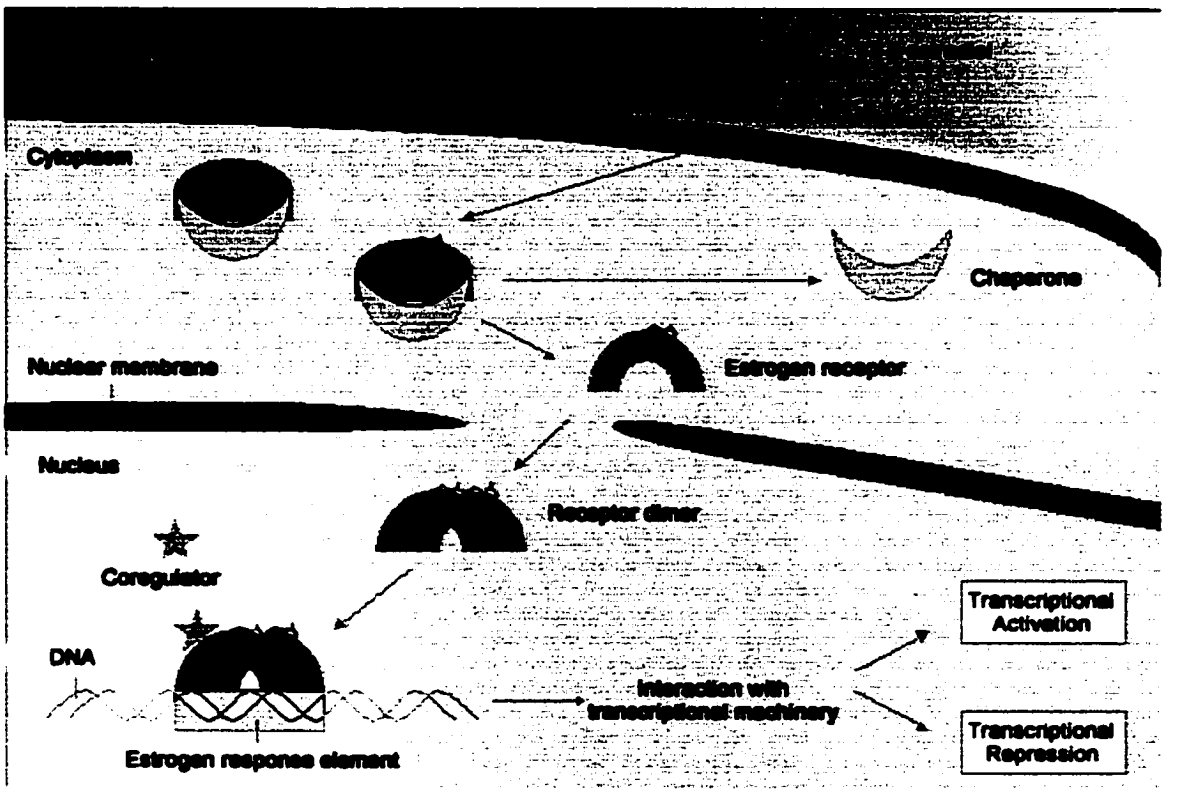

120
100
80
60
40
20
0
% homology to primary structure of ERα
ERα
ERβ
NHD
DBD
Hinge
LBD
F
Receptor domain

Figure 5. Overview of ER mechanism of action. A generalized scheme of ER mechanism of action is represented. 17-β estradiol is a steroid hormone, and is hydrophobic. This allows 17-β estradiol to pass easily through the cell membrane and bind cytoplasmic ERs. The binding of 17-β estradiol to the ERs results in conformational changes the consequence of which is the release of the chaperone proteins associated with the ER. The ligand-bound receptor dimerizes and enters the nucleus where it can bind specific response elements in the regulatory region of a gene, and encourage transcriptional activation or repression. The ER often acts in concert with other transcription factors and coregulatory proteins.


Cytoplasm
Chaperone
Nuclear membrane
Estrogen receptor
Nucleus
Receptor dimer
Coregulator
DNA
Transcriptional Activation
Interaction with transcriptional machinery
Transcriptional Repression
Estrogen response element

proteins associated with the ER (53). The ligand-bound receptor then homodimerizes or heterodimerizes (54,55) and enters nucleus where it binds to DNA at specific sequences in the promoter regions of genes (56). In classical 17-β estradiol signaling, the receptor binds a palindromic sequence known as the estrogen response element (ERE). The ERE, is an enhancer sequence, usually located upstream of the promoter region (12). When the ER dimer binds the enhancer sequence, it is able to influence transcription of the gene in question (50). ER-mediated transcription also relies on interactions with coregulatory proteins, the binding of which can tip the scales towards transcriptional activation or repression of a particular gene (50,58,59,60). Interaction with coregulatory proteins normally occurs via a LXXLL sequence in the coregulatory protein (61). The ERs bind both general steroid receptor coregulators such as SRC-1 (steroid receptor coactivator 1) (62,63,64), transcriptional coactivator p300 (65), and coactivator GRIP-1 (glucocorticoid receptor interacting protein) (66) and ER-specific coregulators such as REA (repressor of estrogen activity)(67). The ERE is by no means the only path for estrogen-mediated transcription (12). ERs can also bind DNA by association with activating protein 1 (AP-1) enhancer elements and Sp1 enhancer elements (12,57).

At this time, evidence is amassing which bolsters the notion of cell surface estrogen receptors (68,69,70,71,72,73,74). These are thought to be associated with plasma membrane caveolae, specific detachable membrane invaginations that play a role in signal transduction (75,76). Binding of the ligand to the putative surface ER could ultimately result in extracellular regulated kinase (ERK)

activation. This could account for rapid-onset effects triggered by estradiol, such as nitric oxide mediated vasodilation (77). Figure 6 shows a simplified speculative model of surface ER signal transduction.

A functional ER (ERα) was first shown to be expressed in human VSMCs in 1994 (78,79). In 1995, in-vitro work with rat arterial VSMCs provided the first evidence of 17-β estradiol synthesis in vascular cells by the enzyme, aromatase, bolstering the notion of vascular cells as legitimate 17-β estradiol targets (11).

ER and the vessel wall

In animal models of arterial injury, the formation of a lesion was found to be impeded in the presence of estrogen (endogenous or exogenous) (80,81,82). ERβ was discovered in rat ovarian tissue in1996 (51,83). In 1998, increased expression of ERβ mRNA in male blood vessels was noted after vascular injury (84). This key observation stimulated interest in ER β in the vascular system.

Table 1 shows the effects of estrogen treatment on various animal models of atherosclerosis. The variation in response to estradiol between the animal models, is due not only to differences in physiology and experimental conditions, but also to the specific vascular characteristics investigated by the researchers, and definitions of "protective" cardiovascular effects. The relative significance of vascular traits that manifest as a consequence of hormone treatment, is often a matter of debate. The overwhelming number of studies and the variety of models available adds to the complexity of the field. The major point of consensus

Figure 6. Cell surface ER. This figure was adapted from reference (12), figure 4. 17-β estradiol binds the cell surface estrogen receptor, which is associated with cell membrane caveola. Subsequent activation of extracellular regulated kinases can lead to rapid-onset effects in the cell.

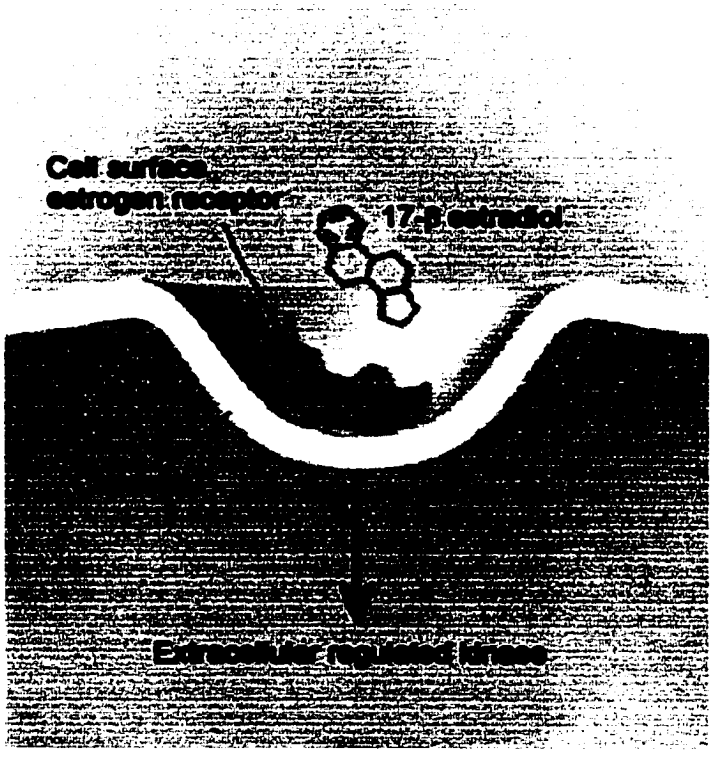

Cell surface
estrogen receptor
17-β estradiol

Table 1. The effect of estrogen treatment on the vascular wall for animal models of atherosclerosis.

ANIMAL MODEL OF ATHEROSCLEROSIS	SUMMARY OF RESULTS
ERα KO mouse	• Reendothelialization after carotid injury (85) • Attenuated medial thickening and VSMC proliferation after arterial injury and treatment with 17-β estradiol (86,87)
ERβ KO mouse	• VSMC proliferation, medial thickening, after arterial injury and treatment with 17-β estradiol (86,87) • Increased vasoconstriction with 17-β estradiol treatment and increased hypertension in treated and untreated animals (88)
ERβα KO mouse	• VSMC proliferation with 17-β estradiol treatment (86,87)
ApoE KO mouse	• Decreased fatty streak formation in castrated animals fed low-fat diet and treated with 17-β estradiol (89,90)
Rat	• Ovariectomized rats, arterially injured and treated with subcutaneous 17-β estradiol have improved reendothelialization (91) • Inhibition of intimal thickening after arterial injury and treatment with 17-β estradiol (92) • Upregulation of ERβ after arterial injury, no change in ERα (84)
Rabbits	• Reduced serum triglycerides, reduced VSMC proliferation in ovariectomized, cholesterol-fed and estradiol-treated animals(93,94) • Smaller lesions in cholesterol-fed, ovariectomized, estradiol-treated animals (95) • Reduced intimal thickening in ovariectomized, cholesterol-fed, estradiol-treated animals (96)
Cynomologus monkeys	• Ovariectomized monkeys fed an atherogenic diet and treated with 17-β estradiol, experience 50% decrease in the extent of coronary atherosclerosis, but does not regress established disease (97) • Pregnancy, female gender, and oral contraceptives result in reduced number of lesions in monkeys with normal diet (98,99,100)

seems to be that 17β-estradiol does have a significant impact on the vascular wall.

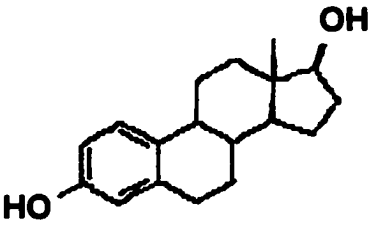
Research performed in our lab has shown that the ERs are expressed in male and female, healthy and diseased arteries. Semi-quantitative RT-PCR, Southern blot analysis, Northern blot analysis, and immunohistochemical studies of paired, normal and diseased, human male and female, vascular tissue, demonstrated the expression of ERβ in all samples (101,102).

The results from the knockout studies, the known correlation between vascular properties and 17-β estradiol levels, and the synthesis of estradiol within the vascular wall, all serve to underline the undeniably significant role of estrogens in the cardiovascular system.

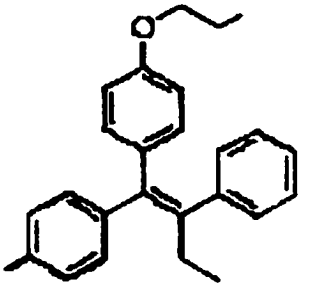
In addition to the physiological ligand 17-β estradiol, several man-made estrogens are able to bind the ERs. This property has been exploited most notably in the treatment of ER positive breast cancer with the drug tamoxifen (103). (Figure 7). 4-hydroxytamoxifen is the active metabolite of the drug tamoxifen, and is highly stable ER antagonist (104). It stimulates the receptors in some cells, while blocking the receptor in some other cells (103). It is believed that these differences can be attributed to the coregulating proteins which differ from cell to cell. Further pharmaceutical research has generated several other ER modulators including faslodex (105,106). Faslodex is a drug developed by Imperial Chemical Industries (ICI 182, 780) with a chemical structure analogous to 17-β estradiol, yet sufficiently different to elicit opposite transcriptional outcomes. It is capable of binding both ERα and ERβ.

Figure 7. Chemical structure of specific ER ligands. Structure A depicts 17-β estradiol. Structure B is 4-hydroxytamoxifen. Structure C is faslodex.

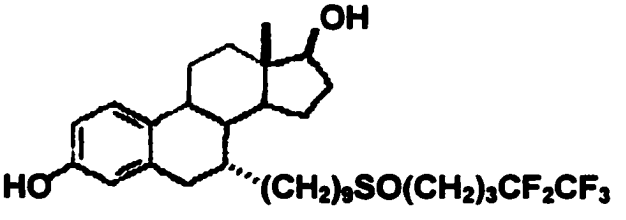
A



B



C



An increasing awareness of the differing properties of the various antiestrogens has stimulated increased research into SERMs (selective estrogen receptor modulators) and various plant-derived hormones or phytoestrogens. SERMs are characterized by their ability to produce different cellular effects as a function of the tissue into which they are introduced (107,108).

The body of this thesis will address the experimental results and their interpretation. Expression of ERβ and ERα was verified in primary VSMCs in the presence or absence of 17-β estradiol. Immunocytochemical analysis revealed predominant expression of ERα in the nucleus, and ERβ in the cytoplasm. VSMC uPA activity was observed after treatment with 17-β estradiol, 4-hydroxytamoxifen, faslodex and antisense to ERβ, the most recently discovered ER. The addition of ER ligands 4-hydroxytamoxifen and faslodex resulted in a substantial increase in the activity of uPA. To determine if the effects of these ligands on uPA activity involved transcription, translation, or secretion, we used the inhibitors actinomycin D (a blocker of mRNA synthesis), cycloheximide (a blocker of *de novo* protein synthesis), and tunicamycin (a blocker of N-linked glycosylated secretion). In the presence of these inhibitors, the previously observed effects of the ligands were significantly reduced. The specific pathway of ER activation was investigated by means of a transfected reporter construct with an estrogen response element (ERE) controlling the expression of a fluorescent protein. The results of the reporter assay suggest that these effects are not mediated via the ERE. While the physiological ligand, 17-β estradiol, seems to exert little effect on uPA activity, specific antiestrogens appear to

modulate uPA in VSMCs in a manner that is predictive of an increased incidence of plaque rupture.

Materials and Methods

1 – Cell culture

Cell lines used: HeLa cells, known to express neither ERα nor ERβ, generous gift of Dr. Heidi McBride. MCF-7 breast cancer cell line, known to express both ER subtypes, the generous gift of Dr. Christine Pratt. Male and female aortic VSMCs were purchased from BioWhittaker. Cells were maintained in low glucose Dulbecco's Modified Eagle Medium (GIBCO/INVITROGEN), phenol red-free (phenol red is fluorescent over the same range as EGFP and is estrogenic (109,110), 10% FBS, 2mM L-glutamine, 50μg/ml gentamycin sulphate, 25μg/ml amphotericin B, and glucose to render final concentration of 4.5g/L. In experiments involving dosage with 17-β estradiol, faslodex or 4-hydroxytamoxifen, cells were grown in media with charcoal stripped fetal bovine serum (FBS). FBS was charcoal stripped (111) with hydrochloric acid-washed charcoal (Sigma). Cells were rinsed with phosphate-buffered saline (PBS). At passage, cells were treated with 0.25% Trypsin/PBS (Sigma). VSMCs were passaged at subconfluence. Cells reached subconfluence in 7 to 9 days and were split 1:3 in 100mm Petri dishes (Corning). Cells were grown at 37°C, 5% CO_2, in a humidified incubator.

2 – Immunofluorescence

VSMCs were grown to subconfluence on Lab Tek 8-well slides, then fixed in 4% paraformaldehyde/PBS. Slides were rinsed in PBS twice, for a period of five minutes. Next, cells were immersed in 0.2% Triton-X100/PBS for fifteen

minutes, and subsequently rinsed in PBS for two five minute periods. The primary antibody was applied for a total of one hour at 37°C, in a moist environment. For the mouse anti-human ERα the dilution was 1/50 (Novocastra), for the rabbit anti-human ERβ, the dilution was 1/500 (Affinity Bioreagents), and for the mouse anti-human smooth muscle cell alpha-actin, the dilution was 1/500 (Dako). Cells were then rinsed in PBS for two five minute periods. The secondary antibody was then applied. A dilution of 1/100 was used for the horse anti-mouse fluorescein-conjugated secondary antibody, and for the goat anti-rabbit Texas Red-conjugated secondary antibody (Vector). The secondary antibodies were allowed to incubate with the slide for thirty minutes at room temperature. This was followed by washing with PBS for two five minute periods. A 1/500 dilution of a 2mM Hoechst stain 22358 (Sigma) solution was applied for fifteen to thirty minutes. Slides were then mounted using glycerin (2:1 solution of PBS:glycerin). Slides were observed by fluorescent microscopy and images were captured using a SPOT digital camera system.

3 – Western blotting

Cell lysates were collected using a "RIPA" lysis buffer consisting of phosphate buffered saline pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 10mg/ml phenylmethylsulfonylfluoride, 30μg/ml aprotinin, and 1mM sodium orthovanadate (112). Bradford assays were performed (113) on total cell lysate using BioRad Bradford assay reagent. The standard curve was established using bovine serum albumin (Sigma) and the

absorbance at 595nm was measured using a Hewlett-Packard UV-vis spectrophotometer. Sample buffer containing SDS, glycerol, tris buffer, and 1% bromophenol blue, was added to protein lysates. The presence of bromophenol blue allowed tracking of the leading edge of the lysate. Samples were boiled for 5 minutes prior to loading into gel. NEB broad range protein marker was loaded into gels to estimate migration position of specific size bands. Protein was subjected to SDS-PAGE in a BioRad vertical electrophoresis apparatus. The resolving gel was 10% acrylamide (v/v) and the stacking gel was 3% acrylamide (v/v). Both gels were made using acrylamide, 10% ammoniumpersulfate, tris buffer, and polymerization was initiated by the addition of 9.5µl TEMED after 15 minutes of degassing. The voltage used in the stacking phase was 40V and the voltage used in the resolving phase was 120V (112). The running buffer was a Tris-glycine buffer at pH 8.4. Electrophoresis was halted after the bromophenol blue ran out of the gel and into the running buffer. The proteins were transferred to a nitrocellulose membrane overnight in transfer buffer at 4 degrees Celsius at 15V using a BioRad electrobotter apparatus (114). The transfer buffer consisted of 20% methanol in tris-glycine running buffer. Proper transfer was verified by staining the gel with Coomassie Brilliant Blue (115). For Western blotting, the nitrocellulose membranes were placed in 50ml centrifuge tubes, into which 5ml of Blotto A was added. Blotto A consisted of 5% (w/v) Carnation Instant Skim milk in Tween-Tris buffered saline (TTBS). TTBS consisted of 10mM Tris-HCL pH 8.0, 150mM NaCl, and 0.05% Tween-20. The membranes were incubated with blotto A for 1 hour at room temperature at 6rpm in a hybridization oven. The

blocking solution was then discarded and the membranes were incubated with primary antibody in blotto A for 1 hour at room temperature. Monoclonal mouse anti-human ERα primary antibody (Novocastra) was used at a dilution of 1:100. Polyclonal rabbit anti-human ERβ primary antibody (Affinity Bioreagents) was used at a dilution of 1:1000. After incubation with the primary antibody, the membranes were washed three times with 25ml TTBS, for 10 minutes. Following the washes, the membranes were incubated with secondary antibody for 1 hour at room temperature. Polyclonal goat anti-rabbit horseradish peroxidase conjugated and polyclonal goat anti-mouse horseradish peroxidase conjugated antibodies (Chemicon) were used as secondary antibodies at a dilution of 1:5000. The membranes were washed three times with 25ml TTBS, for 10 minutes. The membranes were damp dried and proteins were detected using the ECL Western blotting system (Amersham Pharmacia Biotech). Chemiluminescent substrate was added to the membrane and ECL hyperfilm was exposed to the membranes overnight.

4 – Reverse transcription-polymerase chain reaction

All solutions used in RT-PCR were made with DEPC-treated water; and RNase- free tips and tubes were used, and treated by UV-irradiation. RNA was isolated from confluent 100mm dishes of VSMCs using Tri Reagent (Invitrogen). The concentration and purity were estimated using spectrophotometric readings at 260nm and 280nm. 1-2μg of RNA was treated with DNase I (Amersham Pharmacia Biotech) for fifteen minutes at room temperature to eliminate residual

DNA contamination. The DNase I was inactivated using 2mM EDTA. First strand-synthesis was initiated using 200 units of SuperScript II RNase H^- reverse transcriptase (Invitrogen) and oligo dT (New England Biolabs). The reverse-transcribed samples were amplified by PCR using *Taq* DNA polymerase (Amersham Pharmacia Biotech). Optimal results were obtained when the concentration of $MgCl_2$ was 2mM. Controls contained DEPC-treated water in lieu of template. GAPDH primers were used to normalize the results of ERβ amplification, for quantification. All primers were used at a final concentration of 1μM each in the final reaction volume (please refer to Table 2 for primer sequences). The reaction was given a hot start, and the annealing temperature was 57.5°C. The GAPDH amplicons generated were 131bp in length, and the ERβ amplicons generated were 282bp in length. The amplicons were run on a 4% NuSieve agarose (BioWhittaker) gel. The gels were stained with ethidium bromide (Sigma) and photographed under UV illumination (BioRad Gel Doc System). The bands were analyzed using the Molecular Analyst software (BioRad). The quality of RNA isolates was verified by RNA agarose/formaldehyde gel electrophoresis, ethidium bromide staining, and UV-visualization and image capture, using standard procedures (116).

Table 2. Sequences of primers used in RT PCR. These primers were synthesized at Alpha DNA (Montreal, QC) and designed by Bingyi Han (former graduate student in the lab).

Primer	Sequence
GAPDH1 (23mer)	AAC AGC CTC AAG ATC ATC AGC AA
GAPDH2 (19mer)	CAG TCT GGG TGG CAG TGA T
B1 (20mer)	CAG CCA TTA TAC TTG CCC AC
B2 (20mer)	AGT GAC ATT GCT GGG AAT GC

5 – Antisense

An 18-mer antisense phosphorothioate oligonucleotide to ERβ and a scrambled control were synthesized by Alpha DNA (Montreal, QC) (see Table 3 for sequences). The ER antisense oligonucleotides had been previously shown to reduce ER mRNA and protein levels (in vitro) (117,118). The first three and last three bases in each oligonucleotide were phosphorothioated. The substitution of sulfur at phosphorous for one of the nonbridging oxygen increases stability and nuclease resistance, while maintaining solubility and charge (119,120). Cells were seeded in 6-well culture dishes and grown to confluence in DMEM supplemented with 10% FBS, gentamycin sulphate, amphotericin B and L-glutamine, and glucose (as indicated in Cell Culture section) and used between passage 6 and 10. The media was replaced with non-supplemented DMEM at confluence, and the cells were treated with 2.5μM antisense ERβ. At the end of 24h the serum, glutamine and antibiotics were reintroduced. This antisense protocol is from Sirois et al. (121). The antisense technique that I have employed

relies on the design of a single stranded DNA that is complementary to the transcriptional start site region of the mRNA of the protein of interest.

Table 3. Antisense oligonucleotide sequences

Small case letters designate replacement of the phosphodiester backbone with a phosphorothioate backbone.

Oligonucleotide	Sequence
ER beta antisense (18mer)	cat CAC AGC AGG GCT ata
ER beta scrambled (18mer)	gat CTC AGC ACG GCA aat

6 – uPA assay

Human aortic VSMC uPA activity was assessed using a uPA Assay Kit (Chemicon). The kit was tested and optimized with appropriate positive and negative controls to verify its applicability to this study. Cells were grown to confluence in 6-well plates in 10% FBS DMEM. At full confluence, cells were switched to 10% charcoal-stripped FBS DMEM and treated with one of the following: 1μM 17-β estradiol, 1μM Faslodex, 1μM 4-hydroxytamoxifen, antisense ERβ, scrambled ERβ. For studies with the following blockers: actinomycin D (2μg/ml), cycloheximide (10μg/ml), and tunicamycin (1μg/ml), the cells were treated with the blocker +/- ER antagonist. Actinomycin D prevents the transcription of new mRNA, cyclohexmide prevents the synthesis of new protein, and tunicamycin inhibits the secretion of most N-glycosylated proteins by blocking the first glycosyltransferase in the dolichol pyrophosphoryl oligosaccharide precursor synthetic pathway. Where appropriate, vehicle controls were run. Tunicamycin, actinomycin D and cycloheximide have all been

successfully used with aortic VSMCs at the concentrations I employed (122,123,124,125,126,127,128,129). After 6h incubation, the uPA tripeptide substrate was added to the media, and incubation continued for an additional two hours. Cell media was then used to evaluate uPA activity according to the manufacturer's instructions. O.D. 405nm readings were taken in a BioRad microplate reader to assess uPA activity. Results were quantified based on a purified uPA standard curve. All experiments were performed in triplicate.

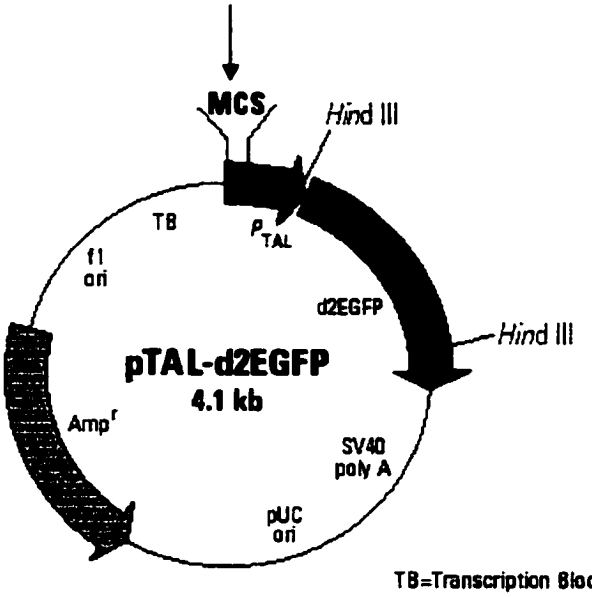
7 – Reporter vector design

A reporter vector was designed to monitor ER-mediated transcription. The reporter gene employed was a destabilized enhanced green fluorescent protein (EGFP) in a Clontech parental vector (see Figure 8). The EGFP was destabilized (i.e. has a shorter half-life) in order to reduce background signal and render it more fit for quantitative transcription reporter assays (130). An ERE was cloned into the reporter gene promoter region to allow ER-mediated control of transcription. The use of ERE coupled to a fluorescent reporter, has been previously employed successfully to detect chemicals with estrogenic activity (131). The ERE in question is derived from the *Xenopus laevis* vitellogenin A2 gene promoter region. It contains a canonical ERE which has been shown to be highly sensitive to 17-β estradiol and has been used in previous reporter assays (132). The vitellogenin segment was synthesized as a double-stranded DNA segment by Alpha-DNA (Montreal, QC), with restriction enzyme sites designed

Figure 8. Design of the estrogen response element reporter vector. Panel A shows the reporter vector design. A 42bp enhancer element from the *Xenopus laevis* (African clawed frog) vitellogenin A2 gene, was cloned into the multiple cloning site of the pTAL-d2EGFP vector (Clontech). The multiple cloning site of this vector lies within the promoter region, controlling expression of the reporter gene, enhanced green fluorescent protein. The enhancer contains a canonical estrogen response element. The yellow highlighted segment of the enhancer sequence indicates the estrogen response element sequence. Panel B shows the transfection efficiency control vector used in the transfection experiments.

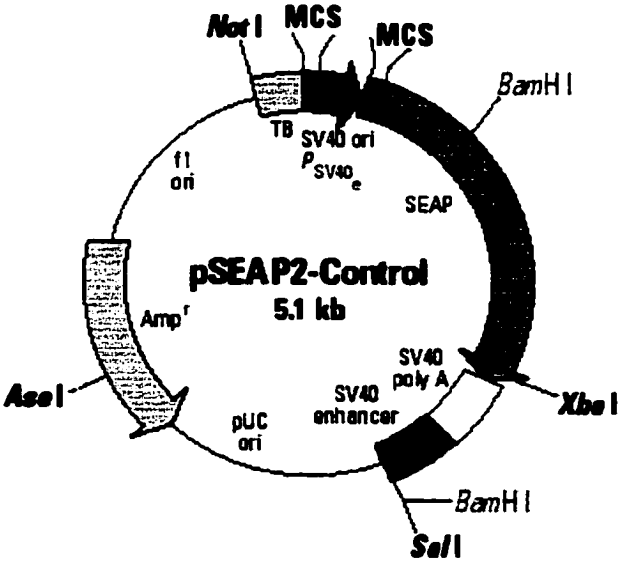
5' **CGC GT**A GGT CAC AGT GAC CT**G ATC AAA GTT AAT GTA ACC TCA** 3'

A



TB=Transcription Blocker

B



into the ends. It was restricted using *Mlu*I and *Bgl*II, and cloned into the d2EGFP Clontech vector. Proper cloning was verified by restriction digest and sequencing. The orientation of the fragment was accounted for by the use of different restriction sites at the ends. The vector has a kanamycin resistance gene, which was used in bacterial selection. Proper functioning of the vector was verified by trial transfections and fluorescent microscopy (see Results).

8 – Transfection and Reporter Assay

VSMCs were seeded at 1 x 10^5 cells/ well in 6 well plates the day before transfection, and counted by haemocytometer. VSMCs were plated in phenol red-free DMEM with 10% FBS so that they were 50-95% confluent on the day of transfection. No antibiotics were used at the time of plating or during transfection. 18h later, cells were transfected with the reporter vector and liposomes combined at a ratio of 12:1 (DNA/liposomes) in polystyrene tubes. The liposome mixture employed was (1:1): 1,2 dioleolyl-3-N, N, N, - trimethylammonium-propane or DOTAP and dioleoylphosphatidylethanolamine or DOPE (Avanti Polar Lipids, Alabaster, Alabama, U.S.A.). Prior to the day of transfection, liposomes were extruded using an Avestin liposome extruder. The use of the extruder creates vesicles that have consistent size and structure, and are unilamellar (133). The cell media was switched to 1ml non-supplemented DMEM and the cells were incubated with the DNA-liposome mixture for a total of 5 hours. At the end of the incubation, cell culture medium components were added to the cell media to achieve normal volume (3ml) and serum concentration

(10%). 24 hours after transfection initiation, the cell media was switched to 10% stripped serum DMEM. At 42h after the transfection initiation, one of the following ligands was introduced: 1μM 17-β estradiol, 1μM faslodex, 1μM 4-hydroxytamoxifen. Samples were collected 6 hours later, removing 110μl of cell media for the SEAP assay, and adding 150μl of RIPA lysis buffer to collect protein lysate for the EGFP assay. The cells were counted at the time of ligand addition and just prior to lysate collection to verify adverse effects of ligand on cell number. Counting was achieved by quadrant counting of dish photographs (please refer to Appendix C). The transfection efficiency was normalized by cotransfection with a secreted alkaline phosphatase (SEAP) overexpression vector (pSEAP2-control by Clontech). The ratio of pSEAP to the reporter was 1:1.24, and approximately 1μg of each vector was used per well. This vector was under the transcriptional control of a different viral promoter than the reporter vector. The production of EGFP was evaluated using the total cell lysate. A fluorometer (Polarstar galaxy) was used to measure emission in the range of 520nm after an excitation pulse at 485nm. The excitation peak of EGPF is 488nm. The emission peak of EGFP is 507nm (134). Polystyrene Nalge Nunc, 384 well plate black, without lids, non-treated, non-sterile, with 120μl wells were used to perform fluorescence readings. Purified EGFP (Clontech) was used to construct a standard curve from which the EGFP concentration of the experimental samples was calculated. These results were normalized to the SEAP results. SEAP samples were collected from the cell media and their luminescence was measured in a luminometer and compared to a standard

curve using Dynex Technologies white microtiter, microlite 96 well plates. The Clontech Great EscAPe chemiluminescent detection kit with CSPD chemiluminescent substrate was used in to produce the luminescence in the SEAP samples. SEAP is a highly sensitive reporter system. SEAP is heat-stable, resistant to most phosphatase inhibitors including L-homoarginine, and well suited for quantification. It has been reported that quantities as small as10-13pg can be detected with use of the chemiluminescent substrate (135,136,137). For details on the calculation for the reporter assay, please refer to Appendix C. The conditions of the transfection experiment (e.g. ratio of DNA to liposome, length of incubation time) were optimized for human aortic VSMCs prior to experimentation.

Results

1. Expression and modulation of ERs in human aortic VSMCs

The expression pattern of ERβ and ERα *in-vitro* was determined by means of Western blotting and immunofluorescence. Antisense oligonucleotide treatments for human aortic VSMCs were optimized and tested to enable subsequent modulation of ER expression.

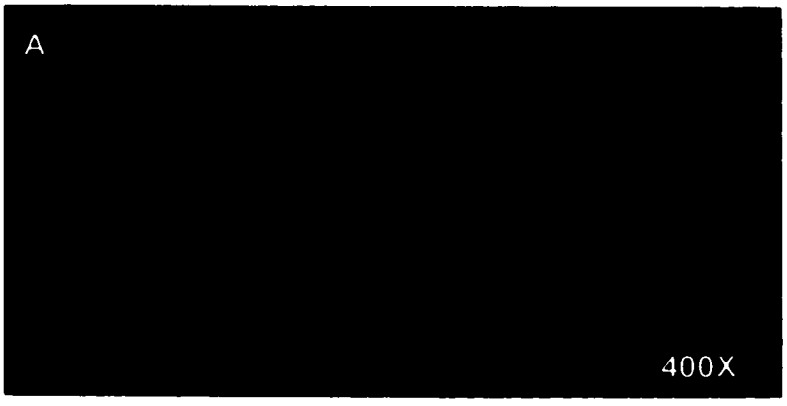
Characterization of human primary VSMCs by immunofluorescence

Primary human aortic VSMCs were characterized for smooth muscle specific markers smooth muscle alpha actin (Figure 9), and caldesmon. As would be expected, the cells were immunopositive in both cases and the actin localized to strands in the cytoplasm. Cells were also characterized for the endothelial cell specific marker von Willebrand Factor, which is not expressed in VSMCs. The immunolabeling was negative. Figure 10 displays positive immunolabeling for ERβ in the cytosol. The expression of ERα is shown in Figure 11. In contrast to the results for ERβ, all immunolabeling for ERα localized to the nucleus. Controls for the secondary antibody revealed essentially no background staining. The identity of the human cell line and the expression of ERs were confirmed by immunocytochemical analysis.

Detection of ERβ and ERα expression by Western blotting

Primary aortic VSMCs were verified for the expression of ERα and ERβ at 6h after the addition of 1μM 17-β estradiol or vehicle (ethanol) (Figure 12). Expression of both ERβ and ERα was observed in both the presence and

Figure 9. Immunofluorescent staining for smooth muscle alpha-actin in human aortic VSMCs. Panel A shows a 400X magnification of cells labeled with a primary antibody to smooth muscle alpha actin and a secondary antibody labeled with fluorescein. Panel B shows a 600X magnification of cells labeled with a primary antibody to smooth muscle alpha actin, a secondary antibody labeled with fluorescein and a Hoechst nuclear stain. Panel C shows a 400X magnification of cells labeled with no primary antibody, but with the fluorescein secondary antibody (control).


A
400X


B
600X

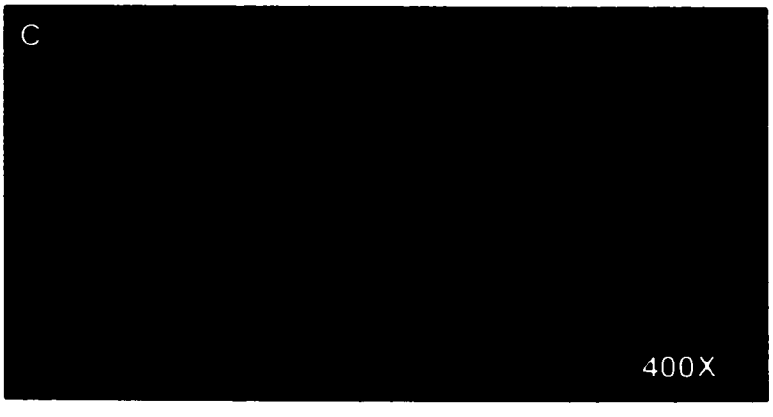
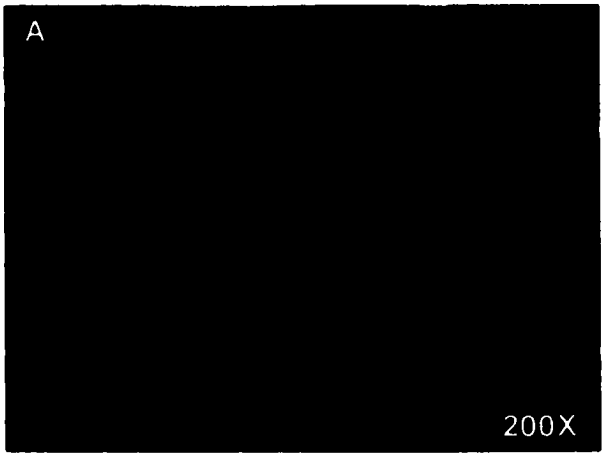
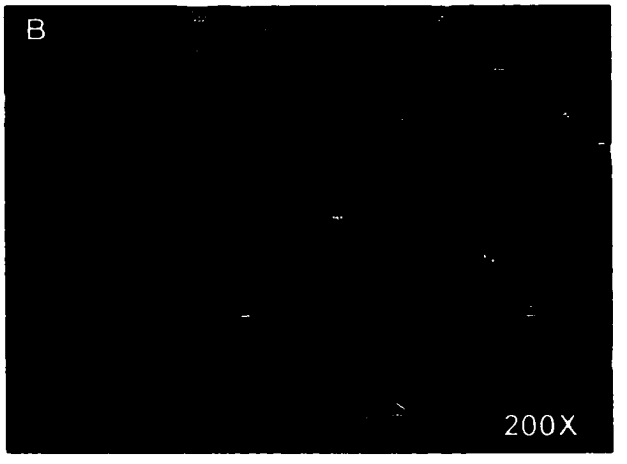

C
400X

Figure 10. Immunofluorescent staining for ERβ in human aortic VSMCs. Panel A shows a 200X magnification of cells labeled with a primary antibody to ERβ, and a secondary antibody labeled with Texas Red. Panel B shows a 200X magnification of the same cells, displaying the Hoechst nuclear stain and labeled with a primary antibody to ERβ, and a secondary antibody labeled with Texas Red. Panel C shows a 200X magnification of cells labeled with no primary antibody, but with the Texas Red secondary antibody (control).


A
200X


B
200X


C
200X

Figure 11. Immunofluorescent staining for ERα in human aortic VSMCs. Panel A shows a 200X magnification of cells labeled with a primary antibody to ERα and a secondary antibody labeled with fluorescein. Panel B shows a 600X magnification of cells labeled with a primary antibody to ERα and a secondary antibody labeled with fluorescein. Panel C shows a 200X magnification of Hoechst-stained cells. Panel D shows a 600X magnification of cells stained with Hoechst. The control corresponding to this experiment is displayed in Figure 9, panel C.

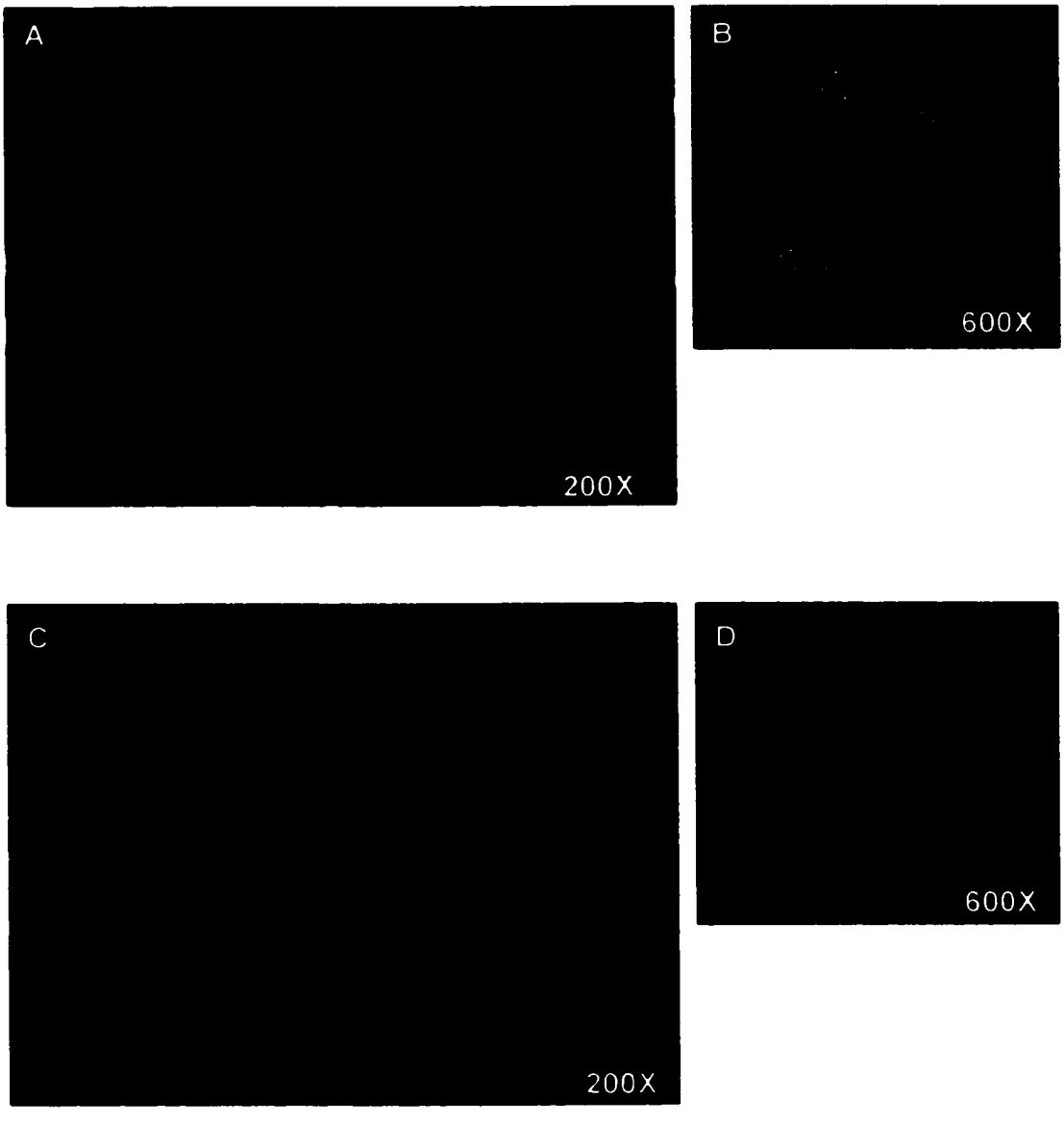
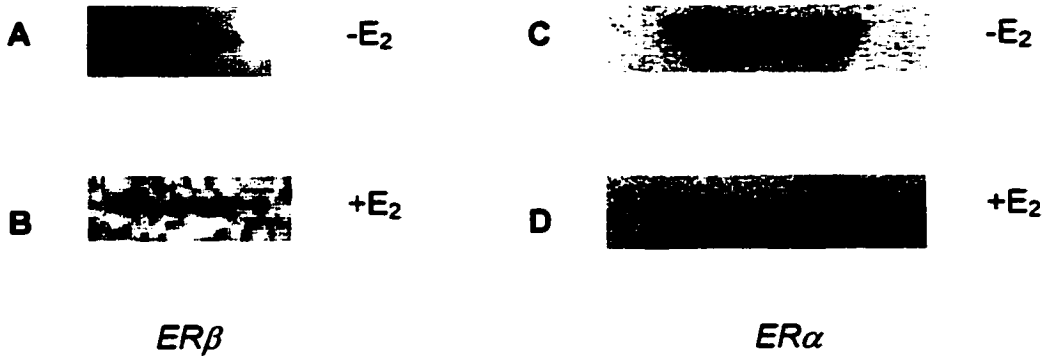

A
200X
B
600X
C
200X
D
600X

Figure 12. Western blots demonstrating ERβ and ERα expression in human aortic VSMCs. Panels A and B show the expression of ERβ at 6h and panels C and D show the expression of ERα at 6h following exposure to 17-β estradiol or vehicle control.. In panel A, cells were grown in the absence of 17-β estradiol and total protein lysates were electrophoresed and blotted onto a nitrocellulose membrane which was subsequently incubated with a primary antibody to ERβ, followed by a peroxidase conjugated secondary antibody. In panel B, cells were grown in the presence of 17-β estradiol, and the total protein lysates were subjected to the procedure described for panel A. In panel C, cells were grown in the absence of 17-β estradiol and total protein lysates were electrophoresed and blotted onto a nitrocellulose membrane which was subsequently incubated with a primary antibody to ERα, followed by a peroxidase conjugated secondary antibody. In panel D, cells were grown in the presence of 17-β estradiol, and the total protein lysates were subjected to the procedure described for panel C. These blots are representative of experiments that were performed in triplicate. ERβ band is at approx. 65kDA. ERα band is at approx. 67kDA. Panel E shows the expression of uPA in the primary VSMC line. Total cell lysate was blotted onto a nitrocellulose membrane and incubated with a primary antibody to the B-chain of uPA, followed by a peroxidase-conjugated secondary antibody. The uPA band is at 33kDA.


A
-E2
C
-E2
B
+E2
D
+E2
ERβ
ERα

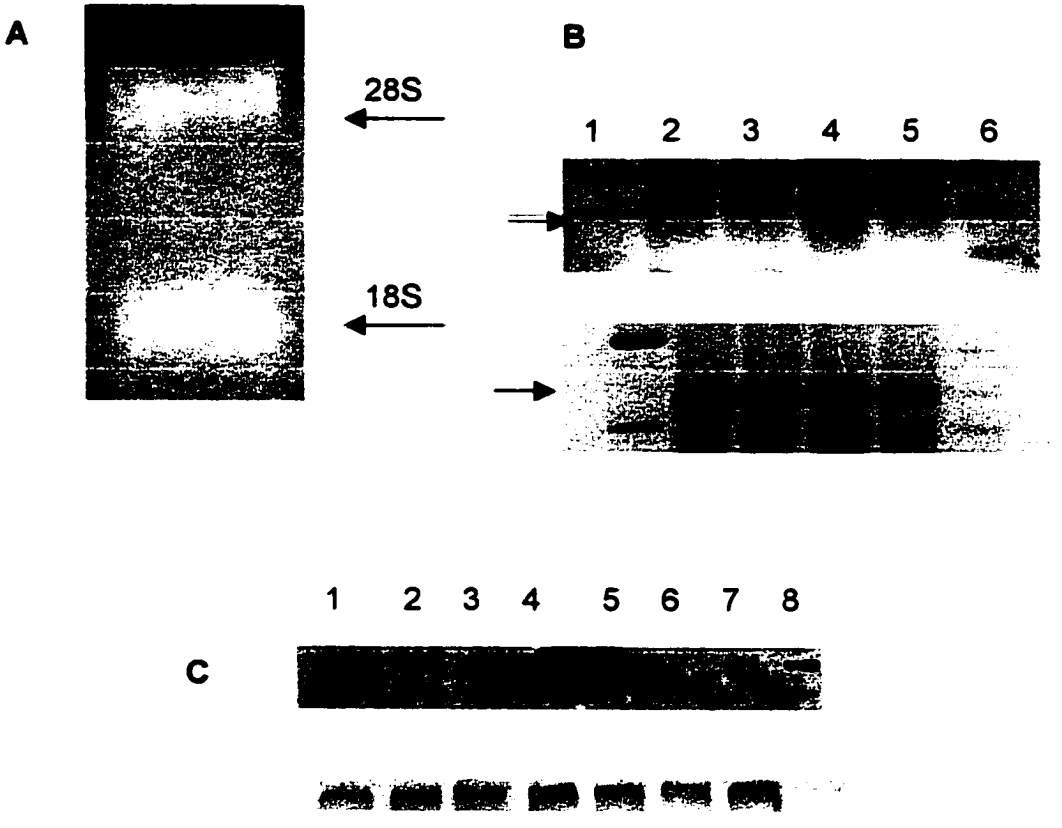

E
uPA

absence of 17-β estradiol. Cells were also characterized for the expression of the uPA B-chain in total cell lysate. The uPA B-chain is detectable in both the active and inactive forms of the protein. Expression of B-chain uPA was detected, as expected. The expression of ERs in the human cell line was further confirmed by Western blotting for a specific time point of interest.

Antisense optimization and testing

Cells were treated with antisense oligonucleotides to ERβ, to suppress the expression of ERβ. The conditions for the antisense treatments were optimized for the aortic VSMCs used. To verify the efficacy of the antisense treatment, RT-PCR studies were employed to evaluate ERβ mRNA production, and Western blotting was used to evaluate ERβ protein levels. Figure 13, panel A, shows successful isolation of total RNA from VSMCs. This step is a prerequisite to RT-PCR. The ribosomal subunit RNA appears as two strong bands on the formaldehyde/agarose gel. The upper band is derived from the 28S subunit, while the lower band is derived from the 18S subunit. Panel B shows a representative agarose gel of the DNA amplicons from the ERβ semi-quantitative RT PCR experiments. Lane 2 clearly shows the reduction of the ERβ mRNA in the antisense treated sample as compared to the baseline sample in lane 4. Parallel GAPDH gels were run for each sample in order to normalize the values for quantitation. The mean reduction in ERβ mRNA was found to be 59±6%. It should be noted that on several occasions, due to the low abundance of the ERβ message in the cells, the signal from the antisense-treated sample was difficult to

Figure 13. Antisense to ERβ. Panel A shows successful isolation of aortic VSMC total RNA. The 28S and 18S ribosomal RNA bands are indicated. The RNA samples were subjected to formaldehyde-agarose electrophoresis. Panel B shows two representative RT PCR gels. The upper gel shows reverse transcribed ERβ mRNA. The arrow indicates the position of the ERβ amplicon. Lanes 1 and 6 show the New England Biolabs100bp ladder, specifically, the 400, 300 and 200bp bands. Lane 2 shows the antisense ERβ band, lane 3 shows the negative control band (water template), lane 4 shows the baseline (untreated) band, and lane 5 shows the scrambled control band. Parallel GAPDH RT PCR gels were run for each ERβ RT PCR gel (gel below). The GAPDH amplicon is located at 133bp. The bands displayed in the lane 1 ladder represent the 100 and 200bp bands. Panel C shows a representative western blot for ERβ. Lane 1 shows the band for untreated cells (baseline sample), lane 2 shows the band for cells treated with antisense to ERβ, lane 3 shows a positive control sample band (rat lung homogenate), lane 4 shows the band for cells treated with scrambled control, lane 5 shows the negative control sample (HeLa cell lysate), lane 6 shows a second positive control band (rat kidney homogenate), lane 7 is empty, and lane 8 indicates positions of bands in the N.E.B. broad range protein ladder. Beneath the blot is a corresponding loading control to β-actin . All experiments were performed in triplicate.


A
28S
18S
B
1 2 3 4 5 6
1 2 3 4 5 6 7 8
C

detect and quantitate properly. Panel C shows a representative Western blot of VSMC lysates probed for ERβ. The ERβ band localizes to approximately 63kDa. Lane 1 shows the baseline (untreated) cell lysates ERβ band, and lane 4 shows the scrambled control ERβ band (which controls for the specificity of the oligonucleotide). In lanes 3 and 6, rat lung and rat kidney homogenates were employed as positive controls, because both of these tissues are known to express ERβ (138,139). As anticipated, both bands are present. HeLa cell lysate was used as a negative control, since HeLa cells express neither ERα nor ERβ, and no band was apparent (140). Reduction in ERβ protein expression was evident in the antisense-treated sample (lane 2). The intensity of the bands were quantified in all blots and the mean reduction in ERβ expression relative to baseline, was determined to be 43±8%. The use of ERβ antisense oligonucleotide resulted in reduced ERβ.

1. The effect of modulation of ER expression on uPA activity.

ER ligands 17-β estradiol, faslodex, and 4-hydroxytamoxifen, and ERβ antisense were used to explore the effect of modulation of ER expression on uPA activity. Further investigation combined the antiestrogens 4-hydroxytamoxifen and faslodex with the inhibitors cycloheximide, actinomycin D, and tunicamycin. Subsequent transfections with an ERE-based reporter vector monitored the production of EGFP in the presence of ER ligands 17-β estradiol, faslodex, and 4-hydroxytamoxifen in addition to antisense to ERβ.

ER ligands and uPA activity

Figure 14 shows the effect of the addition of ER ligands 17-β estradiol, faslodex, and 4-hydroxytamoxifen on uPA activity in the human aortic VSMCs. uPA activity in the cells was very low at baseline, and the addition of 1μM 17-β estradiol did not alter the activity level in any detectable way. In contrast, the addition of 1μM of ER antagonist faslodex or 1μM of partial antagonist 4-hydroxytamoxifen resulted in a fifty-fold elevation in uPA activity levels, relative to baseline. This activity could be reduced by approximately 90%, for both faslodex and 4-hydroxytamoxifen, in the presence of 2μg/ml of actinomycin D, an inhibitor that blocks the production of new RNA (Figure 15). The reduction for the faslodex-treated cells was marginally greater than that observed in the 4-hydroxytamoxifen treated cells. Similarly, the high uPA activity observed in conjunction with faslodex and 4-hydroxytamoxifen treatments was also drastically reduced in the presence of 10μg/ml tunicamycin, an inhibitor of N-linked glycosylation (secretion). Figure 16 shows that this decrease is, yet again, in the order of 90%. The combination of 1μg/ml cycloheximide, an inhibitor of *de-novo* protein synthesis, and the ER ligands, also resulted in a decrease in uPA activity levels of about 90% and is marginally greater for 4-hydroxytamoxifen (Figure 17). Specific ER ligands, but not 17-β estradiol itself, modified the activity of uPA in a manner that was dependent on transcription, translation and secretion.

Figure 14. uPA activity in human aortic VSMCs treated with ER ligands or ERβ antisense. Cells were maintained under baseline conditions or treated with 1μM 17-β estradiol, 1μM faslodex, 1μM 4-hydroxytamoxifen (4-OHT) for 6h, or antisense to ERβ. The faslodex, 4-OHT and ERβ antisense group values were determined to be significantly different relative to the baseline group, with P=<0.001 (indicated with asterisks). All experiments were performed in triplicate.

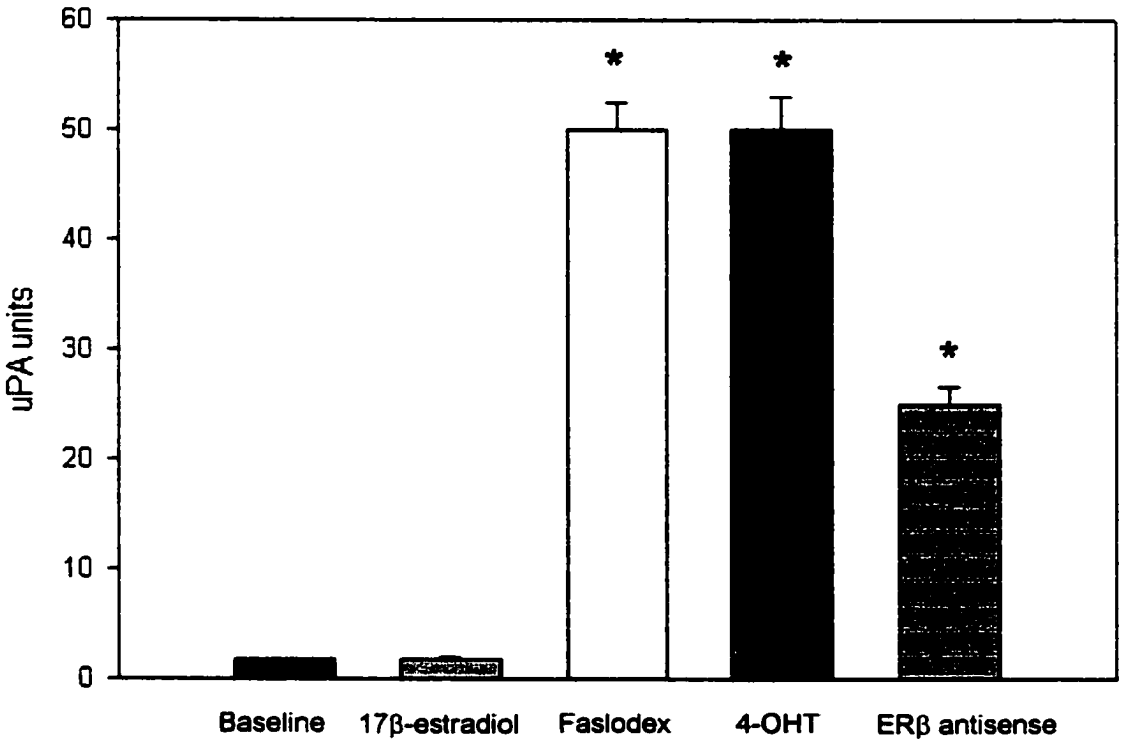

60
50
40
30
20
10
0
uPA units
*
*
*
Baseline
17β-estradiol
Faslodex
4-OHT
ERβ antisense

Figure 15. The effect of actinomycin D on uPA activity in human aortic VSMCs treated with faslodex or 4-hydroxytamoxifen. Cells were treated with 2μg/ml actinomycin D (Act.), actinomycin D + 1μM faslodex (Act.+Faslo), or actinomycin D + 1μM 4-hydroxytamoxifen (Act.+4-OHT), for 6h and compared with the baseline, faslodex and 4-hydroxytamoxifen treated groups. Actinomycin D blocks production of new mRNA. Data for faslodex and 4-OHT are repeated from Figure 14. Values were determined to be significantly different relative to their actinomycin treated counterparts, with $P=<0.01$. All experiments were performed in triplicate.

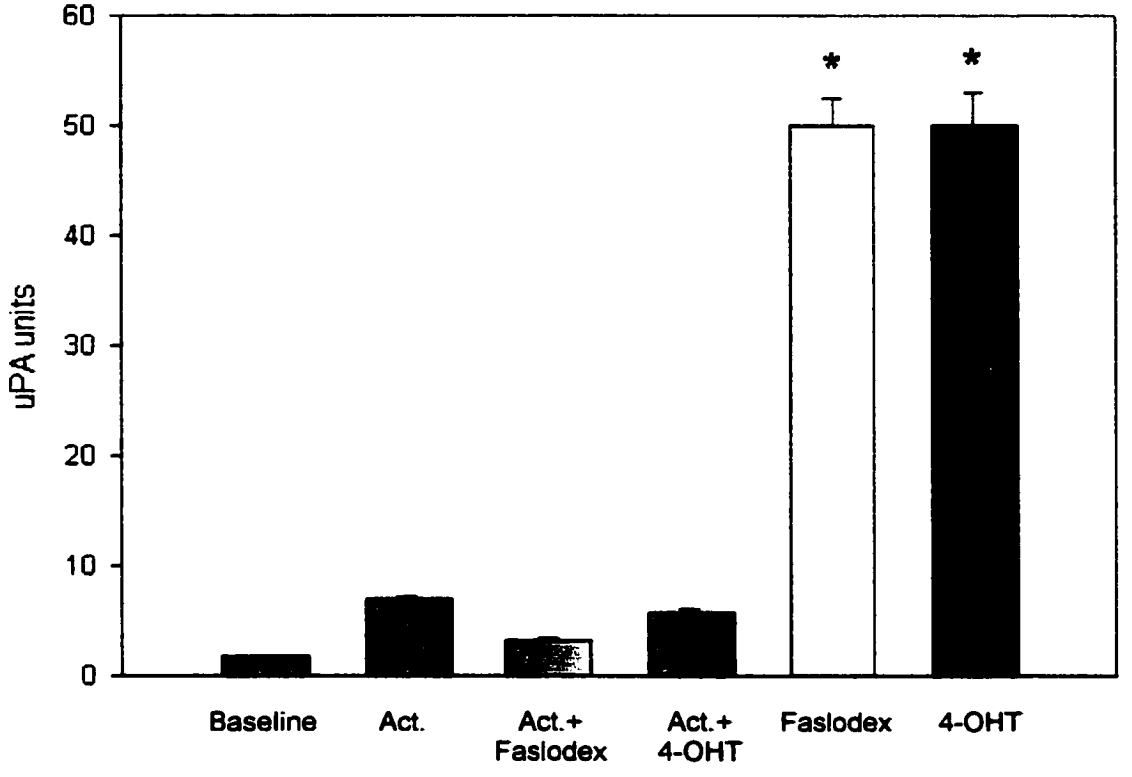

60
50
40
30
20
10
0
uPA units
*
*
Baseline
Act.
Act.+ Faslodex
Act.+ 4-OHT
Faslodex
4-OHT

Figure 16. The effect of cycloheximide on uPA activity in human aortic VSMCs treated with faslodex or 4-hydroxytamoxifen. Cells were treated with 10μg/ml cycloheximide (Cyc.), cycloheximide + 1μM faslodex (Cyc.+Faslo), or cycloheximide + 1μM 4-hydroxytamoxifen (Cyc.+4-OHT) for 6h, as per legend for figure 13. Cycloheximide is use to block *de-novo* protein synthesis. Data for faslodex and 4-OHT are repeated from Figure 14. Values were determined to be significantly different relative to their actinomycin treated counterparts, with P=<0.01. All experiments were performed in triplicate.

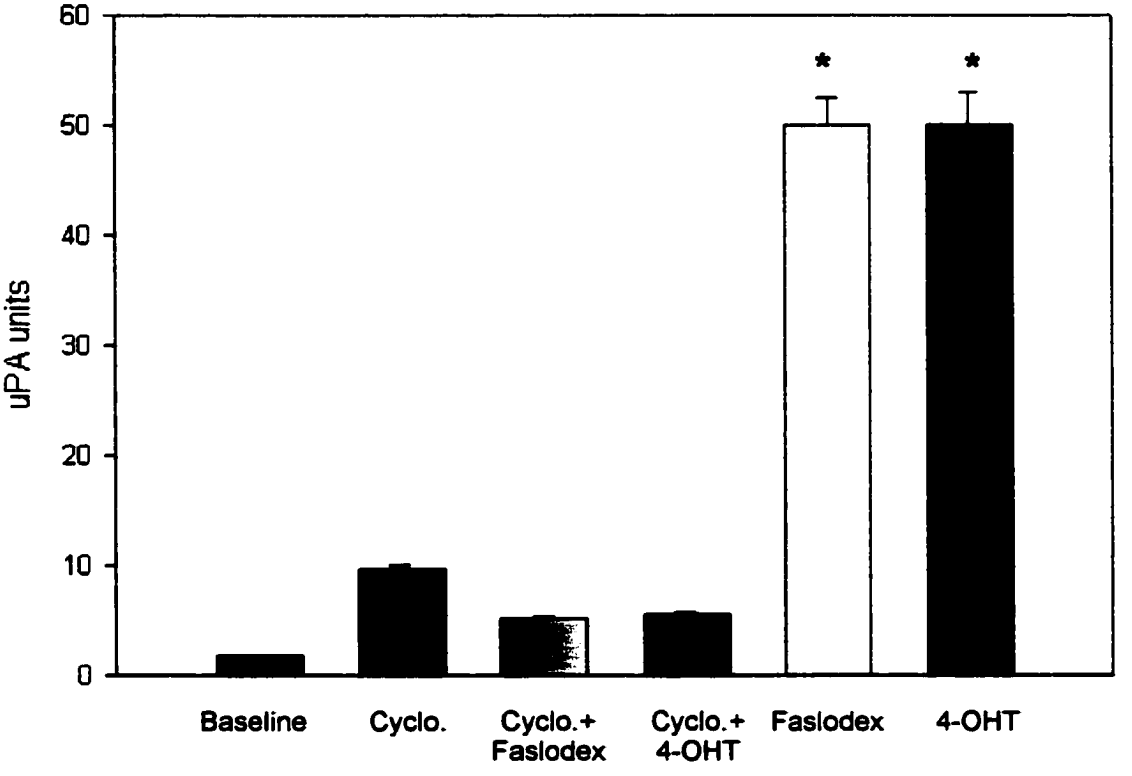
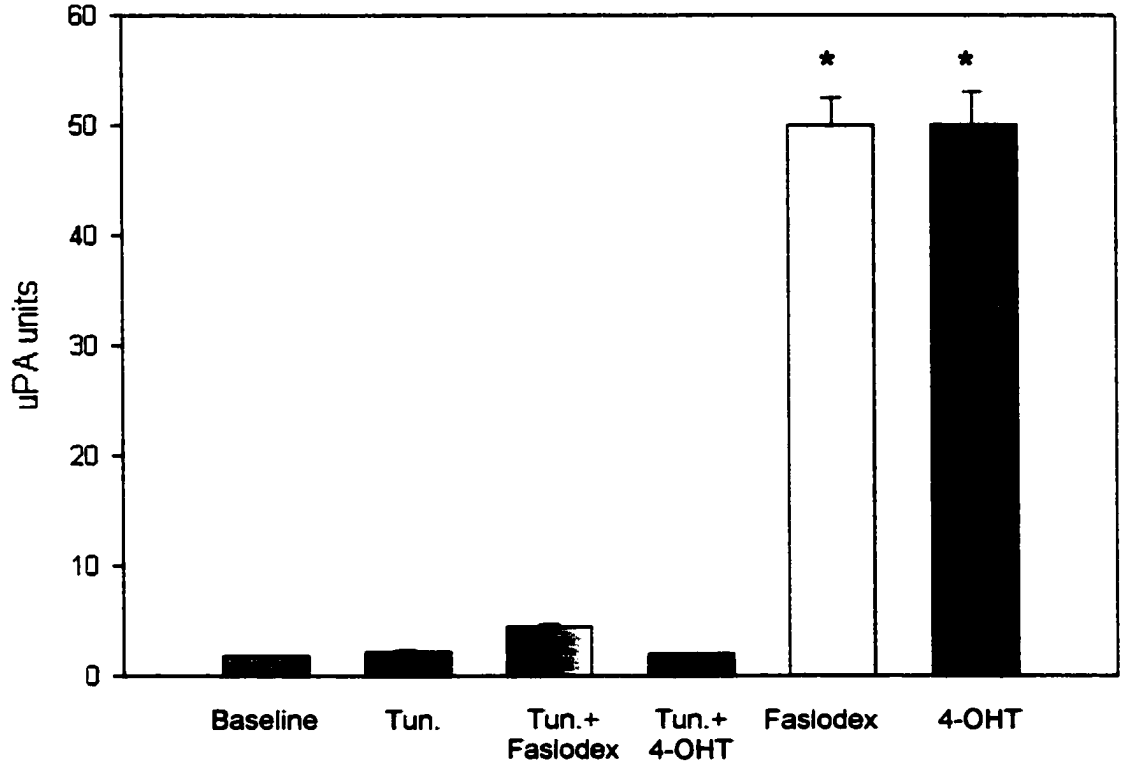

60
50
40
30
20
10
0
uPA units
*
*
Baseline
Cyclo.
Cyclo.+ Faslodex
Cyclo.+ 4-OHT
Faslodex
4-OHT

Figure 17. The effect of tunicamycin on uPA-type plasminogen activator activity in human aortic vascular smooth muscle cells treated with faslodex or 4-hydroxytamoxifen. Cells were treated with 1µg/ml tunicamycin (Tun.), tunicamycin + 1µM faslodex (Tun.+Faslo), or tunicamycin + 1µM 4-hydroxytamoxifen (Tun.+4-OHT) for 6h, as per legend for figure 13. Tunicamycin is used to block N-linked glycosylation in regulated protein secretion. Data for faslodex and 4-OHT are repeated from Figure 14. Values were determined to be significantly different relative to their actinomycin treated counterparts, with $P=<0.01$. All experiments were performed in triplicate.


60
50
40
30
20
10
0
uPA units
*
*
Baseline
Tun.
Tun.+ Faslodex
Tun.+ 4-OHT
Faslodex
4-OHT

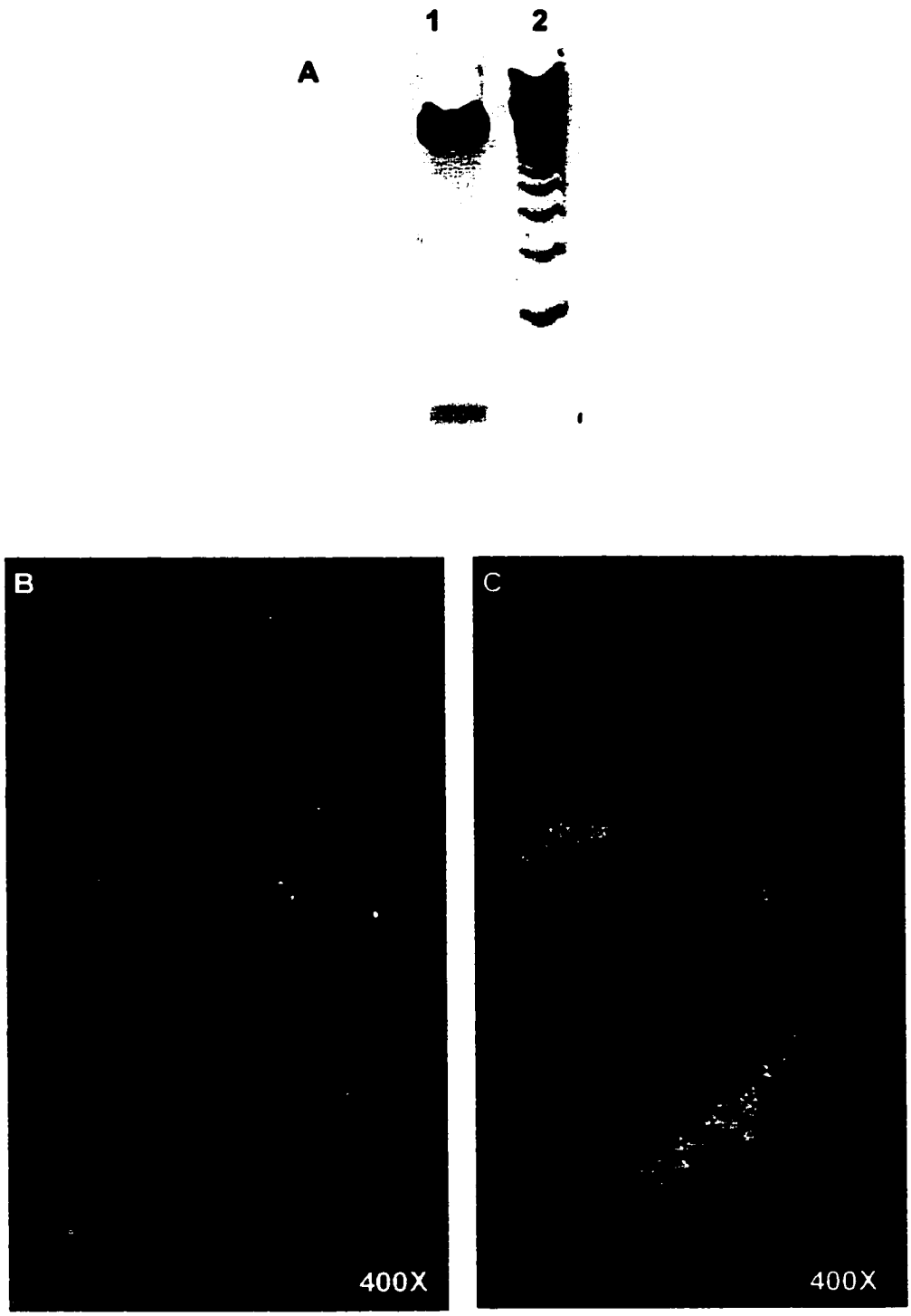
Antisense uPA Assay

VSMCs were treated with antisense oligonucleotides to ERβ, for a period of 24 hours and then evaluated for uPA activity 6 hours later. Scrambled oligonucleotides, consisting of the same nucleotides as found in the antisense oligonucleotides, but in a random, irrelevant sequence, were also employed as controls. The scrambled oligonucleotides produced no change relative to baseline uPA activity levels. Figure14 shows that incubation with antisense to ERβ resulted in an increase in uPA levels to approximately 25 times baseline levels. Therefore, antisense to ERβ results in an increase in the activity of uPA.

Reporter construct design and testing

A reporter vector, with the enhanced green fluorescent protein (EGFP) gene under the transcriptional control of a canonical estrogen response element, (ERE) was successfully designed and cloned. This vector was designed as a means to indicate the effect of ER ligands and antisense on transcription from an ERE-containing promoter. Figure 18 shows the release of the 42 base pair ERE insert from the construct after restriction digest of the purified vector with restriction enzymes *Mlu*I and *Bgl*II. The vector was subsequently introduced into MCF-7 cells by means of transient liposome-mediated transfections, and production of EGFP was observed by fluorescent microscopy (Figure 18, panels B and C).

Figure 18. Development of the estrogen-response element reporter vector. Panel A shows restriction digest of reporter vector pd2EGFPERE, in a polyacrylamide gel. A 42bp *Xenopus laevis* vitellogenin enhancer was cloned into the promoter region of the EGFP reporter vector, conferring estrogen-responsiveness. The reporter construct was digested with *Mlu*I and *Bgl*II to release this fragment, as proof of correct cloning. Lane 1 shows the fragment. Lane 2 shows the New England Biolabs 100bp ladder. Panel B shows a phase contrast image at 400X magnification, of a cell transfected with the reporter vector. Panel C shows a fluorescent micrograph of the same cell, at an identical magnification.


A
1
2
B
400X
C
400X

Ligand Reporter Assay

Figure 19 shows the expression of the reporter gene product, EGFP, in response to treatment of the cells with ER ligands for a period of six hours. The quantification of the reporter was achieved by means of fluorometry against a standard curve of purified protein. Results were normalized to the expression of a co-transfected secreted alkaline phosphatase vector. The negative control consists of a cotransfection of the normalization vector and the parent vector of the reporter plasmid. Expression of EGFP in the negative control was absent. The positive control consisted of a cotransfection of the normalization vector and a vector that constitutively expresses EGFP. As anticipated, elevated levels of EGFP expression were observed in the positive control samples. The addition of 17-β estradiol, the physiological ligand, produced a 95% increase in EGFP production over baseline. Faslodex, a 17-β estradiol antagonist, produced a 70% increase in EGFP expression relative to the baseline level. 4-OHT, a partial antagonist, produced an increase of 50% relative to baseline. The transcriptional activity of the ERs in the VSMCs, as quantified by means of a reporter gene, was modulated by ER ligands.

Antisense Reporter Assay

Figure 20 shows the expression of the reporter gene product, in response to the treatment of the transiently transfected cells with antisense oligonucleotides to ERβ for a period of twenty-four hours. Negative and positive controls for the reporter vector were performed as mentioned above. Scrambled

Figure 19. Expression of EGFP in reporter assay ER ligand experiment. EGFP values are expressed as a percentage of the baseline value. Aortic vascular smooth muscle cells were transfected with the reporter vector, and subsequently treated with a particular estrogen receptor ligand for a period of six hours. The baseline group was not treated with any ligand, the 17-β estradiol group was treated with 1μM 17-β estradiol, the faslodex group was treated with 1μM faslodex, and the 4-OHT group was treated with 1μM 4-hydroxytamoxifen. ANOVA statistical analysis indicated that there was a significant difference between the groups with $P=<0.01$. 17-β estradiol, faslodex and 4-OHT were significantly different relative to baseline according to the Tukey secondary statistical analysis.with $P<0.05$ (indicated with asterisks). All experiments were performed in triplicate.

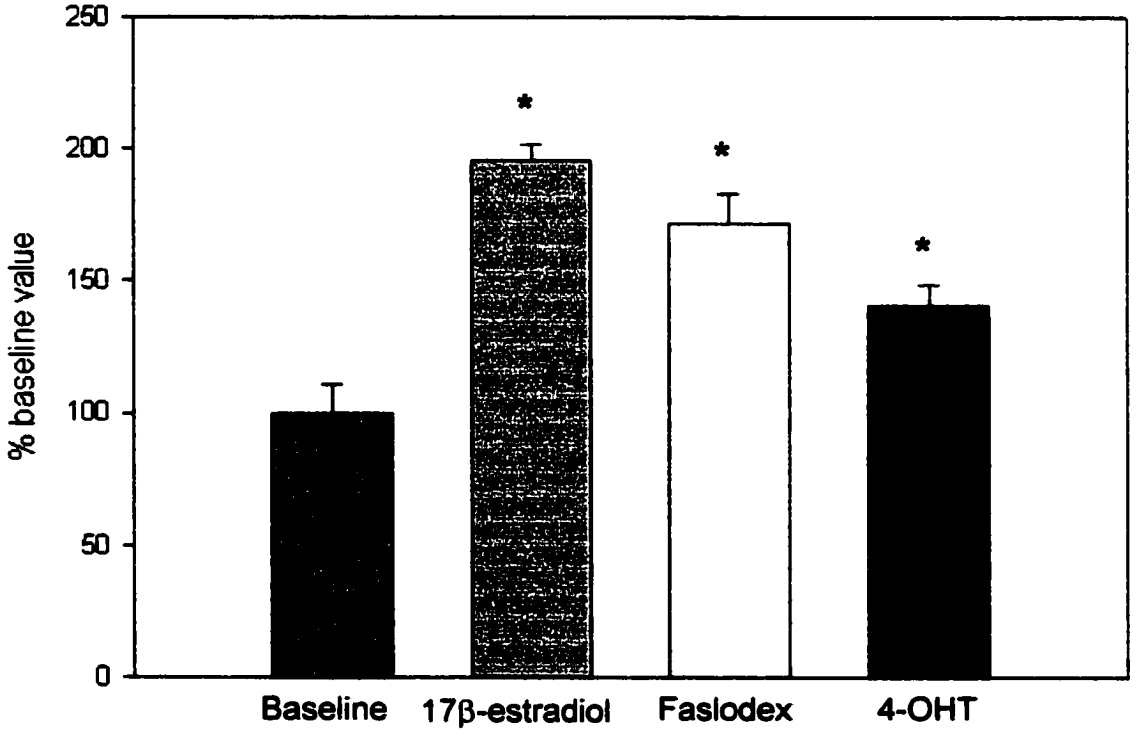
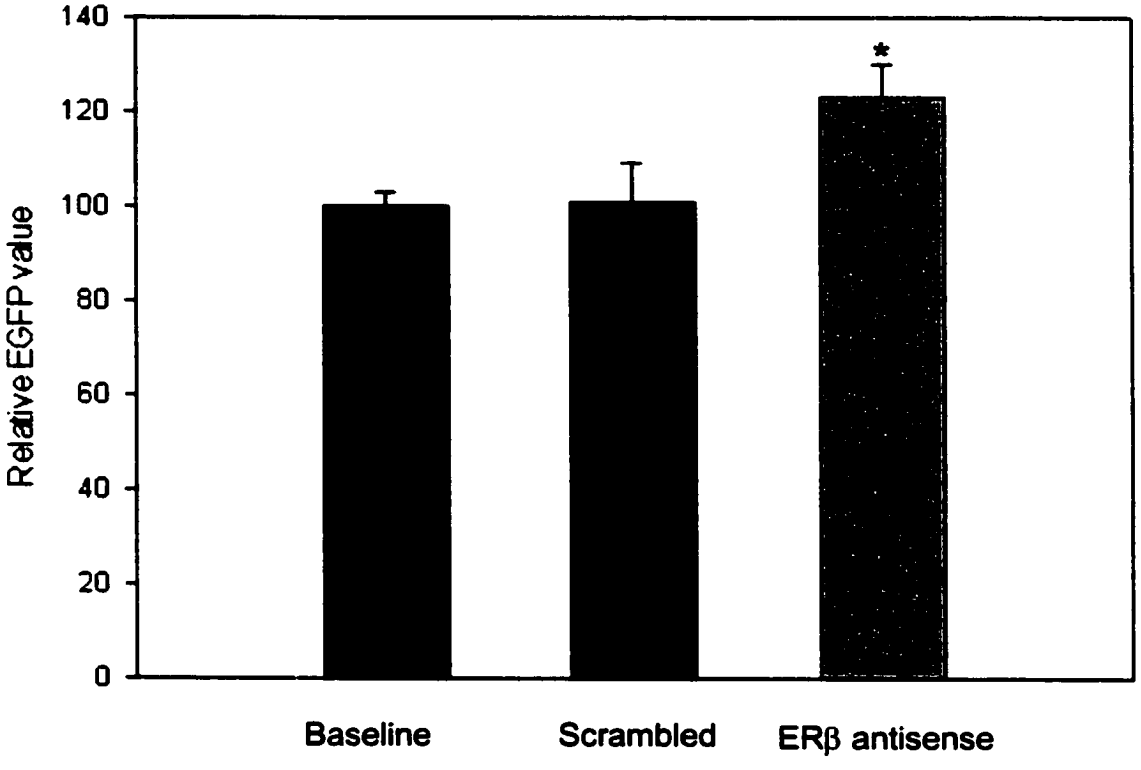

250
200
150
100
50
0
% baseline value
*
*
*
Baseline
17β-estradiol
Faslodex
4-OHT

Figure 20. Expression of EGFP in reporter assay ERβ antisense experiment. EGFP values are expressed as a percentage of the baseline value. Aortic vascular smooth muscle cells were transfected with the reporter vector, and subsequently treated ERβ antisense. The baseline group was not treated with any antisense; while the antisense ERβ group was treated with antisense to ERβ. A scrambled control was also tested to ensure the specificity of the antisense effects (not shown). ANOVA statistical analysis indicated that there was a significant difference between the groups with $P=<0.01$. Antisense ERβ was significantly different relative to baseline according to the Tukey secondary statistical analysis.with $P<0.05$ (indicated with asterisk). All experiments were performed in triplicate.


140
120
100
80
60
40
20
0
Relative EGFP value
*
Baseline
Scrambled
ERβ antisense

oligonucleotides were employed as controls for specificity of the antisense oligonucleotides. The addition of the scrambled oligonucleotides produced no significant changes relative to the baseline value, as expected. The addition of antisense to ERβ produced a small but statistically significant change in EGFP production over baseline. The transcriptional activity of the ERs in the VSMCs, as quantified by means of a reporter gene, was not greatly modulated by the addition of antisense to ERβ.

Discussion

Cellular localization of ERs

The presence of ERα in the nucleus is consistent with previous immunocytochemical analyses (Figure 11) (78). The preferential localization of ERβ to the cytosol has been previously reported in human osteoclasts, rat astrocytes, and rabbit and rat ovarian insterstitial gland cells (141,142,143,144). Since there is increasing evidence for the existence of a cell surface receptor, it is possible that some of the immunopositive fluorescence represented membrane expression of ERβ, however it is not possible to verify this from the immunocytochemical images. Variation in the distribution of the receptor types may hint at a disparity of roles within the cell. It has been shown that the α-α homodimer is the favoured receptor dimer for 17-β estradiol binding, which may explain the presence of ERα in the nucleus (55). The predominance of ERα in the nucleus allows for more immediate access to target gene promoter regions, and hence, the ability to influence transcriptional activation or repression more rapidly. By contrast, ERβ may be implicated with cell surface ER expression, shuttling between the membrane and the cytosol.

uPA activity

The preliminary tests of uPA activity examined several time points. There was a significant drop in uPA activity in the 24h samples compared to the 6h samples (data not shown). This suggests a rapid onset of effects on uPA activity. In the 6h study, the baseline and 17-β estradiol treated cells had

equivalent uPA activities. An extreme boost (50-fold) was observed with the addition of faslodex or 4-OHT (Figure 14). Faslodex and 4-OHT are both ER ligands that can act as ER antagonists (145). In order to elucidate the mechanism of action of this increase in activity, the cells were treated with metabolic inhibitors +/- the antagonists. The addition of actinomycin D severely diminished the previously observed effects of 4-OHT and faslodex. This suggests that transcription is an important mediator of the observed increase in uPA activity. This is not surprising given the role of ERs as nuclear transcription factors (see introduction). The addition of cycloheximide was also able to severely reduce the effects of faslodex and 4-OHT. This suggests that protein synthesis is also implicated. Addition of tunicamycin also ablated the effect of the antagonists. Tunicamycin blocks N-linked glycosylation of proteins, a post-translational modification that occurs on proteins undergoing regulated secretion. It gives a valuable clue as to the possible candidates for this pathway. Notably, uPA itself is N-glycosylated (146).

A proteolytic enzyme such as uPA is under strict regulation at all levels of its production and release. Two mechanisms could potentially explain the effects of 4-OHT and faslodex on uPA activity in human VSMCs. The first possibility is that the regulation of uPA occurs via ER interaction with the uPA gene promoter region; that is to say, regulation is at the level of transcription. This would entail the action of the ERs as transcription factors in coregulatory protein transcriptional complexes associated with AP-1 or Sp1 enhancer elements. The ability of the ERs to modulate uPA transcription would therefore be subject, at

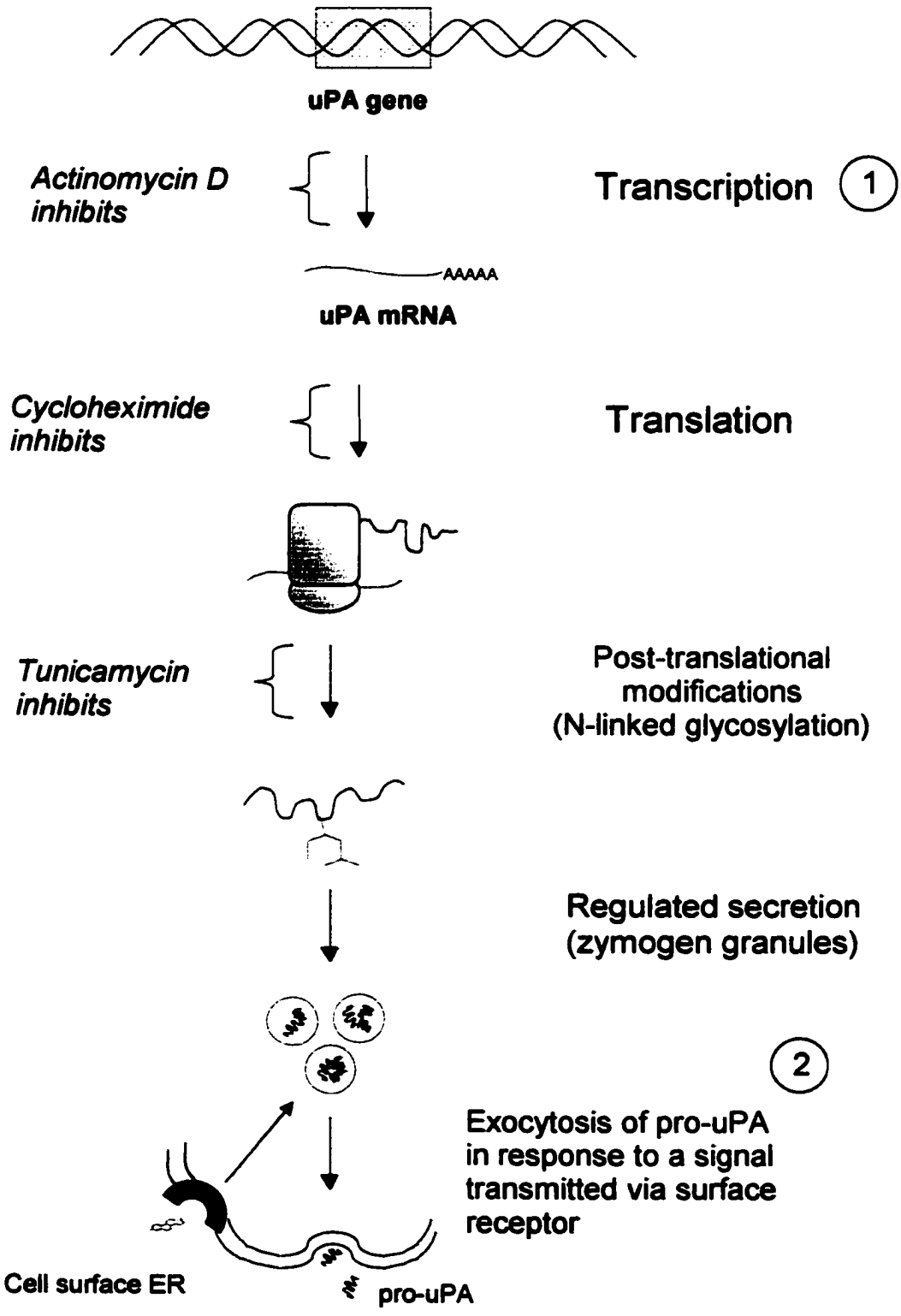
least in part, to the binding of specific ER ligands. The second possibility is that the ER ligands are signaling the exocytosis of pro-uPA zymogen granules by interacting with a cell surface ER; and thus, regulation is at the level of secretion. This would permit a rapid response, as observed in the dramatic increase in uPA activity a mere six hours after the addition of ligand. It is important to note that these two mechanisms are not mutually exclusive. The point of regulation in the uPA synthesis and secretion pathway for each proposal is represented in Figure 21. The first option is represented by the number one, while the second option is indicated by the number two. Both possible explanations are discussed in further detail below.

Transcriptional regulation at the uPA promoter

The possibility of regulation via the ERE enhancer

The reporter transfection experiments suggest that the classical ERE pathway is not directly implicated in the observed effects of ER ligands on uPA. In the uPA experiment, the addition of antiestrogens 4-OHT and faslodex resulted in a dramatic increase in uPA activity, and the addition of 17-β estradiol, the physiological ligand, had no effect relative to baseline conditions (Figure 14). In the reporter transfection experiments, the EGFP production observed in cells treated with these same ligands, varied depending on the ligand, and was significantly higher than baseline in all cases. The highest levels of EGFP production was observed in cells treated with 17-β estradiol, implying that

Figure 21. Potential sites for estrogen receptor-mediated regulation of uPA. Number 1 refers to the possibility of ER-mediated regulation of uPA at the level of transcription via interaction with AP-1 promoter elements. Number 2 refers to the possibility that surface ER conducts the signal for the exocytosis of uPA zymogen granules.


uPA gene
Actinomycin D inhibits
Transcription
1
AAAAA
uPA mRNA
Cycloheximide inhibits
Translation
Tunicamycin inhibits
Post-translational modifications (N-linked glycosylation)
Regulated secretion (zymogen granules)
2
Exocytosis of pro-uPA in response to a signal transmitted via surface receptor
Cell surface ER
pro-uPA

transcription was being induced. However, in the uPA experiment, exposure to 17-β estradiol for an identical length of time had no impact on activity levels. Similarly, no differences were detected between antisense treatments for ERβ and baseline conditions in the reporter transfection experiment, and yet, the addition of antisense to ERβ resulted in a 25-fold increase in uPA activity. Opposite trends in the reporter and uPA assays, are suggestive of ERE-mediated regulation via a repressor of uPA activity, expression or secretion, but this was not observed. Clearly, direct regulation via ERE is doubtful. There are no known EREs in the promoter region of the uPA gene, the PAI-1 gene or the uPAR gene. Nevertheless, the uPA gene promoter contains enhancers with which ERs have been know to interact – namely the AP-1 and Sp1 enhancers (please see introduction for further details).

The possibility of regulation via the AP-1 enhancer

ERα and ERβ are known to indirectly bind at AP-1 enhancer sites via Jun/Fos transcription factors (which form the AP-1 transcription factor). ER deletion studies have determined that the domains necessary for ERα interaction with the AP-1 protein are the N-terminal (a.k.a. A/B, AF-1, TAF-1, NHD) domain and the C-terminal domain (a.k.a. F, AF-2, TAF-2) (147,148,149). Both domains are required for 17β-estradiol-mediated ERα transactivation of AP-1 regulated genes. The interaction of 4-OHT with ERα can mediate transcriptional activation in the absence of the C-terminal region, and to a minor extent in the absence of the N-terminal region (147,148). For ERβ, deletion of

the first 53 amino acids or more of the ERβ N-terminal domain, results in the loss of transcriptional activation at the AP-1 promoter element (147). The loss of the C-terminal domain of ERβ has no effect on regulation of gene transcription via the AP-1 element (147). It is important to note that the domains required for AP-1 based transcription by ERβ and ERα, are those for which there is the least homology between the receptors (please refer to Figure 4). This may help to explain observed differences in regulation at AP-1 by the receptors, in combination with either specific ligands, or each other.

Table 4 summarizes the reported effects of specific combinations of ERs and ER ligands in AP-1 based transcription.

Table 4. Reported effects of ERs of AP-1-based transcriptional activation. In all cases, AP-1 protein is present and assumed to be bound to ER.

LIGAND	RECEPTOR	TRANSCRIPTIONAL RESULT
17-β estradiol	ERα	Activation (148,150)
4-OHT	ERα	Activation (150)
17-β estradiol	ERβ	None (150,151)
4-OHT	ERβ	Greater activation than ERα (148,150,152)
17-β estradiol	ERα & ERβ	Activation, magnitude ↑ when [ERβ] ↓ (147,150)

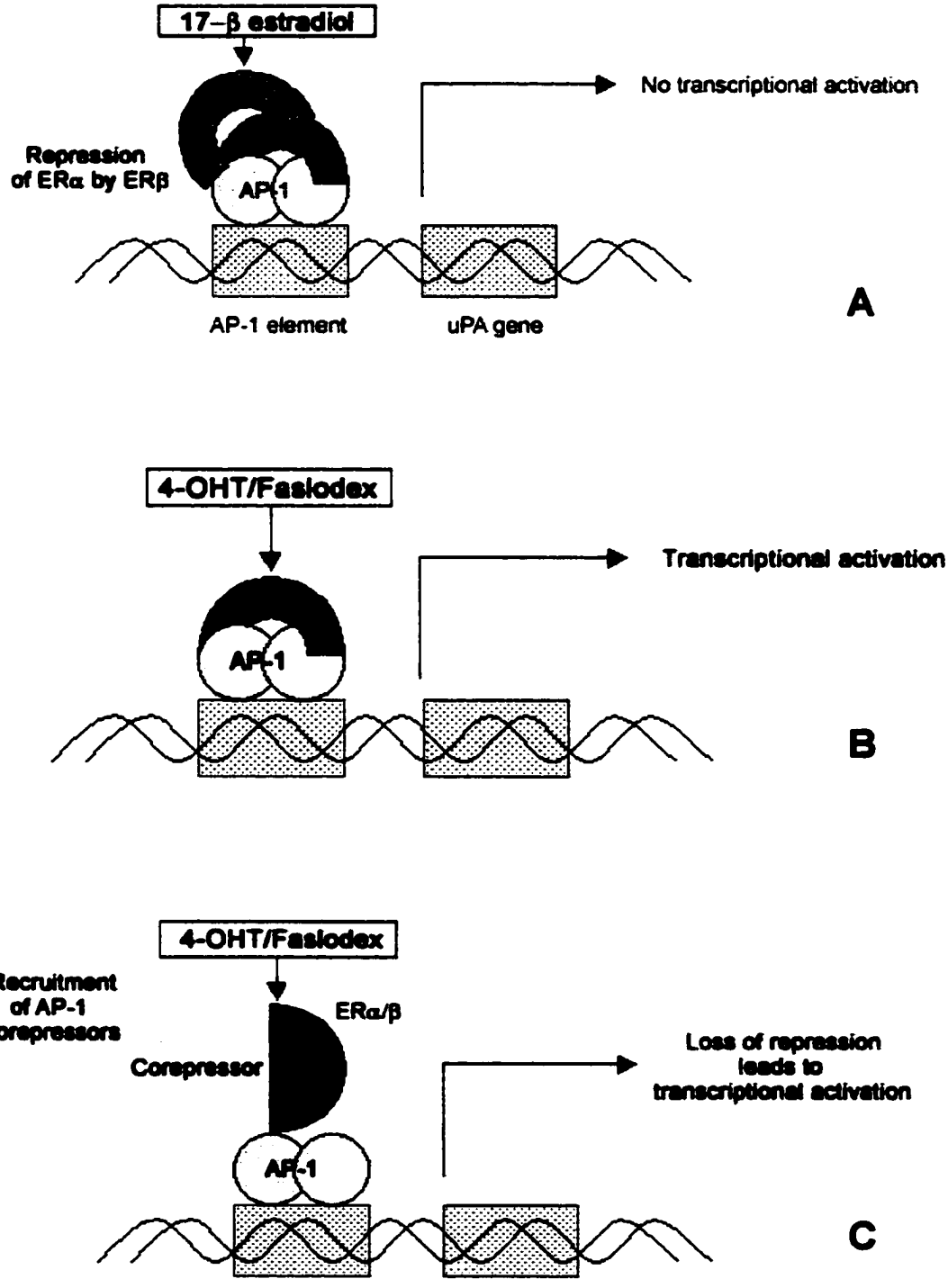
ERβ and ERα can both activate AP-1-mediated transcription in the presence of 4-OHT, but only ERα can stimulate AP-1 based transcription in the

presence of 17β-estradiol. Relative to ERα, ERβ can induce a greater magnitude of activation at AP-1 when treated with 4-OHT (152). When ERα and ERβ are both introduced into a cell, and treated with 17β-estradiol, the level of transcriptional activation via AP-1 will vary with the concentration of ERβ (147). The more ERβ introduced, the lesser the extent of transcriptional activation at AP-1. Thus, ERβ represses ERα in the presence of the physiological ligand (147,150).

The current mechanism proposed for ERα activation at AP-1 involves the recruitment of the p160 coactivator protein GRIP-1 in the presence of 17β-estradiol (148,153). In the case of ERβ transcriptional activation at AP-1 in the presence of 4-OHT, is thought to be encouraged by the sequestration of corepressors. This involves the lifting of repression from the AP-1 element (147,148).

Figure 22 illustrates proposed mechanisms of ER transcriptional regulation of the uPA gene via the AP-1 promoter element. In the absence of ligand, no activation occurs, and ERβ may suppress any non-ligand dependent transcriptional activation that could be mediated by ERα. In the presence of 17-β estradiol, the level of transcriptional activation would depend on the relative concentration of ERβ to ERα. If we assume that there is sufficient ERβ to repress ERα, then transcriptional repression would be similar to the non-ligand treated group. If the concentration of ERβ were to be reduced, as in the antisense ERβ experiment, one could expect an increase in transcriptional

Figure 22. Potential mechanism of uPA gene transcription by ERs in VSMCS at AP-1 element. In panel A, the ERs interacts with the Fos/Jun (AP-1) heterodimer, thereby influencing transcription through the AP-1 promoter element. The repressive action of ERβ on ERα in the presence of 17-β estradiol may prevent transcriptional activation from the AP-1 element of the uPA gene. In contrast, as shown in panel B, the addition of antiestrogens may result in direct activation of the uPA gene via the AP-1 element. The presence of ER antagonists 4-OHT or faslodex, and either ERβ or ERα, may promote transcriptional activation at the uPA gene, as shown in panel C, by recruitment of corepressor proteins away from the transcriptional machinery. This results in a loss of transcriptional repression, enabling transcriptional activation of the uPA gene.


17–β estradiol
No transcriptional activation
Repression of ERα by ERβ
AP-1
AP-1 element
uPA gene
A
4-OHT/Faslodex
Transcriptional activation
AP-1
B
4-OHT/Faslodex
Recruitment of AP-1 corepressors
ERα/β
Corepressor
Loss of repression leads to transcriptional activation
AP-1
C

activation. This was indeed, what we observed. It is not surprising to see activation of transcription in the absence of ligand (as in the case of the ERβ antisense study) when one considers the essential domains for AP-1 based transcriptional activation are not the ligand-binding domains. Therefore, we can assume that while ligands can enhance ER-AP-1 transcriptional regulation and have a significant influence, it is entirely possible that transcriptional activation also occurs in their absence.

When treated with antiestrogens (4-OHT or faslodex), it is possible that both receptor subtypes help activate AP-1 based transcription at the uPA gene (154). 4-OHT acts as a potent activator of ER mediated transcriptional activation at AP-1 sites in certain cell types. One example of this is the collagenase promoter in uterine endometrial cells, where 4-OHT promotes activation of transcription (155). It is also possible that ERβ, or both ERβ and ERα, act to titrate off corepressor proteins. Either scenario would explain the observed increase in uPA activity, and the abrogation of the increase in the presence of actinomycin D, cycloheximide and tunicamycin since transcription of uPA would ultimately lead to translation and N-linked glycosylated secretion.

<u>The possibility of regulation via the Sp1 enhancer</u>

The Sp1 enhancer is another potential site of ER regulation on the uPA promoter (156,157). However, much less is known about the ERs in this context. Nevertheless, there are many genes for which this manner of regulation has been recognized and documented as an important contributor to transcriptional

control. Genes for such proteins as cathepsin D, retinoic acid receptor α, c-fos, and Hsp27, have been shown to contain Sp1 regulatory regions which confer estrogen receptor responsiveness (158,159,160,161,162).

Both ERβ and ERα, require the N-terminal protein domain for binding of Sp1 and transactivation at Sp1 elements (163,164,165). For ERβ, the specific amino acids required are from 79 to 117 (164). This is very similar to the domains required for ER-mediated transcriptional activation through the AP-1 promoter element. Studies of transcriptional regulation have shown that both ERα and ERβ can regulate transcription at Sp1 elements (163,164,165). Table 5 summarizes the reported effects of specific combinations of ERs and ER ligands on Sp1-based transcription.

Table 5. The reported effects of ERs on Sp1-based transcriptional activation. In all cases, Sp1 protein presence and binding to ER(s) is assumed.

LIGAND	RECEPTOR	TRANSCRIPTIONAL RESULT
17-β estradiol	ERα	Activation (164,165)
4-OHT	ERα	Activation (164,165)
17-β estradiol	ERβ	None (164)
4-OHT	ERβ	Minimal activation (163,164)
17-β estradiol	ERα & ERβ	Activation, magnitude ↑ when [ERβ] ↓ (164)

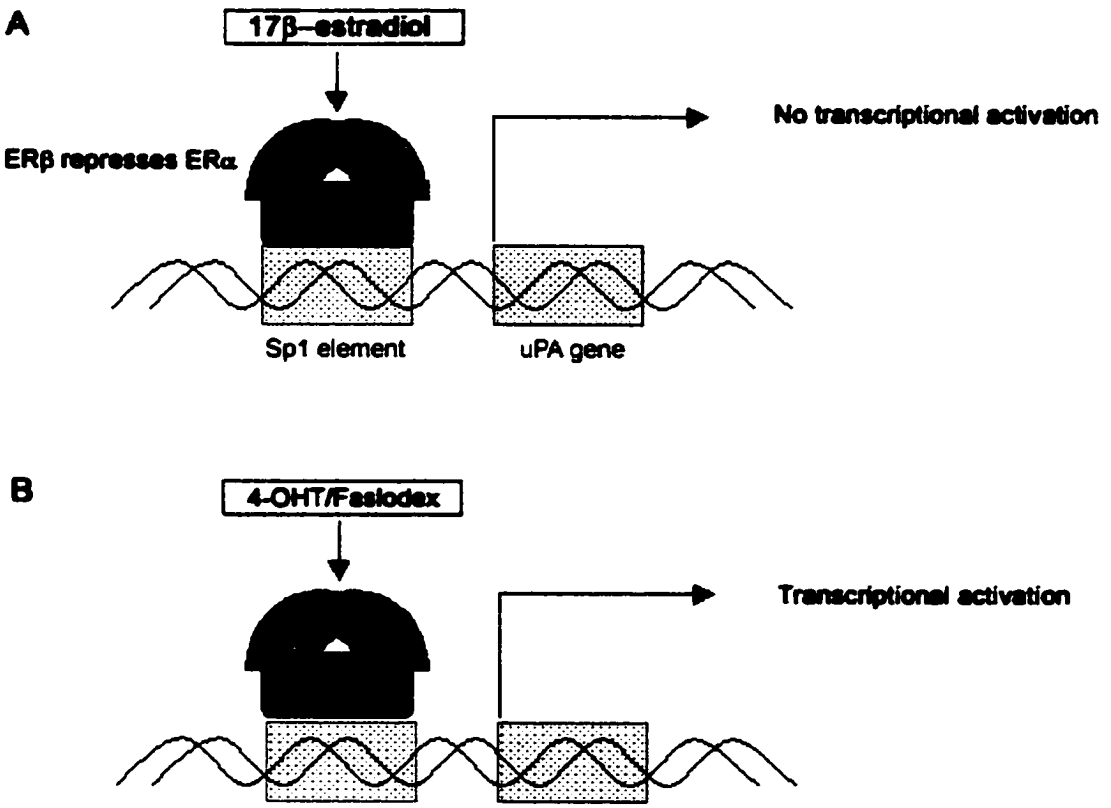
ERα can activate transcription via Sp1 by binding both antiestrogens (such as 4-OHT) and 17-β estradiol (164,165). With regards to ERβ, the addition

of 17-β estradiol has no effect on transcription but the presence of antiestrogens does induce activation at Sp1 to a limited extent (163,164). It has also been shown that when both receptors are present, ERβ represses ERα at Sp1 sites (164). The increase in uPA activity observed in the ERβ antisense study, could be attributed to a loss of strong repression on ERα, since in this situation the ratio of ERα to ERβ is increased. Again, since the domain required for ERα Sp1-mediated transcriptional activation is the N-terminal, non-hormone binding domain, it is possible that unliganded ERα can help mediate transcriptional activation, perhaps via the recruitment of coactivator proteins. The lack of uPA activity in the untreated and 17-β estradiol treated cells may be attributed to the recognized repressive effects of ERβ on ERα, and the role of ERβ as a dominant regulator of ERα activity (166,167) (Figure 23). Addition of antiestrogens has been shown to induce transcription at Sp1 for both receptor types, therefore, the marked increase in uPA activity observed may be the result of ER-mediated transcriptional activation. Alternatively, as has been suggested in the case of the AP-1 promoter element, the antiestrogen liganded ERβ may sequester general transcriptional corepressor proteins away from ERα, further amplifying the normal transcriptional-inducing effects of antiestrogen-bound ERα.

Repression of uPA transcription by ERα unbound, and bound by 17-β estradiol, supports the notion of a protective role for 17-β estradiol in the vascular cells. These proposed mechanisms of uPA regulation could be easily verified by a variety of experiments. Reporter vector constructs incorporating the uPA gene

Figure 23. Potential mechanism of uPA gene transcription by ERs in VSMCS at Sp1 element. The ERs interact with the Sp1 transcription factor. Panel A shows how ERβ may repress ERα in the presence of 17-β estradiol, resulting in a lack of transcriptional activation at the Sp1 promoter element. In contrast, as shown in panel B, the addition of 4-OHT or faslodex may result in agonistic action at the ERs and thereby directly promote transcriptional activation at the uPA gene

.


A
17β–estradiol
No transcriptional activation
ERβ represses ERα
Sp1 element
uPA gene
B
4-OHT/Faslodex
Transcriptional activation

promoter region (normal and mutated at AP-1 or Sp1 sites), could be used to assess the influence of ER ligands on uPA expression. The relative importance of the different ERs in the modulation of uPA activity could be investigated using cell lines from ER knockout animals or by introducing single ER expression vectors into ER-negative cells. The drawback of any approach that requires changing cell type is the possibility that the particular coregulatory proteins expressed in that cell type could engender a different response than that observed in vascular cells.

Regulation at the level of secretion

Little is known about the exocytosis of pro-uPA secretory granules. An unknown signal is transmitted to the cell, and is transduced through cell surface and plasma membrane proteins and enzymes. It is often the release/increase in intracellular Ca+2 levels that is responsible for triggering exocytosis of secretory granules (168). These ultimately result in the modification and interaction with proteins of the secretory granule. The nature of these proteins in both the uPA and VSMC context are not clear. A great deal of research has been conducted on regulated secretion in pancreatic acinar cells. These cells secrete several serine proteases in a regulated fashion. Some important players include SNAREs on the zymogen granules, ion channels (K+, Cl-) (169). Sometimes G proteins are implicated (170), but as it true of cell signaling in general, it is very much cell type dependent. In cultured microglia, uPA release can be triggered by ceramide, and involves protein kinase C in the exocytotic process (171). It

has also been reported that 17-β estradiol caused the release of uPA from endometrial tissue in organ culture (172).

The existence of a cell surface ER has been a matter of speculation for many years, and more solid evidence has just begun to accumulate, though the receptor has yet to be characterized (68,69,70,71,72,73,74) (Figure 6). The notion of a surface receptor was fueled by the well-established rapid onset effects of 17-β estradiol; too quick to be attributable to transcription. Some examples include vasodilation of coronary arteries, release of insulin from pancreatic beta cells, and activation of specific signal pathways in neurons (12). The surface receptor is thought to be a plasma membrane version of ERα. It is associated with caveolae, and functions via calcium-dependent, (extracellular regulated kinases) ERK-1 and ERK-2 mechanism (12). G-proteins have also been implicated in some specific signaling pathways such as NO synthase activation (77,173). In this scenario, faslodex and 4-OHT are assumed to activate the cell surface receptor, while 17-β estradiol has little or no effect. The transduction of the signal could result in the activation of the ERKs and consequent activation of proteins associated with the zymogen granules. Different ER ligands are known to affect ERK activity in human aortic VSMCs (174). The effect of actinomycin, cycloheximide, and tunicamycin would still be felt, if the production of new uPA is more significant than the pre-existing pool. Unfortunately, very little is known concerning the secretion of pro-uPA from VSMCs. In order to investigate this possibility further, signal transduction inhibitors specific to protein kinases, could be used in a fashion similar to the

application of the actinomycin, cycloheximide and tunicamycin, to see if they could reduce the observed increases in uPA activity in the presence of 4-OHT or faslodex.

The possibility that the ERs are affecting multiple proteins or pathways related to uPA, cannot be ignored. The ERs touch on such a wide range of cellular processes and regulatory actions, that it is not inconceivable that they could be controlling several aspects of uPA expression and secretion. The complexity of the uPA system should not be forgotten while focusing so narrowly on a single aspect of its control.

The scope of ER ligands

Similar effects were observed when VSMCs were treated with faslodex or 4-hydroxytamoxifen, despite gross structural differences. Both drugs have been applied to the treatment of ER positive breast cancer. Although faslodex has been shown to have a higher affinity for ER, and does not provoke resistance or increase the risk of endometrial cancer, it lacks the beneficial effects on bone and serum lipid profiles that have been observed with 4-hydroxytamoxifen. Faslodex is considered less toxic, and preferable overall. In relation to heart disease, some trials suggest that treatment with 4-hydroxytamoxifen can decrease the levels of inflammatory proteins implicated in heart disease (such as C-reactive protein and fibrinogen), and improve serum lipid profiles in women (175,176). In January of 2001, a large-scale clinical trial conducted by the Cardiovascular Institute of the University of Pittsburgh found no adverse or

beneficial effects of 4-hydroxytamoxifen on cardiovascular risk. This trial was conducted over a period of seven years with approximately 13,000 postmenopausal women at high risk for breast cancer. 1000 women had had previous coronary heart disease. For all women, the rate of cardiovascular events (defined as fatal and nonfatal myocardial infarction, unstable angina, and severe angina) was not significantly different between placebo-treated and 4-hydroxytamoxifen-treated groups (177). Similar data is not yet available for faslodex, due to its recent inception. Phytoestrogens have also garnered significant attention in recent times, no doubt due, in great part, to the controversy surrounding HRT. Phytoestrogens are plant-based estrogens such as coumestrol (derived from clover) and genistein (derived from soy), which are capable of interacting with the ERs. Their long term effects are still unclear and their full physiological impact requires elucidation. Future work could include a study of the impact of these ER ligands on uPA activity, in light of the results observed for 4-OHT and faslodex. Combinations of the ER ligands would also yield important information with regard to competition for ER binding and potential mechanisms of action.

ERβ versus ERα

The roles of ERβ and ERα differ. Unlike the receptor subtypes of other steroid hormone receptors, ERα and ERβ are not alternate transcripts of a single gene, but are the distinct products of two different genes from two different chromosomes (1). The distribution and tissue expression profile of the receptors

varies, as does the sequence of key domains of their primary structure (12). Moreover, the ERβ knockout mice has several vascular aberrations with regards to vasoconstriction, hypertension and ion channel function which were not observed in ERα knockout mouse (178). It is also ERβ that was observed to be upregulated after arterial injury in the mouse model of atherosclerosis (84). The results of the uPA assay in conjunction with ERβ antisense treatment suggest that ERβ may be involved in the control of extracellular proteolysis in an unexpected way. The precise nature of the involvement of ERβ in the uPA secretion pathway needs to be defined. It is clear that ERβ plays a significant role in cellular regulation and functioning, and is not merely the poor cousin of ERα.

Conclusion

The general goal of this thesis was to determine if 17-β estradiol modulated uPA in VSMCs in a manner that is predictive of a reduced incidence of plaque rupture. The expression pattern of ERβ and ERα in human aortic VSMCs *in-vitro*, and the effect of 17β-estradiol on the expression of these receptors was determined. Antisense to ERβ and ER ligands were used to modulate the expression and activity of ERs in primary VSMCs. It was determined that the application of antagonists of ER and the reduction of ERβ, result in an increase in uPA activity. This increase could be abrogated by the addition of specific inhibitors of transcription, translation and secretion and was independent of ERE-based transcription. The addition of 17-β estradiol did not

increase the activity of uPA. This can be considered predicitive of a reduced incidence of plaque rupture as it is thought that the increased activity and expression uPA, can lead to the degradation and subsequent destabilization of an atherosclerotic plaque. ER ligands known to be antagonists, faslodex and 4-OHT, were shown to dramatically increase the activity of uPA in human aortic VSMCs. This would favour proteolytic degradation, and could be predictive of plaque rupture. Antagonism of the effects of the physiological ligand 17-β estradiol, may be potentially detrimental to the integrity of the extracellular matrix of vascular cells.

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Appendix A – Sources of chemicals

Chemicals and proteins

17-beta estradiol	Sigma
30% acrylamide	BioRad
4-hydroxytamoxifen	Sigma
Acetic Acid	BDH
Actinomycin D	Sigma
Activated charcoal	Sigma
Agar	BDH
Agarose for normal DNA electrophoresis	Gibco BRL
Agarose for RNA	Boehringer Manheim
Agarose, for RT PCR (NuSieve 3:1)	FMC bioproducts
Ammonium persulfate	Sigma
Ampicilin	Novopharm
Aprotinin	Sigma
Bovine Serum Albumin (for restriction digest)	New England Biolabs
Bradford Reagent	BioRad
Brilliant Blue G	Sigma
Broad Range Protein Ladde	New England Biolabs
Bromophenol Blue	Sigma
Butyl alcohol	Sigma
Casein for Western blotting (skim milk powder)	Carnation
Chloroform	BDH

Cycloheximide, ready-made	Sigma
DAB	Sigma
DNA ladder (100bp and 1kb)	New England Biolabs
DNTPs (Ultrapure dNTP set)	Pharmacia Biotech
DTT	GIBCO
Dulbecco's Modified Eagle Medium, phenol red free	GIBCO/Invitrogen
ECL reagents	Amersham Pharmacia Biotech
EDTA	EM Science (Merck)
Ethanol	Commercial alcohols Inc.
Ethidium bromide	Sigma
Faslodex/ICI 182, 280	Sigma
Fetal bovine serum	Wisent Multicell
First strand buffer	GIBCO
Formaldehyde	Invitrogen
Formamide	Gibco
Formic acid	Sigma
Fungizone/Amphotericin B	Bristol-Myers-Squibb
Gentamycin/Garamycin Sulfate	Schering
Glucose	Sigma
Glycerol	Sigma
Glycine	EM Science (Merck)
Hydrochloric Acid	BDH

Hydrogen Peroxide	Sigma
Isopropyl alcohol	Sigma
Kanamycin	Sigma
L-glutamine	GIBCO
Lysozyme	Sigma
Methanol	BDH
MOPS	Boehringer Manheim
Oligonucleotide dT	New England Biolabs
Phenol	Sigma
Polymethylsulfonylfluoride/PMSF	Sigma
Potassium Chloride	BDH
Purified bovine serum albumin	BioRad
Purified recombinant EGF	Clontech
Sodium Chloride	EM Science (Merck)
Sodium Hydroxide	VWR Canlabs
Sodium orthovanadate	Sigma
Taq buffer	Amersham Pharmacia
Biotech	
TEMED	Amersham Pharmacia
Biotech	
Toluene	BDH
TriReagent	Sigma
Tris base	BDH

Triton X-100	Sigma
Trypsin	GIBCO
Tryptone/peptone	Difco
Tunicamycin	Sigma
Tween-20	Fisher Scientific
Xylene cyanole FF	Sigma
Yeast Extract	Difco

Enzymes

Dnase	Gibco
Restriction enzymes: Mlu1, Bgl2, Kpn1	New England Biolabs
Reverse transcriptase (Superscript II)	Gibco
Taq polymerase	Gibco

Software

Molecular Analyst	BioRad
SigmaPlot	SPSS Science
SigmaStat	SPSS Science

Antibodies

Goat anti-mouse peroxidase conjugated	Chemicon
Goat anti-rabbit peroxidase conjugated	Chemicon
Goat anti-rabbit Texas Red (rhodamine) conjugated	Vector

Horse anti-mouse fluorescein conjugated	Vector
Mouse anti-*Aspergillus niger* glucose oxidase	Dako
Mouse anti-human ER alpha	Novocastra
Mouse anti-human smooth muscle alpha actin	Dako
Mouse anti-human uPA B-chain	American Diagnostica Inc.
Rabbit anti-human ER beta	Affinity Bioreagents Inc.
Rabbit anti-human von Willebrand Factor VIII	Dako

Cells

Aortic vascular smooth muscle cells	Clonetics
HeLa	Heidi McBride
MCF-7	Christine Pratt

Machines

Centrifuge (large)	Eppendorf centrifuge 5403
Centrifuge (small)	Eppendorf centrifuge
5415C	
Electrophoretic power source	BioRad PowerPac 200
Electroporator	BTX electroporator
system, electrocell manipulator 600	
Isotemp Incubator	Fisher Scientific
Fluorometer	Polarstar galaxy
UV-Vis chemstation spectrophotometer	Hewlett Packard

Hybridization incubator	Robbins Scientific Mode 1000
GeneAmp 2400 PCR system	Perkin Elmer
Shaking incubator	Lab-line instruments Inc.
CL-100 UV crosslinker	UVP
Microscopes	
CK2 inverted microscope	Olympus
Kits	
uPA assay kit	Chemicon
Maxiprep kit	Qiagen
Vectastain ABC elite kit	Vector
Miscellaneous	
100mm dishes	Corning
6-well dishes	Falcon
ECL Hyperfilm High	Amersham Life Sciences
Bacterial plates	Fisher Scientific
Colorimetry clear 96-well plates	Costar
Cuvettes	Sarstedt
Electrophoretic Apparatus horizontal	Hoeffer

Gel loading pipet tips	Fisherbrand
384 well plate black fluorometry plates	Nalge Nunc International
Pipettors	Eppendorf
Nitrocellulose transfer membrane	Protran
Special tips for RNA work	Axygen
Vertical electrophoresis apparatus	BioRad

Appendix B – List of abbreviations

4-OHT	4-hydroxytamoxifen
AMP	ampicillin
ADP	adenosine diphosphate
E_2	17 beta estradiol
EGFP	enhanced green fluorescent protein
ER	estrogen receptor
ERE	estrogen response element
ICC	immunocytochemistry
KAN	kanamycin
MMP	matrix metalloproteinase
RIPA	RIPA lysis buffer
RT PCR	reverse transcriptase polymerase chain reaction
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEAP	secreted alkaline phosphatase
SM alpha actin	smooth muscle cell alpha actin
uPA	urokinase-type plasminogen activator
uPAR	urokinase-type plasminogen activator receptor
VSMC	vascular smooth muscle cell
EDTA	ethylene diamine triacetic acid
RNA	ribonucleic acid
DNA	deoxyribonucleic acid
DEPC	diethyl pyranocarbanocide

Appendix C – Statistical Analysis

1. uPA assay results

Values were derived from a standard curve based on absorbance at 520nm. The mean and standard deviations were calculated, and all results were subjected to ANOVA analysis. The Tukey test was used as a secondary statistical test. Differences of $P<0.05$ were considered significant.

2. Reporter assay

The SEAP and EGFP values were each derived from their respective standard curves. The final value was obtained by dividing the EGFP value by the SEAP value. The mean and standard deviations were calculated, and all results were subjected to ANOVA analysis. The Tukey test was used as a secondary statistical test. Differences of $P<0.05$ were considered significant.

3. Cell counting by quadrant analysis

A well of a 6 well dish was divided into four quadrants, a random, but representative photograph was capture for each quadrant of the well. Two individuals working independently counted the cells in the quadrant. The total cell number counted was multiplied by 110 to give an estimate of the total number of cells per well, for a given well. The multiplying factor is based on the area of the well represented by the photograph.

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Highlights of Qualifications:

- Enjoys a challenge; quick learner.
- Reliable, responsible, and dedicated.
- Well-developed verbal and written communications skills.
- Bilingual
- Secret Security Clearance
- Creative, resourceful and thorough.

Education:

- MSc. Biochemistry from the University of Ottawa. Research conducted in the Vascular Biology laboratory of the Heart Institute under the supervision of E.R. O'Brien, M.D. on the topic of estrogen receptors and the vascular wall. Projected completion date: December 2002.
- Hon. B.Sc. Biochemistry, *Magna cum Laude* from the University of Ottawa. Research conducted under the supervision of Dr.J.R. Dillon on the subject of bacterial cell division, 1999. Publication: Res. Microbiol. 2001 Nov; 152 (9):781-91.
- Diploma in life science from Héritage College, with award in English, 1996.

Laboratory Skills:

DNA: Agarose and polyacrylamide electrophoresis, PCR, DNA purification, DNA isolation, miniprep and maxiprep, electroporation, transformation of yeast, transfection of mammalian cells by lipofection, transformation of bacteria, primer design, restriction digest, ligation, reporter vector design and construction.

RNA: RT PCR, formaldehyde/agarose electrophoresis, RNA isolation (cells and tissue), antisense.

Protein: Protein isolation, SDS PAGE electrophoresis, western blotting, Coomassie staining, Ponceau-S staining, immunocytochemistry, fluorescent assays (GFP), chemiluminescent assays (alkaline phosphatase), Bradford assay, Yeast-2-Hybrid, beta-galactosidase assays.

Cell culture: Prokaryotes (*N. gonorrhoeae*, *E. coli*), yeast (*S. cerevisiae*), immortal lines (MCF-7, HeLa), mammalian primary and secondary lines (rabbit vascular cells, human vascular smooth muscle and endothelial cells), isolation of primary lines, digital imaging of cells, haemocytometry, gram staining.

Employment Experience:

- Receptionist at Public Works Canada for GTIS (Government Telecommunications and Informatics Services), summer 1999.
- Receptionist for the Director of the Science Policy Branch of Environment Canada, summer 1998.
- Clerk for the Family Orders and Agreements Enforcement Assistance Unit (FOAEA) of the Department of Justice, summer of 1998.
- Interviewer and focus group recruiter for Price Waterhouse in the National Survey Centre, summer of 1997.

Interests and Activities:

- Piano
- Taekwon-Do, second degree black belt
- Founding executive member of Heart Institute Graduate Students Association

References available upon request

Contributions of collaborators

Steve Olsen assisted with the immunofluorescent characterization of cells and helped count cells for the transfection assay.

Harvey Miller assisted with the generation of the reporter vector and prepared the vein graft in-situ hybridization.

Heidi McBride provided HeLa cells for estrogen receptor negative controls.