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Biphasic effect of danazol on human vascular endothelial cell permeability and f-actin cytoskeleton dynamics ☆

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ABSTRACT

Breakdown of endothelial barrier function is a hallmark event across a variety of pathologies such as inflammation, cancer, and diabetes. It has also been appreciated that steroid hormones impart direct biological activity on endothelial cells at many levels. The purpose of this investigation was to explore the effect of the androgen-like steroid, danazol, on endothelial cell barrier function *in vitro*. Primary human endothelial cells exposed to 0.01–50 μM danazol were evaluated for changes in permeability. We found that danazol altered endothelial permeability in a biphasic manner in which nanomolar concentrations enhance barrier function while micromolar concentrations are detrimental. Monitoring of trans-endothelial electrical resistance demonstrated that these barrier enhancing effects were rapid (within 5 min) and lasted for over 24 h. Analysis of intracellular f-actin organization showed that barrier enhancement also correlated with the formation of a submembranous cortical actin ring. Conversely, at higher danazol concentrations, contractile cell phenotypes were observed, represented by stress fiber formation. Competitive binding studies performed using steroid hormone receptor antagonists proved that this activity is the result of androgen and estrogen receptor ligation. These findings suggest that low dose danazol may provide a therapeutic window for diseases involving vascular leakage.

1. Introduction

It has long been clinically appreciated that steroids, especially ovarian sex hormones, provide cardiovascular benefits. Women exhibit a much lower incidence of cardiovascular disease during their childbearing years than men of similar age with a loss of these protective effects after menopause [1]. Despite significant controversy, estrogen replacement therapy in postmenopausal women is, in some studies, associated with a significant reduction in mortality from cardiovascular complications [2,3]. Conversely, men with coronary artery disease present with lower testosterone levels thus implying a possible link between circulating testosterone and the progression of arthrosclerosis [4]. Also, administration of estrogen and testosterone acutely modulates vascular tone, serving mainly as vasodilators but instances of constriction have also been documented [1,4].

An impressive body of evidence also exists suggesting that sex steroids can directly alter endothelial cell (EC) function. The most familiar is the rapid formation of new blood vessels in the endometrium corresponding to the estrogen surge during the menstrual cycle [1]. In addition, estrogen receptor knockout mice are characterized by impairments in vessel formation [5]. Treatment of rats with estrogen attenuates VEGF-induced breakdown of the blood–retinal-barrier while castration has been shown to prevent diabetes-induced permeability complications [6,7]. *In vitro* studies demonstrate that human umbilical vein endothelial cells (HUVEC) exhibit marked increases in proliferation and migration following exposure to estrogen [8]. ECs also express an array of steroid receptors including an orphaned G protein-coupled receptor, reported to be a membrane associated estrogen receptor [9]. Taken as a whole, these reports demonstrate that sex steroids represent a family of vasoactive compounds.

We previously reported that danazol (17a-Pregna-2,4-dien-20-yno[2,3-d]-isoxazol-17), a synthetic analog of 17-α-ethinyl testosterone, alters endothelial function during some of the hallmark

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stages of angiogenesis. In our study, danazol attenuated EC proliferation and prevented the formation of capillary-like structures in a model that mimics tubulogenesis [10]. While these findings are pertinent to the current clinical application of danazol in the treatment of endometriosis, ECs are primarily encountered as intimately linked monolayers on the vessel wall where they provide an essential barrier function. The aim of this study was to explore if danazol affects barrier function in primary EC cultures. We hypothesized that treatment with danazol enhances the adhesive characteristics of these cells in a dose-dependent manner thus altering barrier integrity *in vitro*. Breakdown of this barrier function is a key element in the prognosis of a variety of pathologies such as inflammation, diabetes, and cancer [11,12]. Elucidating these mechanisms may help reduce vascular leakage syndromes and provide additional therapeutic targets for intervention.

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals used in this investigation were obtained from Sigma–Aldrich (St. Louis, MO) unless otherwise noted.

2.2. Cell culture

Primary human retinal (HREC) and brain (HBEC) endothelial cells were purchased from Cell Systems (Kirkland, WA). Human umbilical vein (HUVEC) cells were obtained from Lonza (Walkersville, MD). All cells were cultured in EBM-2 basal medium supplemented with EGM-2 bullet kit (Lonza) at 37 °C and 5% CO_2. Cultures were passaged using trypsin/EDTA (Lonza) as specified by the suppliers and utilized at low passage number (below 10) to retain key characteristics of the endothelial lineage.

2.3. Paracellular permeability assays

1×10^5 cells were seeded into the upper chamber of 24 well cell culture inserts (Thincerts; Greiner, Monroe NC) coated with 10 μg/cm^2 collagen IV and cultured for 24 h to achieve a semi-permeable barrier. Barrier integrity of each insert was confirmed by measuring transendothelial electrical resistance (TEER) using a STX2 "chopstick" electrode connected to an EVOM^2 volt ohmmeter (World Precisions Instruments, Sarasota, FL). Ω^* cm was calculated by dividing the resistance by surface area. The medium was then carefully decanted and replaced with fresh EGM-2 containing compound or vehicle (<0.1% ethanol). Strepavidin-horseradish peroxidase (Thermo Scientific, Rockford IL) was then added to the upper chamber of the system at a final concentration of 25 ng/ml. TEER was then measured again at specific time points. Also, colorimetric analysis of HRP transfer was evaluated at 24 h by drawing 10 μl of the medium from the bottom chamber and mixing with 100 μl tetramethylbenzidine substrate solution (TMB; Thermo Scientific). Reactions were stopped after 5 min by the addition of 100 μl 0.18 M H_2SO_4, and absorbance was measured at 450 nm on a Spectra Max M_2^e microplate reader (Molecular Devices, Sunnyvale, CA). To calculate percent decreases in permeability, the following formula was employed: ((OD vehicle − OD treatment)/OD vehicle)∗100.

2.4. Competitive binding analysis

The permeability assay described above was additionally modified for the competitive analysis of danazol using known steroid receptor antagonists. HRECs were cultured with danazol alone or in combination with the nonsteroidal antagonists hydroxyflutamide and fluvestrant.

2.5. Phalloidin f-actin staining

Clear bottom, black wall 96 well tissue culture plates (Greiner) were coated with collagen IV and seeded with 1.7×10^4 cells per well in a final volume of 200 μl. After 24 h, the medium was aspirated and replaced with Lonza EBM-2 containing 1% BSA, ITS, pen/strep, and 10 ng/ml bFGF. Cells were quiesced in this medium for an additional 24 h then treated with compound for 3 h as indicated. To serve as positive controls for dynamic cytoskeletal changes, additional wells were treated with 25 μM forskolin or 100 ng/ml TNFα. Following exposure, cells were fixed using a 3.6% formaldehyde solution in PBS for 10 min at room temperature. Two 100 μl PBS washes were performed and membranes permeabilized using 100 μl 0.1% Triton X-100 solution in PBS for 5 min. The cells were washed again, and 100 μl 1:50 Rhodamine–phalloidin (Invitrogen, Carlsbad CA) in PBS was added. After 20 min, the cells were placed in 100 μl PBS after a final washing. Following staining, cells were photographed using a Nikon SLR camera (Melville, NY) mounted on a Motic AE31 inverted microscope (Motic AE31, Richmond, British Columbia) with an appropriate filter set.

2.6. Statistical analysis

A paired Student *t*-test was applied to data sets (Microsoft Excel; Redmond, WA) with statistical significance accepted at $p < 0.05$.

3. Results

3.1. Danazol affects endothelial cell paracellular permeability in a biphasic mode

The primary aim of this investigation was to establish if the androgen-like steroid danazol influences vascular permeability. Horse radish peroxidase (HRP) was used as a tracer for the passage of protein across confluent monolayers grown on porous inserts. Permeation is directly proportional to the optical density obtained in the lower transwell chamber after reaction with TMB.

Our results suggest that danazol affects HUVEC monolayer permeability in a biphasic manner in which low doses decrease permeability while higher doses increase the passage of protein across the cells over a 24 h period. Percent decreases in permeability were calculated for three separate experiments, each performed in triplicate and combined to obtain mean ± SEM (Fig. 1A). Peak barrier enhancement was achieved at concentrations of 100 or 500 nM danazol with significant reductions of 36 ± 12% and 31 ± 6% observed, respectively. As the concentration of danazol was increased, however, this effect was incrementally lost. At 25 μM, there was no significant difference between the danazol and controls. Increasing the concentration to 50 μM severely compromised barrier integrity with an increase in HRP permeation of 377 ± 172% detected (Fig. 1B).

3.2. Danazol also decreases permeability across retinal and brain endothelial cells

To determine if the decrease in permeability is unique to HUVEC cells or is a more general phenomenon, other primary ECs were analyzed. Human retinal (HREC) and brain (HBEC) ECs were treated with danazol at final concentrations of 0.05, 0.5, and 5 μM and HRP passage was examined 24 h following treatment.

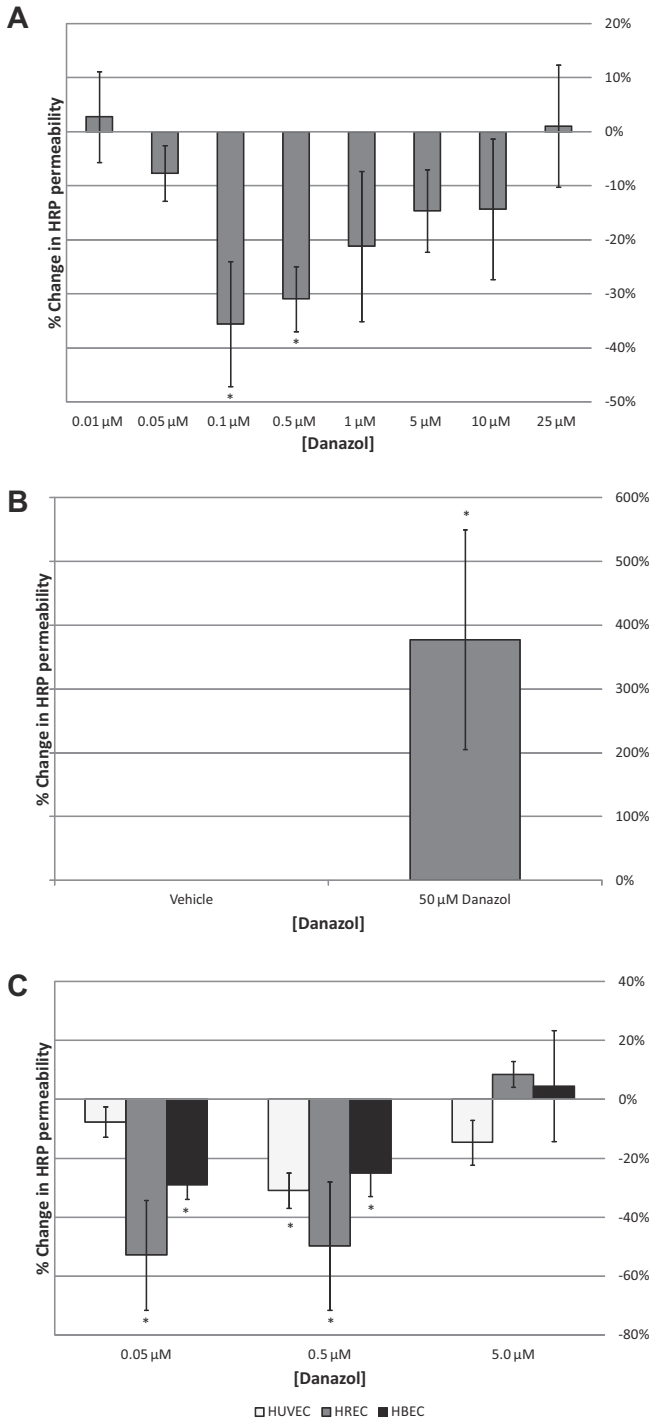

Fig. 1. % Change in permeability. (A) Dose response of danazol effect on HRP permeability of cells treated for 24 h. (B) High dose response (50 μM) increase in permeability. (C) Comparative response of danazol induced barrier enhancement between umbilical, retinal, and brain derived endothelial cells. Data presented as mean ± SEM calculated for three separate experiments each performed in triplicate. * = p value <0.05 vs vehicle.

Percent decreases were calculated and compared to the results observed for HUVEC (Fig. 1C). We found that while danazol still enhanced barrier function in all three cell types, the degree of enhancement and associated doses varied. Of the ECs tested, HUVECs proved to be the least reactive to danazol. As mentioned above, reductions in HRP permeation of 31 ± 6% were achieved across these cells when exposed to a final concentration of 500 nM. By comparison, the decrease in permeability achieved by danazol in HBECs mirrored HUVECs but was accomplished by a 10-fold lower dose (29 ± 5% observed at 50 nM). HRECs were the most responsive cell type tested with a maximal decrease in permeability of 53 ± 18% observed following treatment with 50 nM danazol.

3.3. TEER and the temporal dynamics of danazol induced barrier enhancement

The electrochemical movement of small ionized molecules was evaluated by transendothelial resistance (TEER) to explore the temporal dynamics of danazol induced endothelial barrier alterations. TEER is a tool for the rapid measurement of ion flux across or between monolayers and correlates directly with barrier integrity. ECs exhibiting strong cell-to-cell adhesiveness will restrict the flow of ions between the transwell chambers resulting in higher resistance readings. HRECs were utilized since these cells were most sensitive to danazol and only inserts with similar starting measurements were used.

Significant increases in TEER were immediately measured following challenge with 100 nM danazol (Fig. 2A). In as little as 5 min, danazol significantly elevated monolayer resistance by 30 Ω* cm or 12% as compared to vehicle controls (250 ± 5 Ω* cm for vehicle vs 280 ± 1 Ω* cm for danazol). Resistance increased further to 50 Ω* cm above controls 30 min after exposure (262 ± 3 Ω* cm for vehicle vs 312 ± 7 Ω* cm for danazol), representing an increase of approximately 20%. When measured at 60 min, danazol treated monolayers exhibited resistance levels 62 Ω* cm over controls (251 ± 4 Ω* cm for vehicle vs 313 ± 3 Ω* cm for danazol; 25% increase). This level of TEER enhancement was sustained for over a 24 h period.

3.4. Steroid receptor anatagonists and danazol induced barrier enhancements

To test the possibility that danazol-induced barrier enhancement is the result of an androgen receptor (AR) mediated pathway, competitive analysis was performed using an AR antagonist, hydroxyflutamide. HRECs inserts were treated with combinations of 100 nM danazol and hydroxyflutamide. We found that hydroxyflutamide attenuated the barrier enhancing properties of danazol at all time points tested (Fig. 2B). Intriguingly, hydroxyflutamide alone rapidly reduces TEER measurements and the presence of danazol counteracts this response further supporting competitive interaction at the AR.

For comparative purposes, danazol was evaluated against the estrogen receptor (ER) antagonist, fulvestrant. Initially, estrogen receptor antagonism with fulvestrant reduced danazol TEER increases, but this was eventually overcome, with 24 h measurements of danazol being similar regardless if the antagonist was present or not (Fig. 2C). Fulvestrant treatment alone, however, did reduce TEER but not to the same degree as its ability to reduce danazol enhancements confirming a competitive interaction at the ER as well.

3.5. F-Actin cytoskeletal reorganization is detectable in endothelial cells treated with danazol

To determine if danazol alters f-actin polymerization, HRECs were treated with danazol ranging from 0.1 to 10 μM (Fig. 3). The cells to be investigated were serum starved prior to use in order to minimize background f-actin within the cell (Fig. 3A). Three hours following exposure, the cells were fixed and stained with the filamentous actin specific probe phalloidin. Preliminary experiments suggested f-actin rearrangements peak 3 h after treatment with danazol (data not shown). At lower doses (0.1 and 1.0 μM),

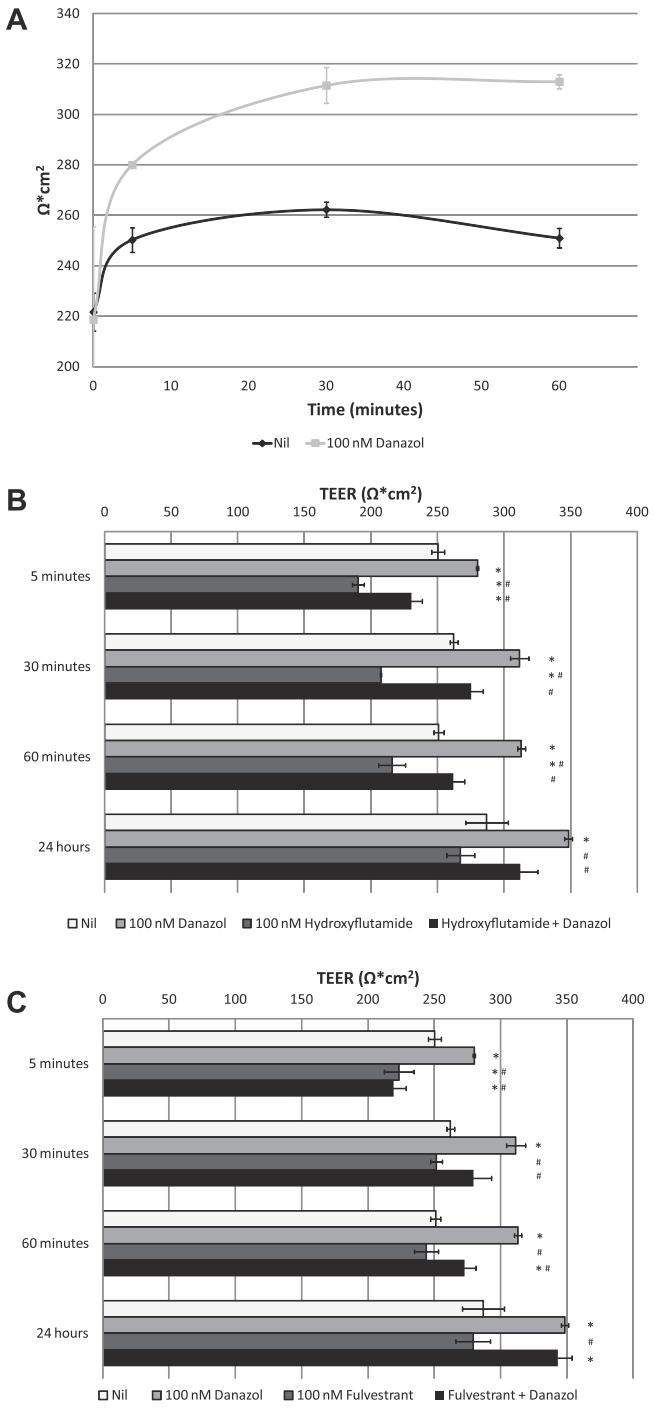

Fig. 2. TEER response of danazol: (A) temporal effect of 0.1 μM vs vehicle. (B) Competitive analysis between danazol and hydroxyflutamide. (C) Competitive analysis between danazol and fulvestrant. Data presented as mean $\Omega^* cm^2 \pm SD$ calculated for three separate inserts at indicated time. ∗ = p value <0.05 vs vehicle control. # = p value <0.05 vs danazol alone.

treatment of ECs with danazol resulted in an increase in f-actin localized in the cortex of the cell (Fig. 3D and E). This type of rearrangement is similar to the cortical actin pattern induced by the adenylyl cyclase activating compound, forskolin (Fig. 3B). Exposure of the cells to 10 μM danazol induced a different f-actin phenotype. Instead of cortical actin, f-actin oriented into contractile stress fiber cables at these doses (Fig. 3F). Stress fiber formation was also observed following exposure to 100 ng/ml TNFα (Fig. 3C) a known inducer of permeability [12].

4. Discussion

The mechanisms by which steroid hormones mediate EC function are poorly understood. Our findings provide three key insights into the biological activity of the androgen-like steroid, danazol. First, we demonstrate that low dose (nM) danazol treatment decreases permeability across a variety of primary EC monolayers. Despite vascular bed variability, the passage of ions as well as high molecular weight protein is restricted following low dose danazol treatment. Second, treatment of ECs with barrier enhancing levels of danazol leads to rapid remodeling of the cytoskeleton into a cortical actin ring. A significant increase in tightly packed f-actin is evident near the surface of ECs treated with low dose danazol potentially providing a lattice work that can interact with the cytoplasmic domain of junctional proteins. Finally, higher concentrations of danazol are detrimental to endothelial barrier function, causing an increase in permeability as well as stress fiber formation inside the cell. This is possibly the result of "hyperstimulation" overriding the barrier enhancing pathways activated at lower concentrations of the drug. It is important to note that all these concentrations exceed the physiological range of androgens (10–25 nM) and could never be reached without pharmacological intervention [4]. Taken together, danazol appears to acutely alter endothelial permeability *in vitro*, and this knowledge may expand our understanding of how steroidal hormones interact with the endothelium.

The classical dogma of steroid signaling suggests that these lipophilic hormones penetrate the cell membrane, bind to a cognate receptor in the cytosol, and, in turn, the resulting complex is translocated to the nucleus to initiate transcription and translation [13]. While still integral to the overall biological activity of steroids, this pathway carries temporal restraints insuring it will take hours, if not days, to run full course. Increasing evidence suggests that important events take place seconds to minutes following treatment with steroids [14]. The rapid nature of danazol-induced endothelial monolayer barrier enhancement would indicate that danazol likely functions through a non-genomic steroidal pathway.

One of the most recognized immediate responses to androgen is an acute increase in intracellular cyclic adenosine monophoshate (cAMP) [14]. This important second messenger molecule controls a variety of cellular responses including changes in cytoskeleton dynamics and permeability. In this investigation, the adenylyl cyclase activating compound, forskolin, induced a thickening of f-actin in the cell periphery demonstrating that cortical actin changes are indeed a downstream consequence of elevated cAMP. This phenotypic response is also reminiscent of what is observed following low dose danazol treatment of ECs. Does this imply a shared pathway?

To establish a working barrier, endothelial cells are "zippered" to neighboring cells through complex interactions between arrays of transmembrane proteins that must anchor to the cytoskeleton for support [11]. The overall adhesive strength of cell-to-cell contacts is the direct result of an additive contribution from two distinct categories of junctional complexes: adherens junctions (AJ) and tight junctions (TJ). Although they are formed by different molecules, they share a common organizational motif that is critical for optimal adhesive integrity. Transmembrane proteins, such as claudins and occludin in TJs or cadherins in AJs, must arrange in homophilic interactions with sister molecules on juxtaposed cells. Scaffold proteins (zona occludin-1 in TJs and beta-catenin in AJs) then couple the cytoplasmic domain of the adhesion molecules to an impressive list of actin binding, capping, nucleating, and severing proteins [11]. This tethering is believed to be essential for optimal binding strength [15]. Supporting evidence for this hypothesis is the fact that mutant forms of VE-cadherin with

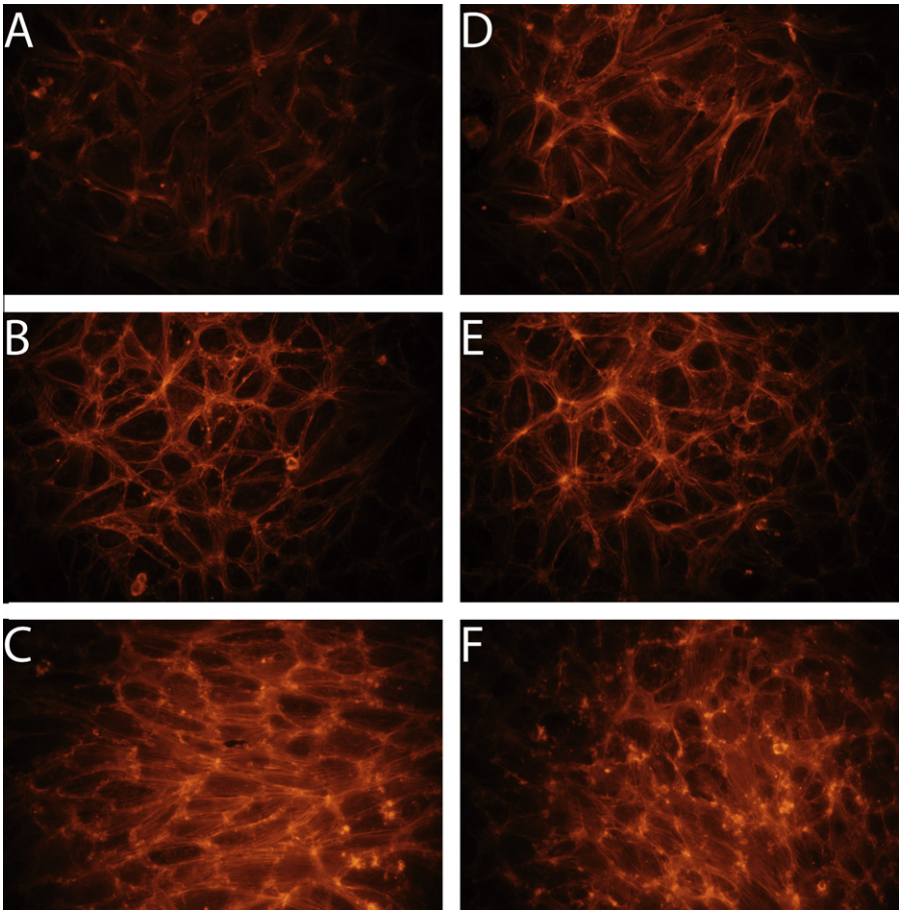

Fig. 3. f-Actin cortical rearrangements and danazol. Retinal endothelial cells treated with increasing amounts of danazol and stained with rhodamine conjugated phalloidin. Cells fixed 3 h after treatment. Treatment groups: (A) vehicle control, (B) 10 μM Forskolin, (C) 100 ng/ml TNFα, (D) 0.1 μM danazol, (E) 1.0 μM danazol and (F) 10 μM danazol.

truncated intracellular binding domains still link in lateral homophilic clusters, but junctional strength is greatly compromised [16]. Therefore, does increased cortical actin provide a catalyst or scaffold for interactions between the cytoplasmic domain of junctional proteins and the cytoskeleton? Sphingosine-1-phosphate, one of the most potent and well characterized barrier stabilizing compounds identified to date, also causes cortical actin rearrangements implying the necessity of these cytoskeletal changes for barrier enhancement [17]. In summary, the importance of cortical actin to barrier function cannot be overestimated, and it is reasonable to suggest that potential elevations in cAMP induced by danazol serving as a classical androgen could have physiological consequences.

In direct opposition to the centripetal forces exerted by cortical actin, cellular contraction will afford free passage of solutes via the paracellular route. One of the cardinal features of endothelial cells challenged with edemagenic agents, such as thrombin or proinflammatory cytokines, is a rapid and severe accumulation of f-actin stress fibers [11]. When cellular microenvironments favor stress fiber formation, myosin motors track across the fibers, sliding actin filaments past one another, inducing contractile forces that cause cell retraction and intercellular gaps [11].

Another well characterized outcome of androgen stimulation is Ca^{2+} mobilization [14] and we propose that this could be the culprit surrounding high dose danazol stress fiber formation and the subsequent increase in permeability associated with these doses. Calcium influx influences a wide range of cellular processes, but, in regards to permeability, the calcium sensing molecule, protein kinase C (PKC) is of key importance. PKC is a known activator of the small GTPase, Ras homolog gene family member A (RhoA) [12]. This intercellular switch, through a series of sequential events, can increase f-actin reorganization into stress fibers and elevate myosin motor activity [12,18]. The effect may also be further exacerbated as danazol levels are driven higher. Rises in intracellular Ca^{2+} stimulate androgen binding to AR as well as activate calcium-dependent kinase pathways, such as ERK and Src, which can phosphorylate AR causing conformational changes that further enhance its activity [14]. Taken together, feed-back loops exist that could drive stress fiber formation under conditions of dramatic calcium influx. Stress fiber formation appears to be a consequence of high dose danazol treatment and these pathways could explain their presence at these concentrations.

In an attempt to confirm that the AR is involved in this response, we carried out competitive binding studies using an AR antagonist in the presence of danazol. It is evident from these experiments that the AR contributes to rapid and dramatic changes in monolayer barrier function and that danazol can augment this activity. The barrier enhancing properties of danazol in our study were blocked by hydroxyflutamide in a competitive manner. In addition, hydroxyflutamide alone reduced TEER demonstrating the importance of functional AR interactions to barrier integrity and that danazol ligation can initiate these downstream effects. Studies designed to track localization of the AR after exposure to testosterone show that a portion of the receptor pool is ferried to the periphery of the cell, placing ligand–receptor complexes in close proximity to cortical events [19].

Contrary to being a known androgen, danazol binding to the ER may also play a role in the observed TEER enhancement.

Unexpectedly, ER antagonism also interferes with the effects of danazol. Danazol has been shown to bind the ER receptor but with very poor affinity [20]. Fulvestrant reduced danazol-induced TEER increases at all time points tested except the 24 h measurement. We suspect that by 24 h, danazol has displaced fulvestrant and inhibition is no longer apparent. Interestingly, a similar biphasic response was documented by another group investigating the action of estrogen on vascular permeability [21]. The ER is obviously a key contributor to vascular barrier integrity as demonstrated by the immediate drop in resistance observed by blockage of the ER with fulvestrant in this model. Any danazol interactions with the ER, weak or otherwise, could have implications on EC barrier function.

A major focus of ongoing research is to establish a biological role for the rapid, non-genomic responses of steroids. Here for the first time we provide evidence that treatment of primary cultured human endothelial cells with danazol can quickly affect cytoskeleton dynamics resulting in altered monolayer permeability. Importantly, danazol's effect appears to be biphasic with low doses promoting the formation of a cortical f-actin ring and stabilizing permeability, while elevated doses cause cell contraction and increased permeation through the paracellular pathway. Current guidelines for the administration of danazol in the treatment of endometriosis load the patient with blood concentrations which exceed the barrier enhancing levels observed in our investigation [22]. We therefore suggest that low dose danazol could potentially serve in the treatment of multiple disorders characterized by pathological vascular permeability. Uniquely, danazol is devoid of the devastating consequences of corticosteroid use [23]. In conclusion, low dose danazol has the potential to provide additional or alternative interventions for the treatment of diseases involving vascular leakage.

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