Estrogen Receptor-α Mediates Gender Differences in Atherosclerosis Induced by HIV Protease Inhibitors*

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As part of highly active antiretroviral therapy, protease inhibitor treatment has significantly increased the lifespan of human immunodeficiency virus (HIV)-infected individuals. Many patients, however, develop negative side effects, including premature atherosclerosis. We have previously demonstrated that in male low density lipoprotein receptor (LDL-R) null mice, HIV protease inhibitors induce atherosclerotic lesions and cholesterol accumulation in macrophages in the absence of changes in plasma lipid levels. We determined that these increases were due to an up-regulation of the scavenger receptor CD36. In the present study, we examined the effects of HIV protease inhibitors in female LDL-R null mice. Female mice given ritonavir and amprenavir (23 and 10 µg/mouse/day, respectively) developed fewer atherosclerotic lesions than males. Furthermore, peritoneal macrophages isolated from ritonavir-treated females had reduced levels of cholesterol accumulation as compared with males, and CD36 protein levels were increased to a significantly lesser degree in females than in males. To investigate the molecular mechanisms of this gender difference, we examined the effect of genetically removing estrogen receptor-α (ERα). In female mice lacking both LDL-R and ERα, the protective effect of gender was lost. Additionally, the reduced levels of cholesterol accumulation in macrophages observed in females was reversed. Furthermore, the absence of ERα resulted in increased expression of CD36 protein in a macrophage-specific manner in mice treated with ritonavir. These data demonstrate that ERα is directly involved in the regulation of cholesterol metabolism in macrophages and plays an important role in the gender differences observed in HIV protease inhibitor-induced atherosclerosis.

Protease inhibitors, as part of highly active anti-retroviral therapy, have greatly increased the lifespan of individuals infected with the human immunodeficiency virus (HIV). Protease inhibitors block the replication of HIV, thereby delaying or preventing the onset of AIDS. Unfortunately, one of the deleterious side effects of protease inhibitor therapy is the potential development of premature atherosclerosis (1). This is particularly true in younger patients (men under the age of 34 and women under the age of 44) (2). HIV protease inhibitor treatment also causes dyslipidemia, which may contribute to the development of cardiovascular disease (3). We have recently demonstrated, however, that HIV protease inhibitors can affect macrophage lipoprotein metabolism and atherosclerotic lesion formation independent of changes in plasma triglycerides and cholesterol levels, suggesting that HIV protease inhibitors can have direct actions on macrophage function (4).

Macrophage recruitment into the vascular wall is one of the earliest events in atherogenesis. The scavenger receptor-dependent uptake of lipoprotein sterols by macrophages in the subendothelial space contributes to the formation of lipid-laden macrophages. The class B scavenger receptor CD36 has been shown to mediate both the uptake and efflux of cholesterol in cell culture models (5, 6) and is the primary mediator of cholesterol accumulation in atherosclerosis induced by HIV protease inhibitors (4).

Many physiological gender differences are the result of the female sex steroid hormones, estrogen and progesterone. Besides the well studied effects of estrogen on the reproductive system, in recent years it has become apparent that estrogen has important actions in other tissues, including non-reproductive areas of the brain and bone and in the immune and cardiovascular systems (7, 8). The mechanisms of action of estrogen in the cardiovascular system are not completely understood, although some protective actions have been attributed to decreasing plasma LDL levels (9). In addition, estrogen has been shown to have a potentially atheroprotective effect by causing vasodilation by increasing nitric oxide production in a non-genomic manner (10, 11). The incidence of cardiovascular disease is greater in men than in women prior to menopause; however, following menopause the risk of cardiovascular disease in women increases dramatically (12). The mechanisms of this difference are not well understood. This lack of understanding is highlighted by the results of the Women’s Health Initiative, which indicate that hormone replacement therapy (HRT) increases cardiovascular disease (13). It remains to be determined whether the negative effects of hormone replacement would be seen with endogenous estrogens (17β-estradiol) instead of the conjugated equine estrogens used in current HRT regimens. Additionally the age at which HRT is initiated is likely a critical factor in the effects of estrogen on cardiovascular disease (14).

Women infected with HIV have a higher rate of gonadal dysfunction resulting in a greater length between menstrual cycles and a higher rate of amenorrhea (15, 16). These symptoms may all be related to a suppression of estrogen levels observed in a subgroup of HIV-infected women (17). A reduction in estrogen in some women could contribute to increasing atherosclerosis, explaining why younger HIV-infected women are no longer protected from cardiovascular disease as compared with males.

The cellular effects of estrogen are primarily mediated through one of two estrogen receptors, α or β (ERα or ERβ). Estrogen receptors are members of the nuclear receptor superfamily and act as ligand-activated transcription factors (18). ERα has been shown to be critical for estrogen atheroprotection in ApoE−/− mice that are prone to develop atherosclerotic lesions (19). In this model of ather-
osclerosis, estrogen lowers plasma lipid levels and reduces atherosclerotic lesion size. However, because estrogen modulates a variety of aspects of cardiovascular physiology, the lipid-independent effects of estrogen on cardiovascular disease are largely unexplored. Such mechanisms were investigated in this study examining the development of atherosclerosis in a model where lesions are induced by HIV protease inhibitors.

MATERIALS AND METHODS

Animals—All animals were housed in the Association for Assessment and Accreditation of Laboratory Animal Care-certified animal facilities at the University of Kentucky. Animals were maintained in constant temperature conditions on a 14:10 light/dark cycle (lights on at 0400 h) and were provided food and water ad libitum. The low density lipoprotein receptor (LDL-R) null and C57BL/6J mice were supplied by The Jackson Laboratory (Bar Harbor, Maine). ERE−/− mice were purchased from Taconic (Germantown, NY). Both LDL-R null and ERE−/− mice are on a C57BL/6J background. To generate mice lacking both the LDL-R gene and ERE, LDL-R null mice were bred with ERE−/+ to produce offspring heterozygous for both alleles (LDL-R−/+; ERE−/+). These mice were then crossed with LDL-R null mice to produce LDL-R−/−; ERE−/+ offspring. Finally, these mice were used as breeding pairs to produce offspring that were homozygous knockouts for both genes (LDL-R−/−; ERE−/−). At 6 weeks of age mice were placed on a normal chow diet and given vehicle control (0.01% ethanol) or protease inhibitors in their drinking water (amprenavir or ritonavir) for 8 weeks. Glaxo Wellcome Inc. (Toronto, Ontario, Canada) provided the amprenavir, and Abbott Laboratories (Abbott Park, IL) provided the ritonavir. This regimen has previously been described to induce atherosclerotic lesions in male LDL-R null mice without altering plasma cholesterol levels (4).

Quantification of Atherosclerotic Lesions—To quantify atherosclerotic lesion area, the aorta from the arch to the ileal bifurcation was collected and fixed in 4% paraformaldehyde. The extraneous tissue was dissected away, and the intimal surfaces were exposed by a longitudinal cut. Isolated aortas were secured with pins and placed under a dissecting microscope equipped with a CCD camera to capture the image of the aorta. Atherosclerotic lesions on the intimal aortic surface appear as opaque white areas compared with the thin, translucent aorta. Areas of intima covered by atherosclerotic lesions were quantified with ImagePro software (Media Cybernetics, Silver Spring, MD) as previously described (20).

Cholesterol and Cholesteryl Ester Mass Quantification—Cholesterol measurements were determined as previously described (21). Mouse peritoneal macrophages were isolated by saline lavage and cell lysates collected (22). The samples were extracted with isopropanol-hexane, and cholesteryl heptadecanoate was added to each vessel preparation to serve as an internal standard. The extracted lipid was derivatized by suspending the dried lipid in N,O-bis(trimethylsilyl) trifluoroacetamide, trimethylchlorosilane, and acetonitrile (89:1:10). The material was heated at 80 °C for 5 min, dried, and suspended in iso-octane. Pure cholesterol (Sigma-Aldrich) was dissolved in iso-octane and used as a standard for the retention time of cholesterol. The samples were injected into a 6890 gas chromatograph G2579A system (Agilent Technologies, Palo Alto, CA). A mass-selective detector (model 5973; Agilent Technologies) was used in both scan and selected ion-monitoring modes to identify the samples.

SDS-PAGE and Immunoblotting—Proteins were isolated from peritoneal macrophages by homogenization in lysis buffer (1× phosphate-buffered saline, 1% Triton-X, 0.1% SDS, 50 mM sodium fluoride, 0.5% deoxycholate with Complete protease inhibitor mixture (Roche Applied Science)). Proteins were suspended in sample buffer containing 0.31 M Tris (pH 6.8), 2.5% (w/v) SDS, 50% (v/v) glycerol, and 0.125% (w/v) bromphenol blue 1.2% (v/v) β-mercaptoethanol and heated at 95 °C for 3 min. 20 μg of total cell protein was separated on a 12.5% SDS-polyacrylamide gel. The separated proteins were then transferred to polyvinylidene difluoride membranes. Each membrane was blocked in Tris-buffered saline containing 5% dry milk for 1 h at room temperature. Primary antibodies were diluted in TBS containing 1% dry milk and incubated with the polyvinylidene difluoride membrane for 1 h at room temperature. Anti-actin IgG was from Sigma-Aldrich, antiamouse CD36 (IgM) was from BioDesign International Inc. (Kennebunkport, ME), and anti-scavenger receptor A was from Serotec Ltd. (Raleigh, NC). Horseradish peroxidase-conjugated IgGs were purchased from Organon Teknika Corp. USA (West Chester, PA). Super Signal chemiluminescent substrate was purchased from Pierce Chemical Co. The polyvinylidene difluoride membrane was washed four times, 10 min each, in TBS plus 1% dry milk. The secondary antibodies (conjugated to horseradish peroxidase) were diluted 1:20,000 in TBS plus 1% dry milk and incubated with the polyvinylidene difluoride membrane for 1 h at room temperature. The membrane was then washed, and the labeled proteins were visualized by chemiluminescence autoradiography. Relative band densities were quantified using ImagePro Software (Medical Cybernetics, Silver Spring, MD).

Northern Blot Hybridization—Total RNA was extracted from cells using TRIzol (Molecular Research Center). RNA was quantified by spectrometry, and 20 μg of RNA was loaded onto a 5% denaturing agarose gel. Following electrophoretic separation, the RNA was transferred to nylon membranes and probed for CD36, peroxisome proliferator-activated receptor γ (PPARγ), and glyceraldehyde-3-phosphate dehydrogenase mRNA as previously described (4).

Statistics—Least squares analysis of variance was used to evaluate the data with respect to treatment and gender or genotype using Statistica v.6 (StatSoft, Tulsa, OK). When appropriate, samples were compared within a given group using the Tukey–Kramer test. Means were considered statistically different at p < 0.05.

RESULTS

Protease Inhibitors Induce Smaller Lesions in Intact Female LDL-R Null Mice as Compared with Males—To determine the effect of gender on the development of atherosclerotic lesions by HIV protease inhibitors, 6-week-old LDL-R null male and female mice were treated with vehicle (0.01% ethanol), amprenavir, or ritonavir (23 or 10 μg/mouse/day, respectively). Following 8 weeks of treatment, aortas were removed and analyzed for the extent of atherosclerotic lesions as we previously described (4). Male mice given amprenavir had a 3-fold increase in lesion area, whereas females had only a 1.5-fold increase (Fig. 1). Male mice given ritonavir had a 5-fold increase in lesion area, whereas females only had a 2-fold increase.

Peritoneal Macrophages from Female LDL-R Null Mice Treated with Protease Inhibitors Accumulate Less Cholesterol than Macrophages from Males—To determine whether the decrease in atherosclerotic lesions was associated with a decrease in macrophage cholesterol accumulation, we isolated peritoneal macrophages from animals given vehicle or ritonavir for 8 weeks (same animals as described in Fig. 1). The mass of total cholesterol (free cholesterol plus cholesteryl esters) was determined by gas chromatography. Peritoneal macrophages isolated from animals receiving ritonavir contained significantly more total cholesterol than animals receiving vehicle only (Fig. 2) (p < 0.01, n = 4). Macrophages from ritonavir-
treated female mice contained significantly less total cholesterol than their male counterparts \( p < 0.01, n = 4 \).

**Peritoneal Macrophages from Female LDL-R Null Mice Treated with Ritonavir Have Reduced CD36 Protein Expression as Compared with Macrophages from Males**—We have previously determined that the scavenger receptor CD36 is responsible for HIV protease-induced cholesteryl ester accumulation in macrophages (4). To determine whether the effects of HIV protease inhibitors on the total amount of CD36 protein in peritoneal macrophages are different in male and female mice, we isolated peritoneal macrophages from animals given vehicle or ritonavir for 8 weeks. Cell lysates were generated from the isolated macrophages and equal amounts of protein resolved by SDS-PAGE and immunoblotted for the scavenger receptors CD36 and SR-A. Fig. 3 demonstrates that peritoneal macrophages isolated from male LDL-R null mice receiving ritonavir contained more CD36 than mice receiving vehicle. SR-A levels were not altered by ritonavir treatment, suggesting that the effect is specific for CD36. Female mice also had an increase in CD36 levels, but it was significantly less compared with males. Blots were normalized for loading by blotting with actin. There was no difference in actin levels among the groups (data not shown). Relative levels were quantified and are represented in Fig. 3B \( p < 0.05, n = 4 \).

**ERα Is Required for Reduced Atherosclerotic Lesion Formation in Female Mice Treated with Ritonavir**—To begin to investigate the molecular mechanisms responsible for the gender difference observed in HIV protease inhibitor-induced atherosclerosis, we investigated the role of ERα in mice lacking both LDL-R and ERα. We used female LDL-R null mice with an intact ERα gene and female LDL-R null mice that we crossed with ERα knock-out mice to produce LDL-R null mice that were also ERα−. Six-week-old female mice were treated with vehicle (0.01%) or ritonavir (25 μg/mouse/day) for 8 weeks and analyzed for atherosclerotic lesions. This dose of ritonavir is greater than that used in the previous experiment examining gender differences. This dose was chosen to maximize lesion formation and does not alter plasma lipid levels (Table 1). Ritonavir treatment induced a significant increase in lesion area \( p < 0.001, n = 10 \) as we had observed previously. Mice lacking ERα, however, had an even greater increase in lesion area \( p < 0.001, n = 10 \).

**HIV Protease Inhibitors Do Not Cause Dyslipidemia in LDL-R Null/ERα−/− Mice**—We have previously demonstrated that at low doses, ritonavir can induce atherosclerosis without causing changes in plasma levels of triglycerides or total cholesterol (4). To confirm that the same is true in the mice we generated that lack the ERα gene in addition to the LDL-R gene, we measured plasma levels of glucose, insulin, triglycerides, and total cholesterol from mice treated for 8 weeks with 25 μg of ritonavir/day (Table 1). Additionally, we monitored weight gain over...
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TABLE I
Physiological parameters of mice treated with ritonavir

* significantly different from vehicle-treated mice (p < 0.01), #, significantly different from LDL-R null ritonavir-treated mice (p < 0.01).

|                          | Vehicle LDL-R null | Vehicle LDL-R null/ERα− | Ritonavir LDL-R null | Ritonavir LDL-R null/ERα− |
|--------------------------|-------------------|--------------------------|----------------------|---------------------------|
| % body weight gain       | 39.7 ± 0.022      | 19.0 ± 0.025*            | 46.3 ± 0.018         | 33.4 ± 0.052*            |
| Glucose (mg/deciliter)   | 81.2 ± 0.014      | 81.4 ± 1.477             | 82.0 ± 0.803         | 81.9 ± 1.130             |
| Insulin (ng/ml)          | 0.32 ± 0.007      | 0.32 ± 0.004             | 0.33 ± 0.009         | 0.33 ± 0.007             |
| Total cholesterol (mM/liter) | 8.9 ± 0.35     | 9.2 ± 0.41               | 8.7 ± 0.21           | 9.4 ± 0.56               |
| Triglycerides (mM/liter) | 2.83 ± 0.014      | 2.82 ± 0.013             | 2.80 ± 0.009         | 2.81 ± 0.014             |

FIGURE 4. Ritonavir promotes atherosclerotic lesion formation in female mice lacking the ERα gene to a greater degree than in female mice with an intact ERα gene. ERα+ or ERα− mice were treated with vehicle (0.01% ethanol) or ritonavir (25 μg/day) for 8 weeks. Following 8 weeks of treatment, aortas were removed and atherosclerotic lesions quantified. ERα− mice had a significantly larger area of atherosclerotic lesions compared with the ritonavir-treated ERα+ mice. Bars represent mean ± S.E., n = 10. *, significantly different from vehicle, p < 0.001. #, significantly different from ERα+ mice treated with ritonavir, p < 0.001.

FIGURE 5. Peritoneal macrophages isolated from female ERα− mice treated with ritonavir accumulate more total cholesterol mass than those isolated from female mice expressing ERα. Female mice were treated with vehicle (0.01% ethanol) or ritonavir (25 μg/day) for 8 weeks. Peritoneal macrophages were isolated by saline lavage and processed to quantify total cholesterol mass by gas chromatography. Bars represent the mean ± S.E., n = 4. *, significantly different from vehicle, p < 0.001. #, significantly different from ERα+ mice treated with ritonavir, p < 0.001.

Ritonavir Induces a Greater Increase in CD36 Protein Levels in Peritoneal Macrophages in the Absence of ERα—Because we have previously determined that CD36 mediates the accumulation of cholesterol esters in macrophages exposed to protease inhibitors, we investigated whether ERα is involved in the regulation of CD36 protein expression. Peritoneal macrophages were isolated from animals given vehicle or ritonavir for 8 weeks. Cell lysates were generated from isolated macrophages and adipocytes and equal amounts of proteins resolved by SDS-PAGE and immunoblotted for the scavenger receptors CD36 and SR-A. Fig. 7 demonstrates that peritoneal macrophages isolated from mice receiving ritonavir contained more CD36 protein than mice receiving vehicle. SR-A protein levels were not altered. In mice lacking both ERα and LDL-R, ritonavir had a greater increase in CD36 protein levels, although there was no difference in baseline CD36 expression. Additionally, SR-A levels were not changed. Blots were normalized for loading by blotting with actin. There was no difference in actin levels among the groups. The effect on CD36 was specific to macrophages, as there was no effect in adipocytes with either ritonavir or the absence of ERα.

Ritonavir Induces a Greater Increase in CD36 and PPARγ mRNA Expression in Peritoneal Macrophages from ERα− Mice—We have previously demonstrated that PPARγ plays a critical role in regulating the expression of CD36 in macrophages (4). To determine whether ERα mediates its expression, we examined PPARγ mRNA levels in peritoneal macrophages isolated from animals treated with ritonavir for 8 weeks. Total RNA was isolated and separated on a 5% denaturing agarose gel. The RNA was transferred to a nylon membrane and probed for CD36 and PPARγ using radiolabeled DNA. Glyceraldehyde-3-phosphate dehydrogenase mRNA served as a loading control. The absence of ERα had no effect on the baseline expression of CD36 mRNA; however, treatment with ritonavir greatly increased the levels of CD36 mRNA, and these increases were further enhanced in ERα− mice (Fig. 8). This...
increase paralleled that seen with the levels of CD36 protein. The levels of PPARγ mRNA are similarly elevated in the response to ritonavir in both male and female patients, especially in young patients (1–3). Healthy young women are generally protected against atherosclerosis as compared with men. However, following menopause this protection is lost, suggesting a protective role of the female hormonal milieu (23). The mechanisms of this gender difference are not completely understood, but estrogen appears to have a beneficial role in reducing plasma LDL levels as well as numerous direct effects on the vascular wall (11, 24). In light of recent data from the Women’s Health Initiative concerning the negative effects of the current HRT regimens on cardiovascular disease (13), the mechanism of action of endogenous estrogens needs to be elucidated. Although the Women’s Health Initiative investigated the actions of conjugated equine estrogens used in HRT, a better understanding of the mechanism of action of endogenous estrogens will eventually lead to improved hormonal replacement therapies.

We have previously shown that HIV protease inhibitors can induce significant atherosclerosis in male mice prone to developing lesions (LDL-R null and Apoe−/− mice) at doses that do not increase plasma lipid concentrations (4). In the present study, females developed lesions following treatment with HIV protease inhibitors, but to a greatly reduced extent as compared with males. Furthermore, the source of this gender difference involves ERα. Genetic removal of the receptor completely reverses the protective effect in females and even worsens atherosclerosis induced by ritonavir without altering plasma lipid levels or metabolic markers associated with insulin resistance commonly seen in patients on HIV protease inhibitor therapy. Reduced cholesteryl ester accumulation, CD36 protein, mRNA and PPARγ mRNA expression in macrophages from female mice require ERα and suggest a novel role of estrogen and ERα in the development of atherosclerosis.

Atherosclerosis is a multifaceted, complex process, but macrophage cholesterol accumulation plays a critical role in the development of foam cells and fatty streaks. Because we have used a model where atherosclerosis develops in the absence of changes in plasma lipid levels, we are able to remove that complicating influence from our studies. Our data suggest that the gender differences observed occur, at least in part, at the level of cholesterol accumulation in macrophages. Peritoneal macrophages from female mice contain less cholesteryl ester mass and have reduced alterations in the scavenger receptor system responsible for cholesterol uptake. The effect on CD36 expression is specific to macrophages as demonstrated by a lack of an effect in adipocytes. Furthermore, macrophages from ER mice have increased cholesteryl ester accumulation in response to ritonavir treatment in vitro. These data suggest that in females estrogen can influence cholesterol accumulation directly at the level of the lipoprotein metabolism machinery in macrophages.
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In several animal models of diet-induced atherosclerosis, females tend to have larger lesions (25). The different effect in females in these models and the current model of atherosclerosis induced by HIV protease inhibitors is likely due to the pharmacological initiation of atherosclerosis in the absence of increase plasma lipid concentrations. The female gonadal steroid hormone 17β-estradiol has been shown to protect against atherosclerosis in ApoE−/− mice (19). In that study, ERα was responsible for atheroprotection, and this may be in part due to the positive effects on plasma lipid levels. In the study we have presented here, the absence of ERα exacerbated lesion size following the HIV protease inhibitor insult, reversing the normally protective effect of gender. These data confirm and extend the findings that ERαs is involved in the gender differences observed in different animal models of atherosclerosis and that estrogen action in the cardiovascular system occurs by multiple mechanisms.

The levels of circulating 17β-estradiol in ERα mice are elevated because of a lack of negative feedback (26). Elevated estrogen levels may influence atherosclerosis progression by the antioxidant properties of estrogens (27). Similar levels of 17β-estradiol in osteoblasts and adipocytes (34). Additionally, ritonavir may directly alter estrogen receptor activity. Ritonavir has been shown to inhibit the activity of the proteasome (35). Interestingly, proteasomal degradation of ERα is required for efficient receptor activity (36). In the case of PPARγ, if ERα is not efficiently degraded the negative effects on PPARγ mRNA expression could be sustained in the presence of ritonavir. This is a positive effect in terms of cholesterol accumulation in macrophages. The signaling events stimulated by ritonavir treatment that result in an increase in CD36 mRNA expression could be counteracted in the presence of ERα.

The data presented here demonstrate a novel role of ERα in regulating proteins involved in lipoprotein metabolism in macrophages. This role of estrogen and ERα is critical for the prevention of atherosclerosis induced by HIV protease inhibitors. This may be of particular importance in patients as HIV and HIV protease inhibitor therapy can alter gonadal hormone levels as well as the metabolism of estrogen (17, 37). Alterations in endogenous estrogen levels may contribute to the loss of the protection against cardiovascular disease in females on HIV protease inhibitor therapy.

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