Estrogen-Mediated Cytoplasmic and Nuclear Distribution of Rat Cardiovascular Estrogen Receptors

Alan L. Lin and Sydney A. Shain

We used either the synthetic estrogen R2858 (moxestrol) or estradiol-17β to characterize estrogen receptors in cytoplasmic (R2858) and nuclear (estradiol-17β) preparations from rat aorta and myocardium. Relative steroid specificity studies showed that only estrogens were effective inhibitors of R2858 or estradiol-17β binding to aortic and myocardial estrogen receptors, whereas androgens, progestins, and cortisol were ineffective inhibitors. Low ionic strength sucrose density gradient analyses showed that myocardial estrogen receptors that localized in the cytoplasmic fraction migrated as macromolecules with sedimentation coefficients of 8S to 9S. In contrast, two binding components of sedimentation coefficients 8S to 9S and 10S to 11S were characteristic of the estrogen receptors localized in aortic cytoplasmic preparations. High ionic strength sucrose density gradient analysis showed that aortic and myocardial estrogen receptors localized in the nuclear fraction migrated as macromolecules with sedimentation coefficients of 4S to 6S. Saturation analyses showed that aortic and myocardial cytoplasmic preparations from intact young mature male rats contained 50.6 ± 12.9 (mean ± SD) and 51.0 ± 14.1 fmol receptor/mg DNA, respectively. The respective R2858 dissociation constants were 0.42 and 0.15 nM. Estrogen receptors could not be demonstrated in nuclear preparations from cardiovascular of intact males. Estradiol-17β injection of intact young mature male rats caused "depletion" of aortic and myocardial cytoplasmic fraction estrogen receptors and resulted in the appearance of 51.9 ± 21.0 and 36.9 ± 9.5 fmol receptor/mg DNA in the corresponding nuclear fractions. The respective estradiol-17β dissociation constants were 1.56 and 0.71 nM. Increased estrogen receptor content of cardiovascular nuclear fractions of estradiol-17β injected male rats correlated well with the concomitant decreased cytoplasmic fraction receptor content. The ability of estradiol-17β to affect localization of cardiovascular estrogen receptors between cytoplasmic and nuclear fractions suggests these estrogen receptors are physiologically functional and indicates that estrogen may directly regulate cardiovascular cell function.

(Arteriosclerosis 5:668–677, November/December 1985)

The higher incidence of coronary heart disease (CHD) in postmenopausal women as compared to premenopausal individuals and in oral contraceptive users as compared to nonusers and the decreased mortality of estrogen-only users as compared to nonusers has suggested a possible etiologic role for estrogens in CHD. This impression is supported by reports of higher plasma estrogen content in males who survived a myocardial infarction or have documented coronary artery occlusion in the absence of an infarction. These clinical observations have led some investigators to conclude that hyperestrogenemia is an additional risk factor for CHD in males. Plasma estrogens are elevated in individuals who have symptoms of CHD without angiographically demonstrable evidence of vascular occlusion. Moreover, individuals with hypergonadotrophic hypergonadism (Klinefelter's syndrome, 47 XXY) and plasma estrogen levels at least as high as in men with CHD do not experience death rates greater than that predicted for the general population. Additionally, plasma estradiol levels of cases and controls were identical.
during longitudinal evaluation of low risk and high risk males who subsequently did or did not develop CHD. These latter studies in males indicated that hyperestrogenemia may not be a uniform risk factor for CHD because many hyperestrogenemic individuals do not experience increased episodes of CHD compared to males with normal plasma estrogen content. Moreover, the suggested protective effect of estrogen in females is opposed to its apparent absence in males implies complex effects of estrogens in CHD that may be gender-specific.

Rodent and chicken aortic collagen and elastin content is diminished by exogenous estrogen treatment. Gonadectomy of female rats increases both the aortic glycosaminoglycan (GAG) content and the activity of GAG synthetic enzymes and decreases the activity of enzymes associated with GAG degradation. These effects are reversed by estradiol treatment. Cultured bovine aortic smooth muscle cell collagen production is diminished by estrogens, whereas estrogens increase prostacyclin synthesis by cultured rat aortic smooth muscle cells. Prepubertal ovariectomy decreases left ventricular function in mature female rats. Both biochemical and autoradiographic data establish the presence of estrogen binding components in cardiovascular tissue of the rat, rabbit, canine, and baboon (Lin and Shain, unpublished data). However, none of these studies describe nuclear estrogen binding components, establish an in vivo effect of estrogens on estrogen binding component distribution between nuclear and cytoplasmic fractions, or well establish a relationship between tissue estrogen-binding component content and/or distribution and modification of physiologic function.

It is well documented that breast and uterus contain estrogen-binding components, which are not estrogen receptors. These Type II binding components may be distinguished from estrogen receptors, Type I binding components, on the basis of differences in estrogen binding kinetics (both cytoplasmic and nuclear components) or by the inability of estrogens to cause depletion of cytoplasmic Type II estrogen binding components. Because autoradiographic studies do not permit identification of either the binding component or the retained steroid and because prior preliminary biochemical characterizations have not differentiated Type I and Type II estrogen binding components or have quantitated only steroid unoccupied cytoplasmic estrogen binding components, there is concern about the nature or possible physiologic significance of previously described cardiovascular estrogen binding macromolecules. In this report, we describe characterization of estrogen receptors in the cytoplasmic and nuclear fractions of rat aorta and myocardium, and we document in vivo effects of estrogen on receptor localization that suggest that these binding components are physiologically functional.

Methods

Animals

Inbred mature male AXC rats were from the colony at Southwest Foundation for Biomedical Research. The origin and maintenance of this colony and tissue collection are described in the preceding report. Selected intact male rats were injected intraperitoneally with 10 µg of estradiol-17β in ethanolic saline (20:80, vol/vol) and were sacrificed 1 hour later. Males were lightly anesthetized with ether and sacrificed by cervical dislocation. Animal protocols were performed in accordance with guidelines established by the Department of Health and Human Services for the care and maintenance of laboratory animals. All animal protocols were approved by the Animal Research Committee at Southwest Foundation.

Chemicals

[118-Methoxy-3H]R2858, moxestrol (specific activity 87 Ci/mmol), radioinert R2858, [2,4,6,17H]-estradiol (specific activity 134 Ci/mmol), radioinert R5020 (promegestone), and radioinert R1881 (methyltrienolone) were obtained from New England Nuclear Corporation, Boston, Massachusetts, and were used as provided by the manufacturer. Sources for other chemicals used in this study were previously described.

Preparation of Cytoplasmic Extracts and Quantitation of Total Estrogen Receptor Content by Saturation Analysis

Extracts of aorta and myocardium were prepared in buffers containing sodium molybdate and phenylmethylsulfanyl fluoride, according to previously described methods with the exception that triamcinolone acetonide was not added to the cytoplasmic extracts. Duplicate incubations were performed at 30°C for 2 hours in a final volume of 220 µl buffer containing 1 mg/ml BSA to minimize adsorption of R2858 to glass.

Preparation of Nuclear Extracts and Quantitation of Total Estrogen Receptor Content by Saturation Analysis

Nuclear extracts were prepared as previously described. Duplicate incubations were performed at 30°C for 2 hours in a final volume of 440 µl Buffer BBPP [20 mM sodium barbital, 1.5 mM EDTA, 150
mM KCl, 5 mM dithiothreitol, 5 mM pyridoxal-5'-phosphate, 20% glycerol (vol/vol), pH 8.0 at 20°C containing 200 μl nuclear extract and various concentrations of radiolabeled estradiol-17β (0.5 to 10 nM). Nonspecific binding was determined in a parallel series of identical duplicate incubations that additionally contained 1.0 μM radioinert estradiol-17β. Specific binding was calculated as described in the preceding section.

Other Methods

Bound and free radioligand were separated by the hydroxylapatite adsorption method previously described.35 Specific binding data were evaluated by the method of Scatchard38 and as double reciprocal plots.37 Linear sucrose density gradient centrifugation analyses to characterize rat myocardial and aortic estrogen receptors were performed in the Sorvall vertical tube rotor (TV 865) using conditions previously described.35 Unless otherwise stated, at least two independent analyses were performed. Protein was determined by the procedure of Lowry et al.40 using bovine serum albumin as standard. DNA was quantitated by the fluorometric method of Vytasek41 using salmon testes DNA as standard. Sample preparation for quantitation of radioisotope was as previously described.35

Results

Effect of Incubation Interval on Radiolabeled R2858 Binding in Rat Myocardial and Aortic Cytoplasmic Extracts

To identify optimal conditions for characterization and quantitation of rat cardiovascular total (steroid-occupied and steroid-free) estrogen receptors, three different incubation protocols were evaluated. Maximum binding of R2858 to myocardial estrophiles was detected during incubation of tissue extracts at 39° C for 2 hours (Figure 1). By comparison, the quantity of detected estrophiles was diminished when extracts were incubated either at 24° C for 20 hours or 37° C for 1 hour (Figure 1). Subsequent binding time course determinations showed that maximum binding of R2858 to rat myocardial or aortic estrophiles occurred during 30 to 60 minutes of incubation and was maintained through at least a 3-hour incubation at 30° C (Figure 2). When we added a 200-fold excess of radioinert R2858 (final concentration 200 nM) to rat myocardial or aortic tissue extracts which had been incubated with 1 nM radiolabeled R2858 for 30 minutes at 30° C and continued incubation at 30°C, we observed rapid displacement from myocardial and aortic estrophiles of radiolabeled R2858 by radioinert R2858 (Figure 2). Exchange of radioinert and radiolabeled R2858 occurred with a t½ of approximately 60 to 90 minutes in both myocardial and.

Figure 1. Effect of incubation interval and temperature on R2858 binding to estrogen receptors in myocardial cytoplasmic preparations. Incubation mixtures contained 0.1 to 4 nM radiolabeled R2858. A. Observed specific binding: (•) = 30° C, 2 hours; (•) = 24° C, 20 hours; (•) = 37° C, 1 hour. B. Scatchard plots of specific binding data in A. Separation of bound and free steroid by HAP adsorption was performed at 2° C. Specific binding was determined as described in the text. Reported data have had background subtracted.

Figure 2. Effect of incubation interval and inclusion of radioinert R2858 on radiolabeled R2858 binding to estrogen receptors in rat aortic (A) and myocardial (B) cytoplasmic preparations. Incubation mixtures initially contained 1 nM radiolabeled R2858 (•). After incubation for 30 minutes at 30° C, a parallel series was made 200 nM in radioinert R2858 and incubation at 30° C was continued (•). Other procedures were described in Figure 1. Reported data have had background subtracted.
aortic extracts. Incubation of myocardial extracts at 60° C for 1 hour in the absence of radiolabeled probe and sodium molybdate caused complete inactivation of R2858 binding components (data not shown).

Characterization of Relative Steroid Specificity of R2858 and Estradiol-17β Binding to Rat Myocardial and Aortic Estrogen Receptors Localized in Cytoplasmic and Nuclear Preparations

Single concentration determinations of the ability of selected steroids to inhibit R2858 binding to estrogen receptors in rat myocardial and aortic cytoplasmic preparations (Table 1) showed that estradiol-17β and diethylstilbestrol were effective inhibitors whereas progesterone, R5020, testosterone, R1881, and cortisol were ineffective inhibitors of R2858 binding. Because estrogen receptors could not be detected in nuclear fractions prepared from cardiovascular tissue of intact males, we used estradiol-17β injection as a means of attempting to generate nuclear estrogen receptors. Estradiol-17β injection consistently caused appearance of estrogen receptors in extracts prepared from treated male aortic or myocardial nuclear fractions. Evaluation of the relative ability of selected steroids to inhibit estradiol-17β binding to rat myocardial and aortic nuclear fraction estrogen receptors showed (Table 1) that only estrogens were effective inhibitors whereas androgens, progestins, and cortisol were ineffective inhibitors of estradiol-17β binding.

Sucrose Density Gradient Characterization of Rat Myocardial and Aortic Estrogen Receptors

Low ionic strength linear 10% to 30% sucrose density gradient analyses (Figure 3) showed that myocardial cytoplasmic extracts contained a major peak of R2858 binding with a sedimentation coefficient of 8S to 9S which effectively was eliminated by 100-fold excess radiolabeled estrogen, while R1881 and R5020 (100-fold excess) were ineffective inhibitors. Low ionic strength linear 10% to 30% sucrose density gradient analysis (Figure 4 A) showed that rat aortic cytoplasmic extracts contained a dispersed area of R2858 binding in the 8S to 9S region of the gradient and a peak of binding activity sedimenting at 10S to 11S. Both areas of binding activity were effectively eliminated by excess radiolabeled R2858. Identical results were obtained for four independent determinations performed in duplicate. When aortic cytoplasmic R2858 binding components were characterized on high ionic strength linear 20% to 40% sucrose gradients, a single 4S to 6S peak of binding activity was identified, which effectively was diminished by excess radiolabeled R2858 (Figure 4 B). High ionic strength density gradient analyses showed that rat myocardial and aortic nuclear extracts contained a major peak of estradiol-17β binding with sedimentation coefficient of 4S to 6S (Figure 5). These aortic and myocardial nuclear estradiol binding components effectively were eliminated by excess radiolabeled estradiol-17β (Figure 5).

<table>
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<th>Nuclear†</th>
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Table 1. Relative Steroid Specificity of Estrogen Receptors in Rat Cardiovascular Cytoplasmic and Nuclear Preparations

Data are the means of triplicate (myocardium: cytoplasmic, nuclear), duplicate (aorta: nuclear) independent determinations, or are a representative determination (aorta: cytoplasmic) made by using tissues from male rats. NP = determination not performed.

* Radiolabeled R2858, 2 nM, was used as probe of estrogen receptors in cytoplasmic preparations. Competitor concentration was 200 nM. Incubation was at 30° C for 2 hours.

† Radiolabeled estradiol-17β, 3 nM, was used as probe of estrogen receptors in nuclear preparations. Competitor concentration was 300 nM. Tissues were obtained 1 hour postinjection (i.p.) of 10 μg estradiol-17β in ethanol: saline (20:80). Incubation was at 30° C for 2 hours.
Figure 3. Sucrose density gradient characterization of estrogen receptors in rat myocardial cytoplasmic preparations. Tissue extracts were incubated at 30°C for 2 hours with \( {}^{3}H\)-R2858 (0.5 nM) or with \( {}^{3}H\)-R2858 (0.5 nM) plus radiolabeled competitors (50 nM). Incubates were cooled to 2°C and treated with DGCC before application to linear sucrose density gradients (10% to 30% sucrose) which were developed by centrifugation at 370,000 g (TV 865 vertical rotor) at 2°C for 2 hours. Data from a single experiment are divided into two sets (A and B) for clarification. The specific binding peak in both panels contains 1.02 fmol R2858 and the concentration of receptor in this preparation was 7.42 pM. Reported DPM have had background subtracted. Sedimentation is from left to right. The sedimentation coefficient of human \( \gamma \)-globulin (arrow) is 7S.

Figure 4. Sucrose density gradient characterization of estrogen receptors in rat aortic cytoplasmic preparations. A. Conditions of assay and definitions are described in Figure 3. The sum of R2858 binding in the 8S to 9S and 10S to 11S peaks is 1.85 fmol and the concentration of binding species in this preparation was 13.5 pM. B. Aortic cytoplasmic extracts were labeled by incubation at 30°C for 1 hour with \( {}^{3}H\)-R2858 (0.5 nM) or with \( {}^{3}H\)-R2858 (0.5 nM) plus radiolabeled R2858 (500 nM). After separating free and bound ligand by HAP adsorption, receptors bound to HAP were eluted with 1 M phosphate buffer as described in the text. Eluates were applied to linear sucrose density gradients (20% to 40% sucrose containing 15% glycerol) which were developed by centrifugation at 370,000 g (TV 865 vertical rotor) at 2°C for 12 hours. The specific binding peak contains 4.58 fmol R2858 and the concentration of the binding species in this preparation was 4.58 pM. Sedimentation is from left to right. The sedimentation coefficient of human \( \gamma \)-globulin (arrow) is 7S. Reported DPM have had background subtracted.
Figure 5. Sucrose density gradient characterization of estrogen receptors in rat aortic (A) and myocardial (B) nuclear preparations. Tissues were obtained 1 hour after injection (i.p.) of 10 μg estradiol-17β in ethanol/saline (20:80, vol/vol). Nuclear extracts were prepared and labeled by incubation at 30°C for 1 hour with 1H-estradiol-17β (1 nM) or with 1H-estradiol-17β (1 nM) plus radioinert estradiol-17β (1 μM). HAP adsorption and gradient centrifugation (9 hours) were described in the legend to Figure 4B. The specific binding peaks in A and B contain 4.30 and 3.21 fmol estradiol-17β, respectively, and the concentration of binding component in these preparations was 1.78 and 2.39 pM, respectively. Reported DPM have had background subtracted.

Quantitation of Rat Myocardial and Aortic Estrogen Receptors Localized in Cytoplasmic and Nuclear Fractions

To assess the reproducibility and accuracy of the analyses, we prepared a single cytoplasmic and nuclear extract of rat myocardium and divided each preparation into three aliquots. Each homogenous preparation was used for independent, simultaneous determination of site content by saturation analysis. The saturation (Figure 6) and Scatchard plots (Figure 7) of these data establish that ligand binds to a single class of limited capacity, high affinity binding sites. Cytoplasmic, 10.3 ± 0.4 fmol/ml (mean ± so), and nuclear, 1.82 ± 0.28 fmol/ml. Site content determinations are highly reproducible and have intraassay coefficients of variation of 3.76% and 15.4%, respectively. Site content quantitated by saturation analysis is representative of that estimated at the same ligand concentration during sucrose gradient characterization of myocardial receptors (Figures 3 and 5B).

Typical saturation data (Figures 7 and 8) demonstrate that R2858 is binding to limited capacity, high affinity binding sites in aortic and myocardial cytoplasmic extracts. Cytoplasmic receptors are saturated at 2 to 4 nM ligand. Saturation analyses of nuclear extracts prepared from myocardium or aorta of estradiol-17β injected intact males consistently showed evidence of limited capacity, high affinity binding of estradiol-17β that saturated at 3 to 6 nM ligand, whereas identical extracts prepared from myocardium or aorta of intact males consistently failed to have demonstrable, saturable nuclear estradiol-17β binding components (Figure 8).

Estrogen-Mediated Localization of Estrogen Receptors in Cytoplasmic and Nuclear Fractions of Rat Cardiovasculature

Injection of intact males with estradiol-17β (10 μg) caused a 65% reduction in aortic cytoplasmic fraction estrogen receptor content which was quantitatively accounted for by localization of estrogen receptors in the nuclear fraction (Table 2). The total aortic estrogen receptor content of estradiol-17β-injected males was not significantly different from that of control males, p < 0.8 (Table 2). Identical treatment of intact males also caused a 65% reduction of myocardial cytoplasmic fraction estrogen receptors and a concomitant increase in estrogen receptors localized in the nuclear fraction (Table 2). The total myocardial estrogen receptor content of control and estradiol-17β-injected rats was not significantly different, p < 0.5. The precision of these determinations (Table 2), as assessed by the coefficient of variation for the individual measurements, is comparable to that determined for intraassay variation (Figures 6 and 7). The R2858 dissociation constants of cytoplasmic aortic and myocardial estrogen receptors were 0.42 ± 0.32 (mean ± so) respectively, and 0.15 ± 0.04 nM, whereas the respective values for estradiol-17β binding to nuclear estrogen receptors were 1.56 ± 0.19 and 0.71 ± 0.33 nM.
Figure 6. Saturation plots of R2858 binding to cytoplasmic and estradiol-17β binding to nuclear estrogen receptors as determined in replicate analyses. Cytoplasmic or nuclear extracts were prepared from rat myocardium and each was divided into three aliquots. These were used for independent saturation analyses in which total and nonspecific binding were determined in duplicate at each ligand concentration. Specific binding, calculated from mean values, is reported. For each ligand concentration, the percent difference between the mean value and individual determinations was calculated. These data were used to calculate a mean for all determinations. The values for cytoplasmic total and nonspecific binding data were 2.4% ± 2.2% (mean ± so) and 2.8% ± 2.4%, respectively, whereas those for nuclear determinations were 2.5% ± 1.6% and 3.2% ± 2.9%, respectively.

Figure 7. Scatchard plots of specific binding data reported in Figure 6.
Discussion

We used R2858 and estradiol-17β as probes to characterize estrogen binding components in cytoplasmic and nuclear fractions prepared from AXC rat aorta and myocardium. These estrogen binding components are estrogen receptors as established by the following evidence: 1) relative steroid specificity (Table 1) which shows that only estrogens in effective inhibitors of R2858 or estradiol-17β binding, whereas androgens, progestins, and cortisol are ineffective; 2) sedimentation properties on linear sucrose gradients, which establish that myocardial cytoplasmic R2858 binding components are macromolecules with sedimentation coefficients of 8S to 9S and that aortic cytoplasmic R2858 binding components migrate at 8S to 9S and 10S to 11S in low ionic strength gradients (Figures 3 and 4), whereas estradiol-17β binding components in nuclear preparations are macromolecules with sedimentation coefficients of 4S to 6S in high ionic strength gradients (Figure 5); 3) the properties of limited capacity, high affinity (Figure 6) and readily exchangeable binding (Figure 2); 4) estradiol-17β-mediated localization of estrogen receptors in nuclear and cytoplasmic tissue preparations (Table 2, Figure 8); and 5) temperature instability.

Concomitant detection of 8S to 9S and larger 10S to 11S estrogen-specific binding components in cytoplasmic fractions prepared from estrogen target tissue is unusual. Our observations (Figure 4) may represent aggregate formation by 8S to 9S receptor with resultant formation of the 10S to 11S binding component. This possibility is consistent with the known ability of steroid hormone receptors to form aggregates (see reference 42). Alternately, the 10S to 11S binding component may be the native form of aortic cytoplasmic estrogen receptors and the diffuse binding at 8S to 9S may be 10S to 11S degrada-

Table 2. Effect of Estradiol on Localization of Estrogen Receptors in Male Aortic and Myocardial Cytoplasmic and Nuclear Preparations

<table>
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<th>Treatment*</th>
<th>Aortic</th>
<th>Myocardial</th>
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<td></td>
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<tr>
<td>Estradiol-17β</td>
<td>18.1 ± 4.8†</td>
<td>51.9 ± 21.0†</td>
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</table>

Data are the means ± SD of three or four independent determinations. Each independent determination was performed with cytoplasmic and nuclear preparations from the same tissue pool. Site content values were obtained by linear regression analysis of double reciprocal plots of the saturation data. Regression correlation coefficients for cytoplasmic and nuclear preparation site content were 0.961 ± 0.02 (mean ± SD), aorta; 0.952 ± 0.04, myocardium. Receptor content, fmol/mg protein, of cytoplasmic preparations was: aorta: 3.35 ± 0.82 (mean ± SD), control, and 1.27 ± 0.20, estradiol-17β; myocardium: 1.49 ± 0.36, control, and 0.50 ± 0.13, estradiol-17β. Differences were significant, p < 0.01. DNA content of male aorta and myocardium respectively was 2.25 ± 0.29 and 1.80 ± 0.20 mg/g tissue.

*Intact, young-mature (6- to 8-month-old) males either did or did not receive an estradiol-17β injection (see footnote to Table 1).

†Significantly different from control subjects; p < 0.01, one-way analysis of variance.
tion products. Our studies do not permit distinction between these possibilities. The sedimentation properties of cardiovascular nuclear preparation estrogen binding components, extracted using buffer containing pyridoxal-5'-phosphate, are comparable to those of nuclear estradiol binding components similarly extracted from hen oviduct.43

We show in Table 2 that in vivo administration of estradiol-17β profoundly affects receptor distribution between nuclear and cytoplasmic components of cardiovascular tissue as determined by in vitro fractionation. Previously, such data would have been considered to represent estrogen-mediated, in vivo translocation of cytoplasmic steroid unoccupied receptor subsequent to formation of estradiol-17β receptor complexes.44,45 Two recent studies, which used profoundly different experimental approaches, described complementary data that indicate “in vivo” that estrogen receptors, steroid-occupied and steroid-free, reside in the nucleus of estrogen target cells.46,47 Cytoplasmic, steroid-occupied, estrogen receptors appear to reflect an experimental artifact resulting from buffer extraction of receptor from nuclear sites of weak association.47,48 Our data (Table 2, Figure 8) for the in vivo effect of estradiol-17β on estrogen receptor localization in cardiovascular tissue fractions do not provide new insights into the resolution of this controversy. However, the properties we describe for cardiovascular estrogen-binding components are consistent with the predictions of either model.44-47

In this study we report definitive biochemical characterization of vertebrate cardiovascular estrogen-binding components that have in vivo properties characteristic of physiologically functional estrogen receptors. Our data provide strong support for the hypothesis that vertebrate cardiovascular tissue is an estrogen target tissue whose function is regulated, in part, by the action of estrogens and their receptors.

Acknowledgments

We acknowledge the expert assistance of Roberto Gonzalez, Jr., and the dedicated work of Robert A. Geiger, Jr., in maintaining the AXC rat colony. We are grateful to Linda J. Styles for her aid in the preparation of this manuscript.

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Arterioscler Thromb Vasc Biol. 1985;5:668-677
doi: 10.1161/01.ATV.5.6.668

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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