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SEXUAL DIMORPHISM CHARACTERIZES BABOON MYOCARDIAL ANDROGEN RECEPTORS BUT NOT MYOCARDIAL ESTROGEN AND PROGESTERONE RECEPTORS

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Summary—Using biochemical methods we established that estrogen receptor content and distribution and progesterone receptor content in female and male baboon myocardium did not differ between sexes. In contrast, myocardial androgen receptor distribution between cytosolic and nuclear compartments was sexually dimorphic. Female baboon myocardial androgen receptors were restricted to the cytosolic compartment, whereas male myocardial androgen receptors were distributed between the cytosolic and nuclear compartments. Using human estrogen receptor cDNA we showed that baboon aorta, myocardium and uterus contain a 6.3 kb estrogen receptor transcript. Analyses performed with human progesterone receptor cDNA established that baboon aorta and uterus contain an 8 kb progesterone receptor transcript; however, progesterone receptor transcripts were not demonstrable in baboon myocardial RNA preparations. Because relative hybridization signal intensity reflected known uterine and aortic progesterone receptor content, failure to detect progesterone receptor transcripts in myocardial preparations may reflect sensitivity limitations and the fact that aortic progesterone receptor content is 5-fold greater than that of myocardium. Immunocytochemical analyses demonstrated that baboon myocardial progesterone receptors were present in greater than 25% of myocytes and generally absent from other myocardial cells. Our studies establish that: (1) gonadal steroid hormone receptor gene transcription occurs in cells of the baboon cardiovasculature, (2) these steroid hormone receptors may be physiologically functional, and (3) gonadal steroid hormone receptors may be restricted to specialized cells of the cardiovasculature.

INTRODUCTION

Epidemiologic [1, 2] and clinical studies [3–6] have established sex differences in the risk of cardiovascular disease. Biochemical studies have shown sex and gonadal hormone related differences in cardiovascular cell function [7–10]. Model studies have suggested that gonadal steroids may affect the severity of cardiovascular dysfunction [11–13]. These findings imply that gonadal steroids may affect cardiovascular cell function.

Biochemical and autoradiographic studies demonstrated that rat [14–17], canine [18], and baboon cardiovasculature [19–23] contains androgen, estrogen, and progesterone receptors and suggested that steroid hormones may directly affect cardiovascular cell function. Using biochemical methods, we established that gonadal steroid hormones influenced sex related differences in rat [17] and baboon [23] aortic androgen and estrogen receptor content and distribution. Additionally, we showed that estrogens affected baboon [23, 24] and rat [17] aortic progesterone receptor content. These studies provided direct evidence that aortic androgen and estrogen receptors were responsive to changes in plasma androgen and estrogen content and established that intracellular distribution of aortic androgen [17, 23] receptor and aortic estrogen [17, 24] receptor content differed according to sex.

To determine whether the sex related differences in baboon and rodent aortic gonadal steroid hormone receptor content and distribution were representative of baboon

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myocardium, we examined effects of endogenous hormones on gonadal steroid hormone receptor content and distribution in young mature male and female baboon myocardium. We used immunologic probes to identify cells of the baboon cardiovasculature which contained gonadal steroid hormone receptors and cDNAs to characterize steroid hormone receptor transcripts in baboon cardiovasculature. The results of these studies are detailed in this report.

EXPERIMENTAL

Animals

Young mature male and female baboons (*Papio* sp.) were from the colony at Southwest Foundation for Biomedical Research. They were 7–8-yr-old and had not received prior treatment. Females who had completed three consecutive, consistent estrus cycles were sacrificed, during the next cycle, at the time of maximum sex skin turgescence. The latter occurs essentially at the time of peak plasma estradiol content [25].

Materials and methods

[17α-methyl-$^3H_3$]R1881 (methyltrienolone, 86 Ci/mmol), [1,2,4,5,6,7,16,17-$^3H_8$]5α-dihydrotestosterone (5α-DHT, 208 Ci/mmol), [11β-methoxy-$^3H_3$]R2858 (Moxestrol, 86 Ci/mmol), [2,4,6,7,16,17-$^3H_6$]estradiol, and radioinert R1881 and R2858 were from New England Nuclear Corporation (Boston, Mass). [$^3H_2$]ORG 2058 (16α-ethyl-21-hydroxy-19-nor-[6,7-$^3H_2$]pregn-4-ene-3,20-dione, 40 Ci/mmol) and radioinert ORG 2058 were obtained from Amersham Corporation (Arlington Heights, Ill.). [α-^{32}P]dATP was from ICN Radiochemicals (Irvine, Calif.). Triamcinolone acetonide, DNA (salmon testes, type III) and bovine serum albumin (Fraction 5) were from Sigma Chemical Company (St Louis, Mo.). Radioinert steroids not enumerated above were obtained from Steraloids Incorporated (Wilton, N.H.). Human γ-globulin was from Calbiochem–Behring Corp. (La Jolla, Calif.). Dextran T70 was from Pharmacia Fine Chemicals Inc. (Piscataway, N.J.). Hydroxyapatite (DNA grade, Bio-gel HTP) was from Bio-Rad Laboratories (Richmond, Calif.). Oligo(*d*T)-cellulose was from P. L. Biochemicals Inc. (Milwaukee, Wis.). DNA polymerase I and DNase I were obtained from Boehringer Mannheim (Indianapolis, Ind.) and Worthington Biochemicals (Freehold, N.J.), respectively. Restriction endonucleases were from New England BioLabs (Beverly, Mass). Materials for acrylamide gel electrophoresis were from Bio-Rad (Richmond, Calif.) and agarose, SeaKem, was from FMC Corp. (Rockland, Me).

The two monoclonal antiestrogen receptor antibodies used were D75 and H222 [26]; the latter was generously provided by Abbott Laboratories (Chicago, Ill.). The monoclonal antiprogesterone receptor antibody used was JZB39 [27]. The control monoclonal antibody for immunocytochemistry, affinity-purified antiantigen B, a component of Timothy grass pollen (AT), was kindly provided by Dr A. Malley (Oregon Regional Primate Research Center). Antibody localization in tissue sections was visualized by using an antirat IgG avidin–biotin peroxidase kit (ABC kit, Vector Laboratories, Burlingame, Calif.). Immunocytochemical procedures, including antibody dilution and use of the ABC kit, were as previously described [28]. Plasmids pHOR8 [29] and pHPR54 [30] respectively contain cDNA to human estrogen or progesterone receptor. Other materials were the highest quality reagent grade available from the manufacturer. All solutions were prepared in water purified by reverse osmosis, deionized and redistilled from glass.

Preparation of cytosolic extracts and measurement of total cytosolic androgen, estrogen, or progesterone receptors

Baboons were restrained, sedated by intramuscular injection of ketamine (15 mg/kg) and exsanguinated. The heart was removed, the left ventricle was cut into 1–2 g blocks, wrapped in aluminum foil, sealed in heat-sealed packets, frozen by compression between blocks of dry ice, and transferred without delay to a -90°C freezer where tissue was stored until analyzed. All procedures were performed in accordance with guidelines established by the Department of Health and Human Service for the treatment of laboratory animals and were approved by the Animal Research Committee of the Southwest Foundation for Biomedical Research. Myocardial cytosolic extracts were prepared by using previously described methods [23]. In brief, frozen tissue (left ventricle) was thawed on ice and subsequent procedures were performed at 2–4°C. Thawed tissue was finely minced, and disrupted in 4–5 volumes of buffer TEDS [50 mM Tris–HCl, 0.1 mM EDTA, 10 mM dithiothreitol (DTT), 380 mM sucrose, pH 7.4], by using a Duall (Kontes, Vineland, N.J.)

glass–glass homogenizer. Homogenates were clarified by centrifugation at 50,000 *g* at 2°C for 5 min. Resultant supernatants were made 20 mM in sodium molybdate, 2.2 mM in phenylmethylsulfonyl fluoride (PMSF), and incubated for 10 min at 2°C with the pellet from 0.1 volume of a dextran, γ-globulin-coated charcoal (DGCC) suspension (5% charcoal, 0.5% dextran, 1% γ-globulin, w/v) in buffer TED (buffer TEDS without sucrose). Incubates were clarified by centrifugation for 15 min at 200,000 *g* at 2°C.

To quantify cytosolic receptors, incubations were performed in a final volume of 220 μl buffer TEDS containing 100 μl of tissue extract and various concentrations radiolabeled steroid. For progesterone receptors, incubation mixtures contained radiolabeled ORG 2058 (0.25–5 nM) and a 400-fold molar excess of cortisol to block ORG 2058 binding to corticosteroid receptors. These mixtures were incubated at 2°C for 20–24 h. To quantify androgen receptors, incubation mixtures contained radiolabeled R1881 (0.1–5 nM) and a 100-fold molar excess of triamcinolone acetonide to block R1881 binding to progesterone receptors. These mixtures were incubated at 2°C for 20–24 h. To quantify estrogen receptors, incubation mixtures contained radiolabeled R2858 (0.25–5 nM), and these were incubated at 30°C for 4 h. For each determination, nonspecific binding was measured by incubating a parallel series of identical samples that contained radiolabeled ligand and a 100-fold molar excess of the corresponding radioinert ligand. Separation of bound and free radioligand was by either the DGCC (androgen and progesterone receptors) or hydroxyapatite (estrogen receptors) method previously described [15, 21]. Specific binding data were evaluated by the method of Scatchard [31] and as double reciprocal plots [32]. Two classes of cytosolic progestin binding components, as previously described for baboon aorta [23], were detected in myocardium. These data were analyzed by the method of Scatchard [31] using the correction protocols described by Rosenthal [33] and Feldman [34]. Full saturation analyses were always used to quantify cytosolic receptor content.

Preparation of nuclear extracts and measurement of total nuclear androgen or estrogen receptors

Myocardial nuclear extracts were prepared by using previously described methods [15]. In brief, the 50,000 *g* pellet obtained from preparation of the cytosolic extract was suspended in buffer TEC (10 mM Tris–HCl, 1.5 mM EDTA, 5 mM $CaCl_2$, pH 7.4), passed through a layer of fine nylon stocking, collected by centrifugation, and washed three times with buffer TEC. The washed nuclear pellet was suspended at 4–5 ml/g tissue in buffer BBPP [20 mM sodium barbital, 1.5 mM EDTA, 150 mM KCl, 5 mM DTT, 5 mM pyridoxal-5′-phosphate, 20% glycerol (v/v), pH 8.0 at 20°C]. After an aliquot had been removed for DNA analysis, the pellet was sonicated and then extracted by incubation in the dark at 2°C for 30 min. The nuclear extract was clarified by centrifugation for 45 min at 200,000 *g*. Nuclear androgen receptors were quantified by incubation at 2°C for 18–24 h, whereas nuclear estrogen receptor quantification was by incubation at 30°C for 4 h. Incubations contained 200 μl nuclear extract and various concentrations, 0.5–5 nM, of either radiolabeled 5α-DHT (androgen receptors) or estradiol-17β (estrogen receptors) in a final volume of 440 μl buffer BBPP. Nonspecific binding was determined in a parallel incubation series which also contained a 100-fold molar excess of radioinert ligand. Separation of free and bound radioligand was by the hydroxyapatite method and specific binding was calculated as described in the preceding section.

Sucrose gradient assessment of antibody binding to myocardial estrogen or progesterone receptors

Concentrated receptor preparations were obtained by homogenizing left ventricle in 2–3 volumes of buffer TEDS containing 20 mM sodium molybdate and 2.2 mM PMSF and clarifying the homogenate by centrifugation at 200,000 *g* for 15 min. Clarified extracts were incubated with either 0.5 nM radiolabeled R2858 (estrogen receptor) for 1 h at 30°C or with 1 nM radiolabeled ORG 2058 and a 1000-fold molar excess of radioinert cortisol (progesterone receptor) for 1.5 h at 2°C. Free radioligand was removed by incubating these mixtures for 10 min at 2°C with pelleted DGCC, and incubates were clarified by centrifugation at 100,000 *g* for 10 min. Antibody, 5–10 μg, was added to 300 μl of radiolabeled, clarified extract, and mixtures were incubated on ice for 7 h. As control we used monospecific polyclonal antibody (kindly provided by Dr Walter Heyns, Leuven, Belgium) to rat ventral prostate prostatein. Incubates were then made 0.3 M in KCl and 250 μl were layered onto 20–40%

linear sucrose gradients in 20 mM sodium barbital, 1.5 mM EDTA, 5 mM DTT, 0.3 M KCl and 15% glycerol (v/v), pH 8.0 at 20°C. Gradients were developed by centrifugation in the Sorvall vertical tube rotor (TV 865, DuPont Company, Newtown, Conn.). Human γ-globulin served as sedimentation standard.

Immunocytochemistry

Freshly obtained myocardium was cut into several 1–2 mm cross-sections and these were placed in a drop of O.C.T., Tissue Tek II, Miles Laboratories, Inc. (Naperville, Ill.) on a square of aluminum foil and immediately quenched in liquid propane. Frozen specimens were wrapped in aluminum foil and stored at −90°C until shipped, packaged in dry ice, to Oregon for immunocytochemical processing. Indirect immunocytochemistry was performed as previously described [28] with minor modification to include a post fixation wash in 0.1 M phosphate buffered (pH 7.3) 0.2% picric acid, 2% paraformaldehyde. Antiprogesterone receptor antibody JZB39 was used for progesterone receptor localization, whereas a mixture of antiestrogen receptor antibodies D75 and H222 was used for localization of estrogen receptors.

Isolation of RNA and detection of estrogen or progesterone receptor transcripts

Baboon aorta, myocardium and uterus, which had been snap-frozen in liquid nitrogen and stored at −90°C, were individually pulverized in liquid nitrogen and the frozen powder was solubilized in guanidinium isothiocyanate. Rat ventral prostate RNA was extracted from fresh tissue, obtained from animals maintained in the colony at Southwest Foundation, by disruption in guanidinium isothiocyanate. RNA was isolated from initial extracts by precipitation from guanidine hydrochloride. Integrity of these preparations was monitored by electrophoresis on formaldehyde-agarose gels. Poly(A)$^+$ RNA was prepared from total RNA using two cycles of oligo(*d*T)-cellulose chromatography. Northern hybridization analyses were performed by fractionating denatured poly(A)$^+$ RNA by electrophoresis through 1.5% agarose gels and capillary transfer of RNA to nitrocellulose. Membranes were hybridized with cDNAs which had been purified by acrylamide gel electrophoresis prior to being radiolabeled by nick-translation. An RNA ladder (Bethesda Research Laboratories, Gaithersburg, Md) was used to estimate transcript size. All procedures were performed as previously detailed [35].

Other methods

Protein content was measured using the procedure of Lowry *et al.* [36] with bovine serum albumin as standard. DNA was measured by the fluorometric method of Vytasek [37] with salmon testes DNA as the standard. Plasma estradiol and testosterone content was measured using antibody and methods previously detailed [23]. Reported values were determined in a single assay. Radioisotope was measured in either a Beckman 7800 or 7500 scintillation spectrometer and most samples were counted to 2% precision (at the 95% confidence level). The paired t-test was used to assess significance of observed differences.

RESULTS

Androgen receptor content and distribution in female and male baboon myocardium

Saturation analyses established that baboon myocardium contained a single class of limited capacity, high affinity cytosolic or nuclear androgen receptors (data not shown). The mean dissociation constant for cytosolic or nuclear androgen receptors respectively was 0.14 ± 0.04 ($n = 10$) or 1.7 ± 0.3 nM ($n = 5$). Male baboon myocardium contained both cytosolic and nuclear androgen receptors. In male baboons, approximately 26% of total myocardial androgen receptors were localized in the nuclear fraction (Table 1). In contrast, nuclear androgen receptors were not demonstrable in female baboon myocardium (Table 1). Total myocardial androgen receptor content of female baboon myocardium was significantly greater, $P < 0.05$, than that of males (Table 1). Mean plasma testosterone content in male and female baboons respectively was 569 ± 346 (mean ± SD) and 61 ± 38 ng/dl.

Table 1. Cytosolic and nuclear androgen receptor content of baboon myocardium

	Content (fmol/mg DNA)	
Sex	Cytosolic	Nuclear
Male	107 ± 20	37 ± 17
Female	222 ± 18*	0*

Data are the mean ± SD, $n = 5$. Values were obtained by linear regression analysis of double reciprocal plots of the saturation data.

*Significantly different from the paired male value; $P < 0.05$, paired t-test.

Estrogen and progesterone receptor content of baboon myocardium

Saturation analyses established that baboon myocardium contained a single class of limited capacity, high affinity cytosolic estrogen receptors (data not shown) with mean dissociation constant of 0.35 ± 0.04 nM ($n = 10$). Myocardial cytosolic estrogen receptor content was indistinguishable, $P > 0.05$, in young mature male and female baboons (Table 2). Nuclear estrogen receptors were not detectable in tissue from either male or female baboons. Mean plasma estradiol content of male or proestrous female baboons respectively was 116 ± 70 (mean $\pm$ SD) or 250 ± 208 pg/ml.

Saturation analyses showed that both male and female baboon myocardium contained two classes of progestin binding components (Fig. 1). The dissociation constant for the low affinity type II binding components was 56 ± 54 nM (mean $\pm$ SD, $n = 10$), whereas the value for the high affinity type I binding components was 0.25 ± 0.14 nM ($n = 10$). Low affinity, high capacity type II myocardial progestin binding components ranged from 650 to greater than 5000 fmol/mg DNA. Limited capacity, high affinity myocardial progesterone receptor (type I) content of male and female baboon was not significantly different, $P > 0.05$ (Table 2).

Baboon cardiovasculature contains estrogen and progesterone receptor transcripts

Because baboon myocardial progesterone and estrogen receptor content is much lower than that of baboon uterus [24], a classical progesterone and estrogen target tissue, we presumed that progesterone and estrogen receptor transcript content of myocardium would be much lower than that of uterus. Preliminary analyses (data not shown) confirmed this presumption and indicated that large quantities of myocardial poly(A)$^+$ RNA would be needed

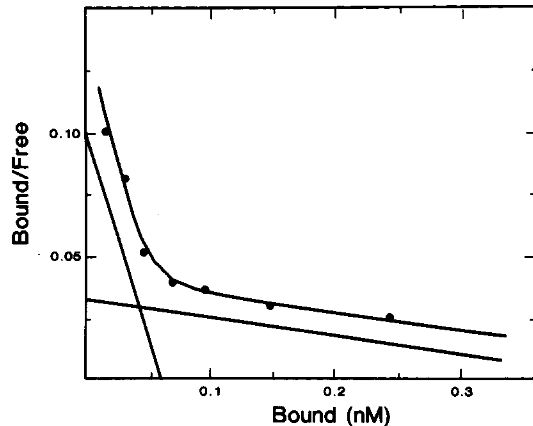

Fig. 1. Representative plot of saturation data obtained during quantification of baboon myocardial progesterone receptors. (●), Scatchard plot of the specific binding data. Solid lines represent the replot of specific binding data after correction using methods described by Rosenthal [33] and Feldman [34].

to detect progesterone or estrogen receptor transcripts. Comparative analysis of progesterone receptor transcripts in poly(A)$^+$ RNA from baboon uterus, aorta and myocardium (Fig. 2) showed a unique band, approx. 8.0 kb, in uterine preparations and a slightly more rapidly migrating band in aorta. Progesterone receptor transcripts were not detected in myocardial poly(A)$^+$ RNA preparations. Comparable analyses showed that baboon uterus, aorta and myocardium contained a single estrogen receptor transcript, approximately 6.3 kb (Fig. 3). No hybridization signal was obtained when either probe was used to analyze a rat ventral prostate poly(A)$^+$ RNA preparation (Figs 2 and 3). The absence of a hybridization signal when the rat ventral prostate poly(A)$^+$ RNA preparation was probed with either human estrogen or progesterone receptor cDNA was not attributable to potential degradation of RNA because the expected hybridazation signal was obtained when this preparation was probed with cDNA for prostatein (data not shown).

Table 2. Cytosolic estrogen and progesterone receptor content of baboon myocardium

	Content (fmol/mg DNA)	
Sex	Estrogen	Progesterone
Male	29 ± 4	115 ± 21
Female	36 ± 10	164 ± 70

Data are the mean $\pm$ SD, $n = 5$. Values for estrogen receptors were obtained by linear regression analysis of double reciprocal plots of the saturation data. Values for progesterone receptors were obtained after applying the correction protocols of Rosenthal [33] and Feldman [34] to saturation data plotted according to the method of Scatchard [31].

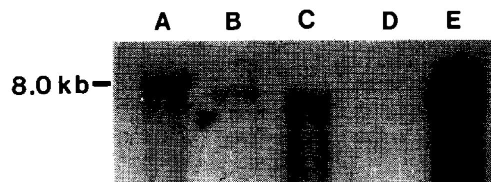

Fig. 2. Northern hybridization analysis of progesterone receptor transcripts. Poly(A)$^+$ RNA was isolated, fractionated on 1.5% agarose, transferred to nitrocellulose, and hybridized with ^{32}P-labeled cDNA prepared from pHPR54. Poly(A)$^+$ RNA content, μg, of each lane was: (A) uterus, 1; (B) aorta from 1.5-yr-old male, 5; (C) aorta from two females of mean age 15 yr, 14; (D) rat ventral prostate, 10; (E) uterus (same as A), 5.

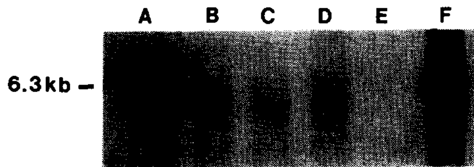

Fig. 3. Northern hybridization analysis of estrogen receptor transcripts. Conditions are as described in the legend to Fig. 2 except that ^{32}P-labeled cDNA was prepared from pHOR8. Poly(A)$^+$ RNA content, μg, of each lane was: (A) uterus, 1; (B) aorta from two females of mean age 15 yr, 14; (C) left ventricle from 17-yr-old female, 32; (D) left ventricle from 6-yr-old male, 32; (E) rat ventral prostate, 10; (F) uterus (same as A), 5.

Monoclonal antibodies to human estrogen or progesterone receptor bind baboon myocardial estrogen or progesterone receptor

Linear sucrose gradient analyses revealed that baboon myocardial cytosolic estrogen receptors migrated at 5.5–6.5S (Fig. 4) during sedimentation through gradients containing 0.3 M KCl. Incubation of the same preparations with 5 μg antiestrogen receptor antibody H222 prior to gradient analysis quantitatively converted estrogen receptors to a complex which migrated with a sedimentation coefficient of 8.5–9.5 S (Fig. 4). Similarly, antiestrogen receptor antibody D75 caused approx. 70% of baboon myocardial estrogen receptor to migrate with a sedimentation coefficient of 8.5–9.5 S (Fig. 4). When the preparation was treated with an irrelevant, monospecific, polyclonal antibody to rat ventral prostate prostatein, estrogen receptor migration was unaltered (Fig. 4).

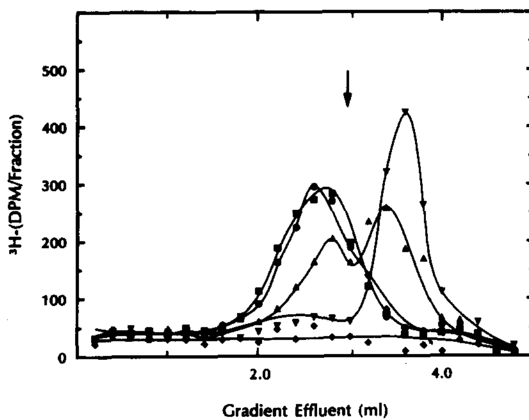

Fig. 4. Antiestrogen receptor antibodies increase myocardial estrogen receptor migration rate during linear sucrose gradient sedimentation. Baboon myocardial estrogen receptors were labeled by 1 hr incubation at 30°C with 0.5 nM radiolabeled R2858 in the presence (◆) or absence (●) of 100-fold molar excess radioinert R2858. After cooling to 2°C, receptor bound and free ligand was separated by DGCC adsorption and aliquots of the clarified supernatant were incubated at 2°C for 7 h with buffer (●), buffer containing 5 μg of either antiestrogen receptor antibody H222 (▼) or D75 (▲), or buffer containing 5 μg of rabbit antirat prostatein antibody (■). These incubates were then made 0.3 M in KCl and 250 μl were layered onto 20–40% linear sucrose gradients which were developed by centrifugation for 11 h at 2°C at 370,000 g (TV 865 vertical rotor). Sedimentation is from left to right. The sedimentation coefficient of human γ-globulin (arrow) is 7S.

Linear sucrose gradient analyses showed that baboon myocardial cytosolic progesterone receptors migrated at 5.5–6.5 S during sedimentation on gradients containing 0.3 M KCl (Fig. 5). When this preparation was incubated with either 5 or 10 μg antiprogesterone receptor antibody JZB39, approx. 20% of myocardial progesterone receptors were converted to a complex which migrated with sedimentation coefficient of 8.5–9.5 S (Fig. 5). Identical treatment of these preparations with antibody to rat ventral prostate prostatein did not alter progesterone receptor migration (Fig. 5).

Immunocytochemistry

Having established binding of human antiestrogen receptor antibody (Fig. 4) or antiprogesterone receptor antibody (Fig. 5) to nondenatured baboon myocardial estrogen or progesterone receptors, we used immunocytochemical methods to identify myocardial cells which contained estrogen or progesterone receptors. When sections of myocardium were incubated with antiprogesterone receptor antibody JZB39, distinct nuclear progesterone

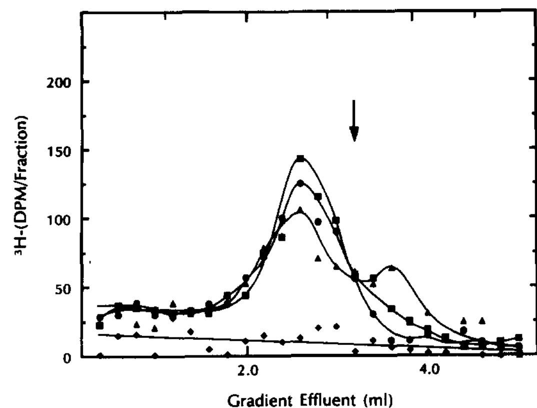

Fig. 5. Antiprogesterone receptor antibody increases myocardial progesterone receptor migration rate during linear sucrose gradient sedimentation. Baboon myocardial progesterone receptors were labeled by 1.5 h incubation at 4°C with 1 nM radiolabeled ORG 2058 in the presence (◆) or absence (■) of 100-fold molar excess radioinert ORG 2058. All incubations additionally contained 1000-fold molar excess of cortisol. Free ligand was removed by DGCC adsorption and an aliquot of the clarified supernatant was incubated for 7 hours at 2°C with buffer (■), buffer containing 10 μg of antiprogesterone receptor antibody JZB39 (▲), or with buffer containing 10 μg antirat prostatein antibody (●). Other conditions are as detailed in the legend to Fig. 4.

receptor staining was readily demonstrated (Fig. 6A). In contrast, nuclei of adjacent sections incubated with control antibody AT were unstained (Fig. 6B). The identity of cytoplasmic and background staining following incubation of sections with either antibody JZB39 or AT (Fig. 6A and B) indicates this is background reaction. Only cardiac myocytes were clearly positive for progesterone receptor; vascular and connective tissue cells generally were negative (Fig. 6A). Pale nuclear staining in other cell types (Fig. 6A and B) is due to hemotoxylin used to visualize histologic structure. Nuclear staining of cardiac myocytes for progesterone receptor was heterogeneous and varied from intense to absence of staining (Fig. 6A). Similar nuclear progesterone receptor staining was observed in baboon aortic tunica media smooth muscle cells (data not shown).

When sections of myocardium were incubated with antiestrogen receptor antibodies H222 and D75, results were essentially identical to those shown in Fig. 6B. Failure to immunocytochemically detect myocardial estrogen receptors suggests that the level of receptors, approx. 1% of that uterus, is below the limit of detection with current techniques. Myocardial progesterone receptor content also is low, approximately 5% of that of uterus; however, the high avidity of JZB39 consistently gives more intense signals in immunocytochemical preparations than either antiestrogen receptor antibody H222 or D75.

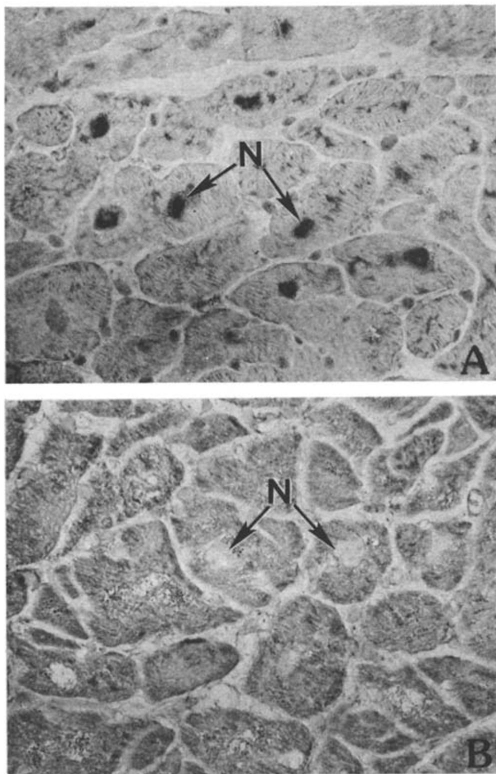

Fig. 6. Immunocytochemical localization of progesterone receptors in baboon myocardium. Sections were incubated with either antiprogesterone receptor antibody JZB39, panel A, or control antibody AT, panel B, and antibody localization was identified by diaminobenzidine deposition. Only cardiac myocyte nuclei (N) show positive retention of antiprogesterone receptor antibody JZB39 (panel A). Note the identity of background and cytoplasmic staining in panels A and B. Magnification, 425 ×.

DISCUSSION

Because cardiovascular steroid hormone receptor content is low, meaningful measurement requires accurate, reproducible methods. We show that the interassay coefficient of variation for male and female baboon myocardial cytosolic androgen or estrogen receptors and male baboon progesterone receptors is 8–28% (Tables 1 and 2). The apparent coefficient of variation for female baboon cytosolic progesterone receptor content is 42%. This value reflects the fact that one female had unusually high, 288 fmol/mg DNA, progesterone receptor content. Mean myocardial progesterone receptor content for the remaining four females was 133 ± 13 (mean $\pm$ SD) fmol/mg DNA, with a coefficient of variation of 10%. The interassay coefficient of variation for nuclear androgen receptor content was 46% which may be largely due to the low receptor content of these preparations. Our results are comparable to those described previously [20, 21, 23] and establish the validity of our assay for the current studies.

Androgen receptor content of female baboon myocardium (Table 1) is similar to that of female baboon aorta [23]. Moreover, androgen receptors of both female myocardium (Table 1) and aorta [23] are exclusively localized to the cytosolic fraction. In contrast, male baboon myocardial androgen receptor content is significantly less than that of both female myocardium (Table 1) and male baboon aorta [23]. In male baboon myocardium (Table 1) and aorta [23], androgen receptors localize in both the nuclear and cytosolic compartments. The difference in receptor distribution in male and female baboon myocardium may be a consequence [15, 17] of differences in endogenous plasma androgen content. However, because androgens up-regulate androgen receptor content of rat prostate [37–39], rat prostate cancer cells [40], human genital skin fibroblasts [41, 42]

and DDT_1MF-2 ductus deferens carcinoma cells [43], the finding that androgen receptor content of male baboon myocardium is less than that of female myocardium is paradoxical. The cause of the dichotomous androgen effects on baboon myocardial androgen receptor content and distribution remains to be examined. Nonetheless, our studies show that baboon myocardial androgen receptor content and distribution are sexually dimorphic. This is in contrast to the baboon aorta where androgen receptor distribution is sexually dimorphic and receptor content is not [23].

Male and female baboon myocardial estrogen receptor content was identical and all estrogen receptors were restricted to the cytosolic compartment (Table 2). The distribution of female myocardial estrogen receptors is identical to that of aorta [23] and implies that cyclic changes in baboon plasma estrogen content also fail to promote nuclear localization of myocardial estrogen receptors. These observations are in contrast to findings for the rat in which physiological, cyclic change in plasma estrogen elevates nuclear aortic estrogen receptor content [16]. The absence of an effect of cyclic change in baboon plasma estrogen content on myocardial (Table 2) or aortic [23] estrogen receptor distribution in intact females also differs significantly from the effects of cyclic estrogen replacement in long-term ovariectomized baboons. In these individuals, myocardial and aortic estrogen receptor content [24] is significantly greater than that of intact females (Table 2) and estrogen replacement causes retention of myocardial and aortic nuclear estrogen receptors. Significantly, mean plasma estradiol content, 189 pg/ml [24], of estrogen replaced ovariectomized baboons was comparable to that of the cycling females used in the present studies. Our findings imply that long-term estrogen depletion may significantly modify subsequent estrogen sensitivity of female baboon myocardium and aorta.

When the one female with unusually high progesterone receptor content, 288 fmol/mg DNA, was omitted from the comparison, mean female baboon myocardial progesterone receptor content, 133 ± 13 (mean $\pm$ SD, $n = 4$), was identical to that of male myocardium (Table 2). When all individuals were included in the analysis, male and female myocardial progesterone receptor content also was not statistically different (Table 2). The essential identity of myocardial progesterone receptor content in male and most female baboons implies that estrogen fails to increase myocardial progesterone receptor content and indicates that estrogen sensitivity of young mature female baboon myocardium is low. However, we have shown that cyclic estrogenization of ovariectomized female baboons promotes relocation of myocardial estrogen receptors to the nuclear compartment and increases myocardial progesterone receptor content [24]. These findings provide additional evidence that long-term estrogen depletion may significantly modify estrogen responsiveness of female baboon myocardium.

Using cDNA to human progesterone receptor, we found that baboon uterus and aorta contained a single 8 kb progesterone receptor transcript. Relative signal intensity reflected both the quantity of poly(A)$^+$ RNA analyzed and relative progesterone receptor content of baboon uterus and aorta [23]. These same analyses failed to identify progesterone receptor transcripts when up to 32 μg of myocardial poly(A)$^+$ RNA were analyzed (data not shown). This may reflect sensitivity limitations and the fact that aortic progesterone receptor content [23] is about 5-fold greater than that of myocardium (Table 2). While it is known that human and rodent androgen receptors [45, 46] share extensive sequence homology with progesterone receptor DNA and steroid binding domains, the absence of a rat ventral prostate hybridization signal (Fig. 2) indicates it is unlikely that the human progesterone receptor cDNA is detecting baboon androgen receptor transcripts. Species specific differences appear to characterize progesterone receptor transcript size. By example, human MCF-7 breast cancer cells contain a major 11.4 kb progesterone receptor transcript and minor transcripts of 5.8, 5.3, 3.5, and 2.8 kb [29]. Rabbit uterus contains progesterone receptor transcripts of 6.6 and 5.9 kb [47]. In contrast, estrogen-withdrawn chick oviduct contains progesterone receptor transcripts of 4.5, 4.0 and 3.9 kb and estrogenization causes appearance of two additional transcripts; one greater than 6 kb and the other about 3 kb [48]. These findings suggest that estrogen status affects the size of progesterone receptor transcripts in various tissues.

Using human estrogen receptor cDNA, we found that baboon uterus, aorta, and myocardium contained a single 6.3 kb transcript. The baboon estrogen receptor transcript is essentially identical in size to the 6.2 kb transcript

demonstrated in human MCF-7 or T47D breast cancer cells [49]. The relative signal intensity for aorta and myocardium (Fig. 3) reflects the similarity in estrogen receptor content of these two tissues [23] (Table 2). Compared to uterus, relative estrogen receptor transcript signal intensity of aortic and myocardial poly(A)$^+$ RNA preparations is considerably greater than would be expected based on relative estrogen receptor content of these tissues. In contrast, we show that relative progesterone receptor transcript content of baboon uterus and aorta (Fig. 2) is similar to progesterone receptor content. The differences between relative estrogen and progesterone receptor content and relative receptor transcript content in these tissues are consistent with the interpretation that sensitivity limitations may account for the present inability to demonstrate progesterone receptor transcripts in baboon myocardium.

Immunocytochemical analysis showed that greater than 25% of cardiac myocyte nuclei contained progesterone receptors, whereas other cardiac cells generally were devoid of progesterone receptors (Fig. 6). Our demonstration that baboon myocardial progesterone receptors are restricted to myocytes is identical to the distribution characteristic of human myocardial progesterone receptors [50]. The immunocytochemical data for baboon myocardium differs from results of an autoradiographic study which showed that about 1% of baboon cardiac myocyte nuclei and less than 5% of interstitial cell nuclei concentrated radiolabeled synthetic progestin [51]. The difference in findings for baboon myocardium may reflect the fact that current studies were performed with tissue from young mature cycling females, whereas prior studies [51] used estrogenized (3 days treatment), long-term ovariectomized females. Despite distinguishing differences, results from these and other studies [23, 50, 51] imply that progestins may directly regulate cardiac myocyte function.

In summary, our current and prior studies show that gonadal steroid hormone receptor content and intracellular distribution in male and female baboon aorta and myocardium are similar. Significantly, interacellular androgen receptor distribution in both aorta and myocardium reflects sex related differences in plasma androgen content. Despite sex related differences in plasma estrogen content, neither estrogen receptor content and distribution nor progesterone receptor content differed in aorta or myocardium of mature, intact female and male baboons. These findings imply that estrogen sensitivity of baboon aorta and myocardium significantly differs from that of accessory reproductive tissue which shows rapid response to change in endogenous hormone levels. It seems likely that any direct effects of estrogen on vascular cell function are the consequence of chronic, as opposed to acute, differences in plasma hormone content. In this regard it is notable that long-term estrogen deprivation significantly enhances female baboon aortic and myocardial estrogen sensitivity [24].

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REFERENCES

1. McGill H. C. Jr and Stern M. P.: Sex and atherosclerosis. *Atheroscler. Rev.* **4** (1979) 157–242.
2. Johansson S., Vedin A. and Wilhelmsson C.: Myocardial infarction in women. *Epidemiol. Rev.* **5** (1983) 67–95.
3. Kannel W. B., Hjortland M. C., McNamara P. M. and Gordon T.: Menopause and risk of cardiovascular disease. The Framingham Study. *Ann. Intern. Med.* **85** (1976) 447–452.
4. Bush T. L., Cowan L. D., Barrett-Connor E., Crique M. H., Karon J. M., Wallace R. B., Tyroler H. A. and Rifkind B. M.: Estrogen use and all-cause mortality. Preliminary results from the Lipid Research Clinics Program Follow-up Study. *J. Am. Med. Ass.* **249** (1983) 903–906.
5. Miller N. E.: The evidence for the antiatherogenicity of high density lipoprotein in man. *Lipids* **13** (1978) 914–919.
6. Goldberg R. B., Rabin D., Alexander A. N., Doelle G. C. and Getz G. S.: Suppression of plasma testosterone leads to an increase in serum total and high density lipoprotein cholesterol and apoproteins A-I and B. *J. Clin. Endocr. Metab.* **60** (1985) 203–207.
7. Altura B. M. and Altura B. T.: Influence of sex hormones, oral contraceptives and pregnancy on vascular muscle and its reactivity. In *Factors Influencing Vascular Reactivity* (Edited by O. Carrier and S. Shibata). Igaku-Shoin, Tokyo (1977) pp. 221–254.
8. Karanian J. W., Sintetos A. L., Moran F. M., Ramey E. R. and Ramwell P. W.: Androgenic regulation of vascular responses to prostaglandins. In *Cardiovascular Pharmacology of the Prostaglandins* (Edited by A. G. Herman, P. M. Vanhoutte, H. Denolin and A. Goossens). Raven Press, New York (1982) pp. 245–258.
9. Fischer-Dzoga K., Wissler R. W. and Vesselinovitch D.: The effect of estradiol on the proliferation of rabbit aortic medial tissue culture cells induced by hyperlipemic serum. *Exp. Molec. Path.* **39** (1983) 355–363.
10. Fischer G. M., Bashey R. I., Rosenbaum H. and Lyttle C. R.: A possible mechanism in arterial wall for mediation of sex difference in atherosclerosis. *Exp. Molec. Path.* **43** (1985) 288–296.

11. Hamm T. E. Jr, Kaplan J. R., Clarkson T. B. and Bullock B. C.: Effects of gender and social behavior on the development of coronary artery atherosclerosis in cynomolgus macaques. *Atherosclerosis* **48** (1983) 221–233.
12. Schaible T. F., Malhotra A., Ciambrone G. and Scheuer J.: The effects of gonadectomy on left ventricular function and cardiac contractile proteins in male and female rats. *Circ. Res.* **54** (1984) 38–49.
13. Adams M. R., Kaplan J. R., Clarkson T. B. and Koritnik D. R.: Ovariectomy, social status and atherosclerosis in cynomolgus monkeys. *Arteriosclerosis* **5** (1985) 192–200.
14. Krieg M., Smith K. and Bartsch W.: Demonstration of a specific androgen receptor in rat heart muscle: relationship between binding, metabolism, and tissue levels of androgens. *Endocrinology* **103** (1978) 1686–1694.
15. Lin A. L., Gonzalez R. Jr and Shain S. A.: Androgen directs apparent cytoplasmic and nuclear distribution of rat cardiovascular androgen receptors. *Arteriosclerosis* **5**, (1985) 659–667.
16. Lin A. L. and Shain S. A.: Estrogen-mediated cytoplasmic and nuclear distribution of rat cardiovascular estrogen receptors. *Arteriosclerosis* **5** (1985) 668–677.
17. Lin A. L. and Shain S. A.: Sexual dimorphism characterizes steroid hormone modulation of rat aortic steroid hormone receptors. *Endocrinology* **119** (1986) 296–302.
18. Horwitz K. B. and Horwitz L. D.: Canine vascular tissues are targets for androgens, estrogens, progestins and glucocorticoids. *J. Clin. Invest.* **69** (1982) 750–758.
19. McGill H. C., Jr, Anselmo V. C., Buchanan J. M. and Sheridan P. J.: The heart is a target organ for androgen. *Science* **207** (1980) 775–777.
20. Lin A. L., McGill H. C. Jr and Shain S. A.: Hormone receptors of the baboon cardiovascular system. Biochemical characterization of aortic cytoplasmic androgen receptors. *Arteriosclerosis* **1** (1981) 257–264.
21. Lin A. L., McGill H. C. Jr and Shain S. A.: Hormone receptors of the baboon cardiovascular system: Biochemical characterization of myocardial cytoplasmic androgen receptors. *Circ. Res.* **49** (1981) 1010–1016.
22. Lin A. L., McGill H. C. Jr and Shain S. A.: Hormone receptors of the baboon cardiovascular system: Biochemical characterization of aortic and myocardial cytoplasmic progesterone receptors. *Circ. Res.* **50** (1982) 610–616.
23. Lin A. L., Gonzalez R. Jr, Carey K. D. and Shain S. A.: Gender and baboon aortic steroid hormone receptors. *Arteriosclerosis* **7** (1987) 248–255.
24. Lin A. L., Gonzalez R. Jr, Carey K. D. and Shain S. A.: Estradiol-17β affects estrogen receptor distribution and elevates progesterone receptor content in baboon aorta. *Arteriosclerosis* **6** (1986) 495–504.
25. Hendrickx A. G. and Kraemer D. C.: Methods. In *Embryology of the Baboon* (Edited by A. G. Hendrick). Univ. of Chicago Press, Chicago (1971) pp 31–60.
26. Greene G. L., Sobel N. B., King W. J. and Jensen E. V.: Immunochemical studies of estrogen receptors. *J. Steroid Biochem.* **20** (1984) 51–56.
27. Greene G. L., Harris K., Bova R., Kinders R., Moore B. and Nolan C.: Purification of T47D human progesterone receptor and immunochemical characterization with monoclonal antibodies. *Molec. Endocr.* **2** (1988) 714–726.
28. McClellan M., West N. B. and Brenner R. M.: Immunocytochemical localization of estrogen receptors in the macaque endometrium during the luteal-follicular transition. *Endocrinology* **119** (1986) 2467–2475.
29. Greene G. L., Gilna P., Waterfield M., Baker A., Hort Y. and Shine J.: Sequence and expression of human estrogen receptor complementary DNA. *Science* **231** (1986) 1150–1154.
30. Nardulli A. M., Greene G. L., O'Malley B. W. and Katzenellenbogen B. S.: Regulation of progesterone receptor messenger ribonucleic acid and protein levels in MCF-7 cells by estradiol: analysis of estrogen's effect on progesterone receptor synthesis and degradation. *Endocrinology* **122** (1988) 935–944.
31. Scatchard G.: The attraction of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51** (1949) 660–672.
32. Shain S. A. and Boesel R. W.: Human prostate steroid hormone receptor quantitation. Current methodology and possible utility as a clinical discriminant in carcinoma. *Invest. Urol.* **16** (1978) 169–174.
33. Rosenthal H. E.: A graphic method for the determination and presentation of binding parameters in a complex system. *Analyt. Biochem.* **20** (1967) 525–532.
34. Feldman H. A.: Mathematical theory of complex ligand-binding systems at equilibrium: some methods for parameter fitting. *Analyt. Biochem.* **48** (1972) 317–338.
35. Shain S. A., Schultz J. J. and Lancaster C. M.: Aging in the AXC/SSh rat: diminished prostate L-ornithine decarboxylase (ODC) activity reflects diminished prostate ODC protein and transcript content. *Endocrinology* **119** (1986) 1830–1838.
36. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193** (1951) 265–275.
37. Vytasek R.: A sensitive fluorometric assay for the determination of DNA. *Analyt. Biochem.* **120** (1982) 243–248.
38. Boesel R. W., Klipper R. W. and Shain S. A.: Androgen regulation of androgen receptor content and distribution in the ventral and dorso-lateral prostates of aging rats. *Steroids* **35** (1980) 157–177.
39. Steinsapir J., Evans A. C., Jr, Bryham M. and Muldoon T. G.: Androgen receptor dynamics in the rat ventral prostate. *Biochim. Biophys. Acta* **842** (1985) 1–11.
40. Ho S.-M., Damassa D., Kwan P. W. L., Seto H. S. K. and Leav I.: Androgen receptor levels and androgen contents in the prostate lobes of intact and testosterone-treated Nobel rats. *J. Androl.* **6** (1985) 279–290.
41. Lin A. L. and Shain S. A.: Androgen modulation of prostate cancer cell androgen receptor content is cell line specific. *Molec. Cell. Endocr.* **63** (1989) 75–83.
42. Kaufman M., Pinsky L., Hollander R. and Bailey J. D.: Regulation of the androgen receptor by androgen in normal and androgen-resistant genital skin fibroblasts. *J. Steroid Biochem.* **18** (1983) 383–390.
43. Gad Y. Z., Berkovitv G. D., Migeon C. J. and Brown T. R.: Studies of up-regulation of androgen receptors in genital skin fibroblasts. *Molec. Cell. Endocr.* **57** (1988) 205–213.
44. Syms A. J., Norris J. S., Panko W. B. and Smith R. G.: Mechanism of androgen-receptor augmentation. Analysis of receptor synthesis and degradation by the density-shift technique. *J. Biol. Chem.* **260** (1985) 455–461.
45. Chang C., Kokontis J. and Liao S.: Molecular cloning of human and rat complementary DNA encoding androgen receptors. *Science* **240** (1988) 324–326.
46. Lubahn D. B., Joseph D. R., Sullivan P. M., Willard H. F., French F. S. and Wilson E. M.: Cloning of human androgen receptor complementary DNA and localization to the X chromosome. *Science* **240** (1988) 327–330.
47. Misrahi M., Atger M., d'Auriol L., Loosfelt H., Meriel C., Fridlansky F., Guiochon-mantel A., Galibert F. and Milgrom E.: Complete amino acid sequence of the human progesterone receptor deduced from cloned cDNA. *Biochem. Biophys. Res. Commun.* **143** (1987) 740–748.

48. Conneely O. M., Sullivan W. P., Toft D. O., Birnbaumer M., Cook R. G., Maxwell B. L., Zarucki-Schultz T., Greene G. L., Schrader W. T. and O'Malley B. W.: Molecular cloning of the chicken progesterone receptor. *Science* **233** (1986) 767–770.
49. Walter P., Green S., Greene G., Krust A., Bornert J.-M., Jeltsch J.-M., Staub A., Jensen E., Scrace G., Waterfield M. and Chambon P.: Cloning of the human estrogen receptor cDNA. *Proc. Natn. Acad. Sci. U.S.A.* **82** (1985) 7889–7893.
50. Ingegno M. D., Money S. R., Thelmo W., Greene G. L., Davidian M., Jaffe B. M. and Pertschuk L. P.: Progesterone receptors in the human heart and great vessels. *Lab. Invest.* **59** (1988) 353–356.
51. Sheridan P. J. and McGill H. C. Jr: The nuclear uptake and retention of a synthetic progestin in the cardiovascular system of the baboon. *Endocrinology* **114** (1984) 2015–2019.