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Specific Binding of Estradiol to Rat Coronary Artery Smooth Muscle Cells

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We report the expression and characteristics of the estrogen receptor in rat coronary artery-derived smooth muscle cells. Polymerase chain reaction analyses of total and poly(A)+ mRNA from rat coronary artery-derived smooth muscle cells indicate the presence of estrogen receptor mRNA. Binding analyses reveal the presence of high affinity binding sites for 17ß-estradiol, with a K_d equivalent to that observed for authentic estrogen receptors in other estrogen responsive tissues. Scatchard and Hill plot analyses of the properties of receptor–ligand binding indicate the presence of a single site, and the absence of cooperative binding. Unlabeled E2 but not testosterone, dexamethasone or progesterone compete with [3H] 17ß-estradiol for binding sites. The affinity, specificity and non-cooperative nature of the estrogen binding sites are identical to those observed in other estrogen-responsive tissues. These cells may provide a novel model in which to study the effects of estrogens on the proliferation, differentiation and function of vascular smooth muscle cells. Copyright © 1996 Elsevier Science Ltd.

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INTRODUCTION

It is becoming increasingly clear that 17ß-estradiol (E2) can function to protect women against coronary heart disease [1, 2]. While E2 can raise plasma HDL levels and reduce LDL levels in humans [3], such indirect actions of E2 likely account for only 50% of its ability to modulate cardiovascular biology [4]. The apparent expression of estrogen receptors (ER) in heart [5] and artery [6] supports the hypothesis that E2 may directly interact with the heart and blood vessel wall. Karas *et al.* [7] demonstrated that cultured vascular smooth muscle cells (VSMCs), derived from human mammary artery, contain transcriptionally competent ER in response to E2 activation. The ability of E2 to regulate gene transcription may account for its ability to inhibit VSMC proliferation [8], increase VSMC PGI_2 production [9], and decrease VSMC collagen synthesis [10]. Should these effects occur *in vivo* they would be expected to reduce the incidence of atheromas and thromboses.

While atherosclerotic lesions occur often in the coronary arteries, including the left anterior descending artery and circumflex, there have been relatively few studies on the effects of E2 upon these vessels. Immunostaining analyses for ER in post-mortem coronary artery specimens from premenopausal women have demonstrated a potential association between the presence of ER in vessels with normal patency and the absence of ER in atherosclerotic tissues [11]. The molecular aspects of E2 function that make ER presence in the coronary artery a seemingly favorable relationship remain unclear. We wished to identify a possible model in which to study ER-mediated events in coronary artery smooth muscle cells (SMC). In this report we describe, for the first time in cultured coronary artery smooth muscle cells [12], both the characteristics of an E2 binding site that are strongly indicative of ER protein and the expression of mRNA specific for the ER. These cells may be used to study the molecular mechanism(s) that mediates E2 effects in coronary artery SMCs.

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Cell lines

The smooth muscle cells were obtained from Dr Clement Diglio (Wayne State University School of Medicine, Detroit, MI), and were originally obtained from the macro- and micro-circulation of perfused rat hearts [12]. E2-dependent MCF-7 human breast cancer cells expressing high levels of ER [13] were provided by Dr Marc E. Lippman (Georgetown University Medical Center, Washington, DC). Cell culture growth medium comprised Improved Minimal Essential Medium containing sodium phenol red and supplemented with 5% (v/v) fetal calf serum (Biofluids, Rockville, MD).

Reverse transcriptase-polymerase chain reaction, ER subcloning and nucleotide sequencing

RNA extraction was executed as previously described [14], by lysing cells in 4 M guanidium isothiocyanate, layering lysate on to a 5.7 M cesium chloride cushion, and the RNA pellet recovered following centrifugation at 36,000 rpm for 18 h at 18°C. The Mini RiboSep™ Ultra mRNA Isolation Kit (Becton Dickinson Labware, Bedford, MA) was used to select the mRNA species from the total RNA pool. The RiboClone cDNA Synthesis System M-MLV (H^-) (Promega, Madison, WI) was used to produce double stranded cDNA from the mRNA. Intron spanning rat ER cDNA specific oligonucleotide primers [15] were used to amplify the rat ER cDNA by initial denaturation at 95°C for 3 min, 40 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 1 min, followed by polymerization at 72°C for 5 min in a Perkin–Elmer Cetus Gene Amp PCR System 9600 (Norwalk, CT). The polymerase chain reaction product was subcloned into pBluescript II SK +/-plasmid prior to DNA sequence analysis by the Promega TaqTrack Sequencing Systems method using M13 forward and reverse primers.

Steroid transport and binding studies

To determine the kinetics of E2 accumulation, cells were incubated at 37°C with [2,4,6,7,16,17-^{3}H] E2 (Amersham, Arlington Heights, IL); specific activity 153.5 Ci/mmol; 5 nM) for the indicated periods. Estimates of the maximal binding (B_{max}) and affinity of binding (K_d) were obtained using a whole cell binding assay similar to that previously described [16, 17]. Nonspecific binding was determined in parallel by incubating cells with [^{3}H] E2 in the presence of a 200-fold excess of unlabeled E2. For both assays, cells were rinsed after incubation, and radioactivity extracted into 1 ml of ethanol. An aliquot (800 μl) was removed and radioactivity was measured in a liquid scintillation spectrometer [16, 17]. Cell number was obtained using a Model ZB Coulter Counter (Coulter Electronics, Hileah, FL) to enable determination of sites/cell. To determine specificity of binding, cells were incubated for 90 min at 37°C with [^{3}H] E2 (0.5 nM, a concentration approximately 5-fold greater than the expected K_d) in the absence or presence of increasing concentrations (1 nM to 10 μM) of competitor (dexamethasone, testosterone, or progesterone).

Statistics

Statistical analyses including estimates of the mean, standard deviation and coefficient of variation were performed using the SigmaStat statistical software (Jandel Scientific, San Rafael, CA). Linear regressions and their coefficients of regression (r) on Scatchard and Hill analyses were obtained from the LIGAND data analysis software [18], and those for NSB data were performed using the functions in SigmaPlot (Jandel Scientific). Non-linear curve fitting, coefficient of determination (r^2), 95% confidence limits, and the estimation of goodness of fit (F-statistic) for the fitted curves were generated using the algorithms in TableCurve 2D (Jandel Scientific).

RESULTS

Reverse transcriptase–polymerase chain reaction analysis of both total RNA and poly(A) + mRNA using rat ER specific oligonucleotide primers clearly indicates the presence of ER mRNA in these rat coronary artery-derived smooth muscle cells (Fig. 1). The size of

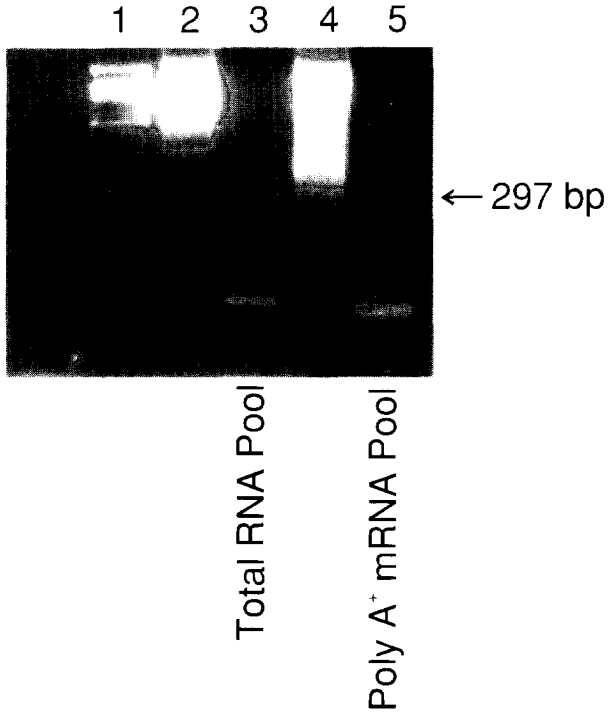

Fig. 1. Electrophoretic size fractionation of the products of the reverse transcription–polymerase chain reaction of RNA from rat coronary artery-derived smooth muscle cells. Lane 1 = markers; lane 2 = bands from reaction using ß-actin primers (total RNA); lane 3 = bands from reaction with ER primers (total RNA); lane 4 = bands from reaction using ß-actin primers (poly(A) + mRNA); lane 5 = bands from reaction with ER primers (poly(A) + mRNA).

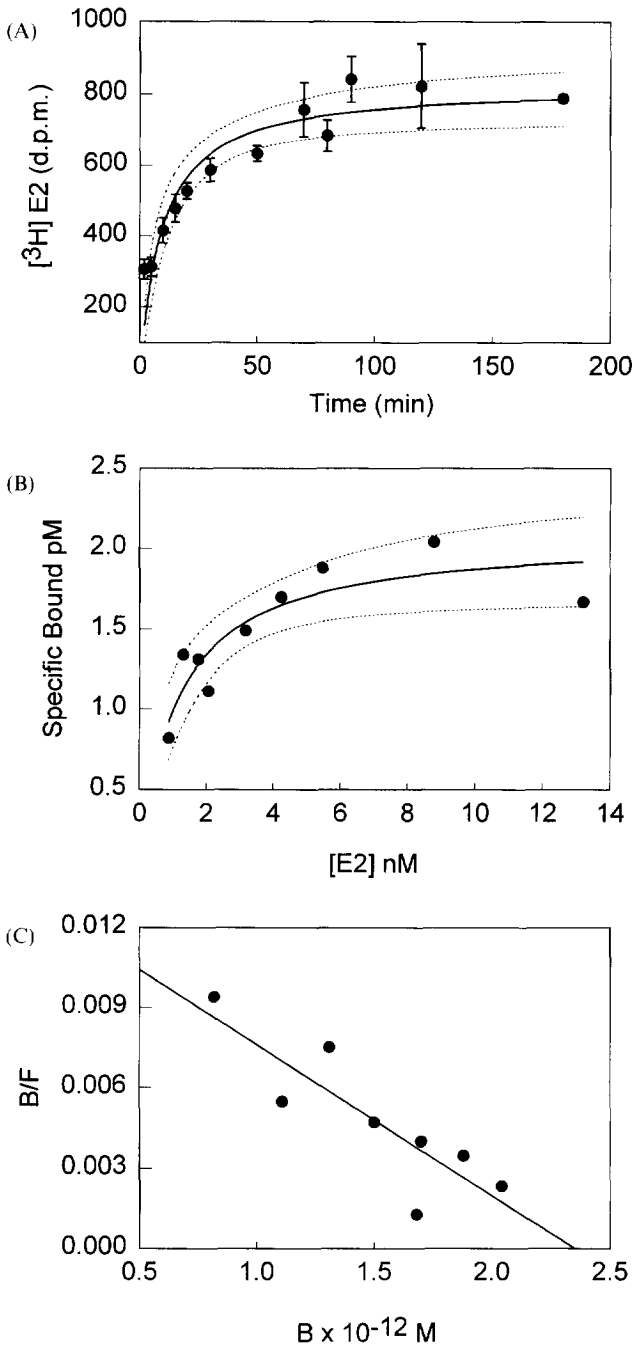

Fig. 2. (A) Kinetics of [^{3}H] E2 accumulation in rat coronary artery-derived smooth muscle cells. The Michaelis–Menten curve was obtained by utilizing the curve fitting functions in SigmaPlot (version 5.0; Jandel Scientific) to fit the curve $y = B_{max}(T/[T+K_{acc}])$ to the experimental data points, where: B_{max} = maximum specifically bound ligand, T = time, K_{acc} = time required to obtain half maximal accumulation. Each data point represents the mean ± SD of three determinations and the apparent absence of error bars indicates that the SD is smaller than the area occupied by the data symbol. The 95% confidence limits for the fitted curve are represented by the dashed lines. (B). A representative saturation plot of [^{3}H] E2 specific binding to rat coronary artery-derived smooth muscle cells *in vitro*. The Michaelis–Menten curve was obtained by utilizing the curve fitting functions in SigmaPlot to fit the curve $y = B_{max}(F/[F+K_d])$ to the experimental data points, where: B_{max} = maximum specifically bound ligand, F = unbound ligand, K_d = concentration required to obtain half maximal binding. The 95% confidence limits for the fitted curve are represented by the dashed lines. (C). A Scatchard plot of the data in (B) (also presented as experiment 2 in Table 1). Data analysis was performed as previously described. The linear transformation was performed by fitting the line $y = (B_{max}/K_d) + (-1/K_d)$ to the transformed data points utilizing the curve fitting functions in SigmaPlot, where B_{max} = maximum specifically bound ligand, K_d = dissociation constant.

the amplified fragment was approximately 300 bp, and is similar to the expected size of 297 bp as determined from the full length rat ER cDNA sequence [19]. The ER origin of the amplified fragment was confirmed by DNA sequence analysis (not shown). However, expression of mRNA alone does not conclusively indicate the presence of appropriate ER protein. To confirm the presence of ER protein, it was necessary to characterize and utilize an appropriate assay.

To determine the experimental conditions that produce equilibrium binding, the kinetics of E2 intracellular accumulation were determined. Since this process could theoretically follow Michaelis–Menten kinetics [20], and the primary data apparently exhibit simple saturation kinetics (Fig. 2a: representative experiment), the Michaelis–Menten curve was initially fitted to the data points using non-linear regression algorithms (TableCurve v 1.0; Levenburg–Marquandt algorithm using the Gauss–Jordan procedure), and the parameters of the fit calculated to determine the time required to obtain equilibrium of binding. To assess the goodness of fit of the model both the coefficient of determination and the F-statistic were determined [21]. The coefficient of determination (r^2) for this representative curve fit is $r^2 = 0.88$ and the F-statistic is $F = 70.18$, both indicating a good fit for the Michaelis–Menten curve to the data. When compared with the other non-linear curve fit models provided in this package (e.g. Gaussian cumulative, logistic dose response, sigmoid), the Michaelis–Menten equation consistently provided the best fit to the data points in each individual experiment. The parameter estimates for this representative experiment (mean ± SE) were $B_{max} = 825 \pm 43$ dpm, $K_{acc} = 9.17 \pm 2.18$ min, and were obtained from data in Fig. 2(A) by analysis using TableCurve for Windows (Jandel Scientific, San Rafael, CA). From these data maximal accumulation was estimated to be achieved by 50–70 min [Fig. 2(A)]. All subsequent estimations of [^{3}H] E2 binding to assess ER expression were performed using 90 min incubations to ensure equilibrium was achieved.

These data suggest that [^{3}H] E2 accumulation in coronary artery-derived smooth muscle cells appears to approximate simple saturation kinetics as described by the Michaelis–Menten equation. While the error of each experimental data point falls within the area described by the 95% confidence limits of the fitted curve, without extensive modeling we cannot exclude

the possibility that a more complex model could provide a better fit [21]. However, such modeling is beyond the scope of the current study. For the purposes of estimating parameters for experimental design, we consider the parameters obtained by the approach described above, and which were used primarily as a guide, to be appropriate for the task.

Having determined the conditions for achieving steady state, we performed a series of binding assays using a range of concentrations of [^{3}H] E2 (0.2–6 nM). The higher concentrations were selected to ensure saturation of any ER present, being 60-fold above the published K_d for the ER in other E2-responsive tissues (approximately 0.1 nM). An accurate estimation of the K_d and B_{max} from Scatchard linear transformations [22] requires that greater than 50% receptor occupancy be achieved [23]. To determine that this requirement was fulfilled, and that we could demonstrate saturation of E2-displaceable binding, we performed a saturation analysis by plotting the concentration of specifically bound [^{3}H] E2 against the concentration of unbound [^{3}H] E2. Since the saturation of specific E2 binding should follow simple mass-action kinetics, the Michaelis–Menten curve was again fitted to these data points (Fig. 2b: representative analysis). The fit for this curve is $r^2 = 0.80$ and $F = 27.29$. Eight of the nine data points fall within the 95% confidence limits of the fitted curve. This analysis clearly indicates that these experimental conditions generate data where a significant proportion of the specific binding occurs at or above an apparent 50% receptor occupancy. While demonstrating the saturability of E2-displaceable (specific) binding, these data and the confidence interval of the fitted curve also reflect the scatter often observed in receptor binding studies.

Linearity and non-saturability are characteristic of non-specific binding sites. The correlation coefficients for non-specific binding data (Table 1) indicate that the estimates of non-specific binding closely fit the predicted linear function, and fulfil the criteria for these binding sites. Thus, the major experimental criteria for performing linear analyses of data obtained from these binding studies were achieved in this whole cell binding assay.

We then used the experimental conditions determined above to determine the number of E2-binding sites per cell, their affinity for [^{3}H] E2, and their specificity, in the rat coronary artery-derived smooth muscle cells (Table 1). We observe the presence of $B_{max} = 1695 \pm 684$ E2-binding sites per cell with an apparent affinity of $K_d = 0.19 \pm 0.08$ nM. There was considerable interexperimental variability (CV for $B_{max} = 40\%$; CV for $K_d = 42\%$; CV = coefficient of variation). Since cell passage number, seeding density and culture conditions were comparable across all experiments, this variability may reflect the low number of binding sites/cell, which we consider to be close to the limit of detection of this assay. The linearity ($r = 0.86$; least squares linear regression) of the representative Scatchard graph [Fig. 2(C)] implies only one class of binding site and no cooperative binding. This is confirmed by the Hill plot analyses (Table 1), which produce a mean Hill coefficient of 0.82 ± 0.08 for the four experiments.

To confirm the ability of this approach to identify ER in cells known to possess high ER levels, we measured the ER levels in the MCF-7 human breast cancer cell line. The experimental conditions were identical with the exception of the time of incubation. We used a 60 min incubation for the MCF-7 cells, since we have previously shown this time to be appropriate for ER determinations in MCF-7 cells [16, 24]. From three experiments using MCF-7 cells the following means ($\pm$ SD) were obtained: $B_{max} = 87\,375 \pm 26\,326$ sites per cell; $K_d = 0.24 \pm 0.12$ nM. These values are equivalent to those previously reported for these cells [14, 24].

The specificity of binding was determined by measuring the ability of unlabeled E2 and other steroid hormones to compete with [^{3}H] E2 for binding to the

Table 1. Characteristics of 17β-estradiol binding sites in rat coronary derived smooth muscle cells in four representative experiments

Rat coronary derived smooth muscle cells: estrogen receptor assays				
Experiment Number	B_{max} (sites/cell)	K_d nM	Hill Coefficient	NSB Regression Coefficient
1	1840	0.17	0.84	0.999
2	2560	0.16	0.70	0.999
3	941	0.11	0.84	0.994
4	1440	0.30	0.89	0.999
Mean $\pm$ SD	1695 ± 684	0.19 ± 0.08	0.82 ± 0.08	0.998 ± 0.0002

B_{max}, K_d and the Hill Coefficient were obtained following analysis of the specific binding data as previously described [18]. A Hill coefficient of > 1.3 or < 0.7 was taken to indicate the presence of significant cooperative binding. The linear transformation for obtaining the Hill Coefficient is given as $y = \log B/(B_{max} - B)$, where: B = specific bound; B_{max} = maximum bound. The NSB regression coefficient was obtained by least squares analysis of the non-specific binding data.

smooth muscle cells [Fig. 3(A)–(D)]. Of the steroids tested, only unlabeled E2 could compete with [^{3}H] E2 for binding sites [Fig. 3(A)–(D)]. However, the other steroid hormones (dexamethasone, testosterone, or progesterone) did not compete with [^{3}H] E2 for binding at physiological concentrations [Fig. 3(A)–(D)].

DISCUSSION

Our data indicate that rat coronary artery-derived smooth muscle cells contain ER mRNA and an E2 binding site. ER-specific oligonucleotide primers were used in a polymerase chain reaction to successfully amplify a cDNA fragment that matched the expected size of the putative ER. The nucleotide sequence of this fragment was nearly identical (98.9%) to that of the rat ER cDNA sequence [19]. Hence, ER mRNA was present in these coronary artery smooth muscle cells. Analysis of the Scatchard and Hill plots indicates there is a single binding site that demonstrates the absence of cooperative binding. The ligand-specific nature of these sites was evident since only E2, but not other steroid hormones could effectively compete with [^{3}H] E2 for binding. A comparison of the affinity, specificity and non-cooperative nature of these sites to ER in established E2 targets indicates that the binding sites identified in this study are ERs. The data clearly indicate that the appropriate experimental conditions for evaluating receptor affinity and number [23,25, 26] were fulfilled for equilibration of binding [Fig. 2(A)], saturation of greater than 50% of binding sites [(Fig. 2(B)], and the linearity/non-saturation of non-specific binding (Table 1).

The data in Table 1 indicate the presence of high affinity E2 binding sites in the coronary artery-derived smooth muscle cells. The affinity (0.19 ± 0.08 nM) is equivalent to that observed for ER in the E2-dependent MCF-7 human breast cancer cells. While the binding of [^{3}H] E2 is readily competed by unlabeled E2, other steroid hormones (dexamethasone, testosterone, and progesterone) did not compete at biologically relevant (1–10 nM) concentrations [(Fig. 3(A)–(D)]. The Scatchard [Fig. 2(C)] and Hill plot analyses (Table 1) of the properties of receptor–ligand binding indicate the presence of a single site, and the absence of cooperative binding. The presence of ER mRNA, and the affinity, specificity, and non-cooperative nature of the E2 binding sites, are equivalent to those observed in other E2-responsive tissues, and strongly suggest that these sites may be authentic ER.

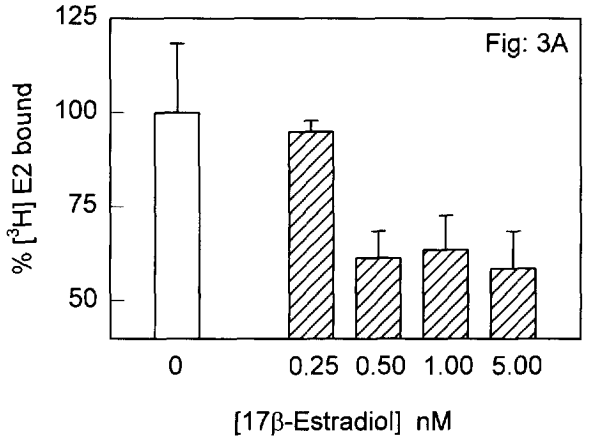

Fig. 3(A).

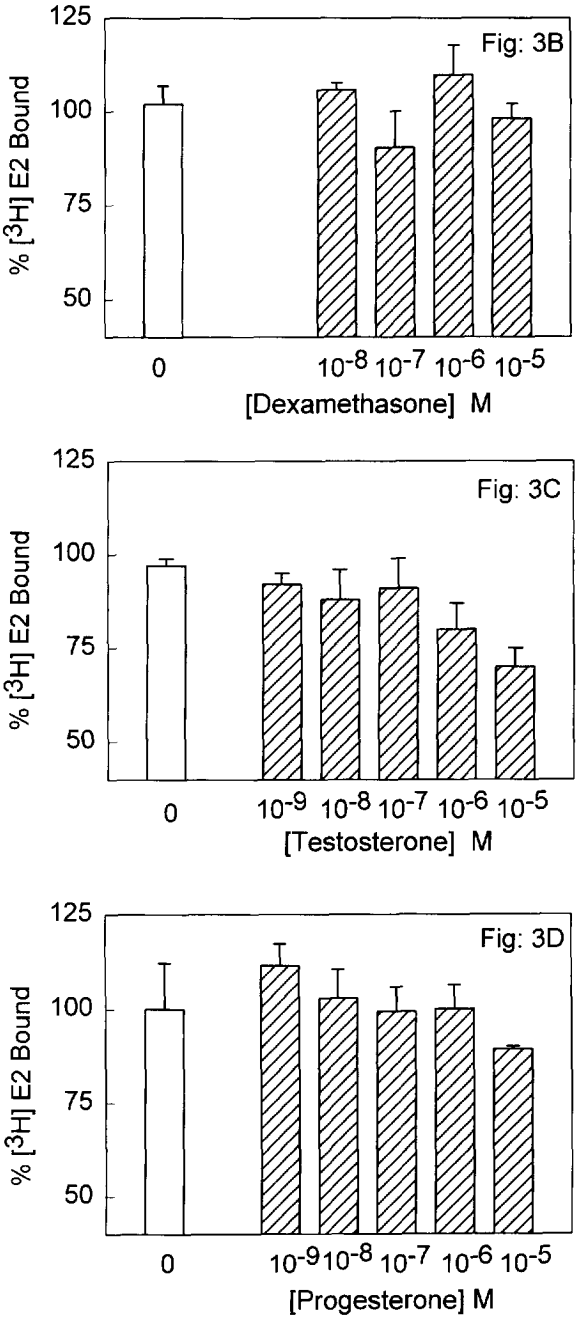

Fig. 3. The ability of 17ß-estradiol (A), dexamethasone (B), testosterone (C) and progesterone (D) to compete with [^{3}H] E2 for binding to rat coronary artery-derived smooth muscle cells. Each data point represents the mean ± SD of three determinations; expressed as a percentage of [^{3}H] E2 bound in the absence of competitor. The apparent absence of error bars indicates that the SD is not sufficiently large to be represented on this scale.

We have not directly addressed the functional capabilities of these receptors in this communication; their affinity for ligand indicates that there are no detectable defects in the steroid hormone binding domain. The presence of ER in these cells suggests a useful model to investigate the effects of estrogens on myointimal proliferation. Identification of ER gene expression and high affinity binding sites for E2 in these cultured vascular smooth muscle cells both supports the role for E2 in the cardiovascular system and suggests that E2 may mediate its physiological effects through direct action upon vascular smooth muscle cells.

In summary, we have characterized these rat coronary artery-derived smooth muscle cells as possessing both ER mRNA and ER protein. The data indicate the value of these vascular smooth muscle cells for studying E2 responsiveness (i.e. cell growth and gene expression).

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REFERENCES

1. Kannel W. B., Hjortland M. C. and McNamara P. M. (1976) Menopause and risk of cardiovascular disease: the Framingham study. *Ann. Intern. Med.* **85,** 452–477.
2. Colditz G. A., Willett W. C., Stampfer M. J., Rosner B., Speizer F. E. and Hennekens C. H. (1987) Menopause and the risk of coronary heart disease in women. *N. Engl. J. Med.* **316,** 1105–1110.
3. Barr D. P., Russ E. M. and Eder H. A. (1952) Influence of estrogens on lipoproteins in atherosclerosis. *Trans. Assoc. Am. Physicians* **65,** 102–113.
4. Bush T. L., Barrett-Connor E., Cowan L. D., Criqui M. H., Wallace R. B., Suchindran C. M., Tyroler H. A. and Rifkind B. M. (1987) Cardiovascular morality and noncontraceptive use of estrogen in women: results of the Lipid Research Clinics Program Follow-up Study. *Circulation* **75,** 1102–1109.
5. Stumpf W. E., Sar M. and Amüller G. (1977) The heart: a target for estradiol. *Science* **196,** 319–321.
6. Malinow M. E., Patterson B. L., Lema B. and Bur G. E. (1963) Vascular and extravascular radioactivity after the injection of estradiol 6,3 ^{3}H in the human being. *J. Clin. Endocrinol. Metab.* **23,** 306–310.
7. Karas R. H., Patterson B. L. and Mendelsohn M. E. (1994) Human vascular smooth muscle cells contain functional estrogen receptor. *Circulation* **89,** 1943–1950.
8. Vargas R., Wrobleska B., Rego A., Hatch J. and Ramwell P. W. (1992) Oestradiol inhibits smooth muscle proliferation of pig coronary artery. *Br. J. Pharmac.* **109,** 612–617.
9. Chang W., Nakao L., Orimo H. and Murota S. (1980) Stimulation of prostaglandin cyclooxygenase and prostacyclin synthetase activities by estradiol in rat aortic smooth muscle cells. *Biochim. Biophys. Acta* **620,** 472–482.
10. Beldekas J. C., Smith B., Gerstenfdeld L. C., Sonenschein G. E. and Franzblau C. (1981) Effects of 17ß-estradiol on the synthesis of collagen in cultured bovine aortic smooth muscle cells. *Biochemistry* **20,** 2162–2167.
11. Losordo D. W., Kearney M., Kim E. A., Jekanowski J. and Isner J. M. (1994) Variable expression of the estrogen receptor in normal and atherosclerotic coronary arteries of premenopausal women. *Circulation* **89,** 1501–1510.
12. Diglio C. A., Grammas P., Giacomelli F. and Wiener L. (1988) Rat heart-derived endothelial and smooth muscle cell cultures: isolation, cloning and characterization. *Tissue and Cell* **20,** 477–496.
13. Soule H. D., Vasquez J., Long A., Albert S. and Brennan M. (1973) A human cell line from a pleural effusion derived from a human breast carcinoma. *J. Natl. Cancer Inst.* **51,** 1409–1416.
14. Clarke R., Brunner N., Katz D., Glanz P., Dickson R. B., Lippman M. E. and Kern R. (1989) The effects of a constitutive production of TGF-α on the growth of MCF-7 human breast cancer cells in vitro and in vivo. *Mol. Endocrinol.* **3,** 372–380.
15. Orimo A., Inoue S., Ikegami A., Hosoi T., Akishita M., Ouchi Y., Muramatsu M. and Orimo H. (1993) Vascular smooth muscle cells as target for estrogen. *Biochem. Biophys. Res. Commun.* **195,** 730–736.
16. Clarke R., Morwood J., van den Berg H. W., Nelson J. and Murphy R.F. (1986) The effect of cytotoxic drugs on estrogen receptor expression and response to tamoxifen in MCF-7 cells. *Cancer Res.* **46,** 6116–6119.
17. van den Berg H. W., Leahey W. J., Lynch M., Clarke R. and Nelson J. (1987) Recombinant human interferon alpha increases oestrogen receptor expression in human breast cancer cells (ZR-75-1) and sensitises them to the anti-proliferative effects of tamoxifen. *Br. J. Cancer* **55,** 255–257.
18. Munson P. J. and Rodbard D. (1980) LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Analyt. Biochem.* **107,** 220–239.
19. Koike S. and Sakai M. (1987) Molecular cloning and characterization of rat estrogen receptor. *Nucl. Acid Res.* **15,** 2499–2513.
20. Neame R. D. and Richards T. G.: Analysis of experimental data. In *Elementary Kinetics of Membrane Carrier Transport.* (1972) 41–55.
21. Sagnella G. A. (1994) Estimation of kinetic constants in ion transport studies: regression analysis and Monte-Carlo simulation. *Clin. Sci.* **87,** 371–381.
22. Scatchard G. (1949) The attractions of protein for small molecules and ions. *Ann. NY Acad. Sci.* **51,** 660–672.
23. Klotz I. M. (1983) Ligand-receptor interactions: what we can and cannot learn from binding measurements. *Trends Pharmacol. Sci.* **4,** 253–255.
24. Clarke R., Brünner N., Katzenellenbogen B. S., Thompson E. W., Norman M. J., Koppi C., Paik S., Lippman M. E. and Dickson R. B. (1989) Progression from hormone dependent to hormone independent growth in MCF-7 human breast cancer cells. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 3649–3653.
25. Bürgisser E. (1984) Radioligand-receptor binding studies: what's wrong with the Scatchard analysis?. *Trends Pharmacol. Sci.* **5,** 142–144.
26. Klotz I. M. (1982) Numbers of receptor sites from Scatchard graphs: facts and fantasies. *Science* **217,** 1247–1249.