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Effects of moexiprilat on oestrogen-stimulated cardiac fibroblast growth

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1 The effects of 2-2-(1-(ethoxycarbonyl)-3-phenylpropyl)-[amino-oxopropyl]−6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline-3 carboxylic acid (moexiprilat), 17β-oestradiol (E_2), oestrone (ES) and angiotensin II (AII) on growth and activation of oestrogen receptors and the immediate-early gene *egr*-1 were investigated in neonatal rat cardiac fibroblasts of female and male origin.

2 In BrdU proliferation assays, oestrone (10^{-7}–10^{-9} M) stimulated cardiac fibroblast growth in a concentration-dependent fashion (maximum at 10^{-7} M, 4.0 fold$\pm$0.14 in female and 3.1 fold$\pm$0.06 in male cells, $n=9$, $P<0.05$), while E_2 (10^{-7}–10^{-9} M) had no effect. Moexiprilat (10^{-7} M) completely inhibited oestrone-induced cardiac fibroblast growth.

3 Angiotensin II (10^{-7} M) induced cardiac fibroblast growth (female 4.1 fold$\pm$0.1/male 3.9 fold$\pm$0.2; $n=9$, $P<0.05$). Angiotensin II induced oestrogen receptor (maximum 21.8 fold at 60 min) and *egr-1* (maximum 47.5 fold at 60 min) expression in a time-dependent fashion.

4 In immunoblot experiments, oestrogen activated oestrogen receptor (ES: 12.8 fold$\pm$2.0; E_2: 14.7 fold$\pm$4.9; $n=3$, $P<0.05$) and *egr-1* (ES: 5.1 fold,$\pm$0.24; E_2: 3.8 fold,$\pm$0.25; $n=3$, $P<0.05$) expression. The induction of oestrogen receptor and *egr-1* protein expression was time-dependent and inhibited by moexiprilat.

5 Our results show that oestrone and 17β-oestradiol reveal a significant difference in their potential to activate cardiac fibroblast growth in female and male cells and that oestrone-stimulated growth is inhibited by moexiprilat. The inhibition of oestrone-stimulated cardiac fibroblast growth by moexiprilat may contribute to the beneficial effects seen in postmenopausal women with hypertensive heart disease treated with ACE inhibitors.

Keywords: Oestrogens; cardiac fibroblasts; moexiprilat; *egr-1*

Introduction

Left ventricular hypertrophy (LVH) in patients with hypertensive heart disease is an independent risk factor for cardiovascular morbidity and mortality and is associated with progressive fibrosis mediated by cardiac fibroblast growth (Brilla *et al*., 1996). This process reveals significant gender-based differences with an increased morbidity in postmenopausal women (Schatzkin *et al*., 1984).

The influence of oestrogens and paracrine substances such as angiotensin II on the progression of cardiac fibrosis during the course of life is not completely understood. Interestingly, angiotensin II, which is a known mitogen of cardiac fibroblasts (Schorb *et al*., 1993), can stimulate oestradiol secretion in target tissues (Kalenga *et al*., 1995), but it remains to be clarified how angiotensin II and oestrogen contribute to the progressive fibrosis found in postmenopausal women with hypertensive heart disease. Recent observations suggest that inhibition of the angiotensin converting enzyme by moexiprilat also affects the synthesis of other paracrine substances i.e. bradykinin in rat heart (Stauss *et al*., 1994). The angiotensin converting enzyme (ACE) inhibitor 2-2-(1-(ethoxycarbonyl)-3-phenylpropyl)-[amino-oxyopropyl]−6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (moexiprilat) (White *et al*., 1994) has been shown to reduce high blood pressure of patients with hypertensive heart disease. However, how ACE inhibition influences the action of sexual hormones, such as oestrogen on the heart and the pathogenesis of left ventricular hypertrophy, is poorly understood.

We therefore investigated the effect of moexiprilat, oestrogens and angiotensin II on cardiac fibroblast growth, the induction of the oestrogen receptor and the immediate-early gene *egr-1*, to elucidate further by what mechanisms ACE inhibition can eventually lead to regression of left ventricular hypertrophy in women with hypertensive heart disease.

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Methods

Cell preparation

Isolation of rat cardiac fibroblasts from neonatal rats was performed according to a modified protocol described by Simpson and Savion (1982). Briefly, the hearts of 1–2 day old rats (Wistar-Kyoto) were isolated and digested with 10 ml of Spinner-solution (composition in mM: NaCl 116, KCl 5.3, NaH_2PO_4 8, $NaHCO_3$ 22.6, HEPES 10, D-glucose 5; pH 7.4) containing 0.1% collagenase (Cytogen, Berlin, Germany) for 10 min at 37°C in eight consecutive steps. After each digestion, the medium containing the suspended cells was removed and an equal volume of Spinner/collagenase solution was added. The cardiac cell suspension was mixed with an equal volume of Ham's F10 (Gibco BRL, Eggenstein, Germany) supplemented with 10% horse serum (HS; Biochrom, Berlin, Germany), 10% oestrogen-free foetal calf serum (FCS; c.c.pro, Hamburg, Germany) and 25 μg ml^{-1} gentamycin (Gibco BRL, Eggenstein, Germany) and stored at 4°C. Cells were centrifuged at 400 *g* for 5 min and the cell pellets were resuspended in 20 ml of Ham's F10 supplemented with 10% HS and 10% FCS and plated on culture dishes. After 75 min the medium which contained the cardiomyocyte fraction of the digested tissue was removed. The dishes were gently rinsed three times to remove remaining cardiomyocytes. The adherent fraction of the plated cells consisted of cardiac fibroblasts. The culture medium for cardiac fibroblasts was changed for DMEM (Gibco BRL,

Eggenstein, Germany) supplemented with 20% oestrogen-free FCS and 25 μg ml^{-1} gentamycin. Purity of cardiac fibroblast culture was assessed by repeated differential plating and microscopic evaluation.

Proliferation assay

After 24 h incubation in serum- and phenol red-free medium (DMEM), neonatal rat cardiac fibroblasts were stimulated with 17β-oestradiol (10^{-7}–10^{-9} M), oestrone (10^{-7}–10^{-9} M), angiotensin II (10^{-7} M) and moexiprilat (10^{-7} M). Cells were harvested after 24 h and cellular proliferation was assessed by BrdU incorporation by use of a colorimetric immunoassay (Boehringer Mannheim, Germany).

Immunoblotting

Neonatal cardiac fibroblasts were lysed in 2 ml of the following buffer (mM): NaCl 50, Tris (pH 7.4) 20, NaF 50, EDTA 50, sodium pyrophosphate ($Na_4P_2O_7$) 20, sodium orthovanadate (Na_3VO_4) 1, 1% triton X-100, PMSF 1, leupeptin 0.6 mg ml^{-1} and aprotinin 10 μg ml^{-1}. Protein content was measured with a standard Bradford assay. Total cell lysates (40 μg/lane) were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 7.5% gel and transferred to a nitrocellulose membrane. Immunoblotting was performed with either a monoclonal oestrogen receptor antibody (Biomol, Hamburg, Germany, 1:500) or an *egr-1* antibody (Santa Cruz Biotechnology, Germany 1:500 dilution), followed by detection with the Enhanced Chemiluminescence technique (ECL, Amersham). Densitometrical analysis was performed on an Epson GT 8000 scanner with the analysis software ScanPack (Biometra, Göttingen, Germany).

All chemicals were obtained from Merck, Darmstadt, Germany and Sigma Chemicals, Deisenhofen, Germany if not otherwise specified.

Statistics

All values are presented as mean ± s.e.mean. Statistical comparisons were made by Student's *t* test with adjustment for multiple comparisons. Statistical significance was assumed if a null hypothesis could be rejected at the $P<0.05$ level.

Results

Proliferation assay

We investigated angiotensin II and oestrogen-induced stimulation of cardiac fibroblast growth in a series of colorimetric immunoassays based on BrdU incorporation. Cells from female and male neonatal rats were investigated. Angiotensin II (10^{-7} M) stimulated cardiac fibroblast growth (female 4.1 fold ± 0.1; male: 3.9 fold ± 0.2; $n=9$, $P<0.05$; Figure 1). 17β-oestradiol had no effect whereas oestrone stimulated cardiac fibroblast growth in a dose-dependent fashion (10^{-7}–10^{-9} M; maximum at 10^{-7} M, 4.0 fold ± 0.14 in female and 3.1 fold ± 0.06 in male cells, $n=9$, $P<0.05$). The effect of oestrone was completely inhibited by moexiprilat (Figure 2).

Immunoblot analysis

We investigated the induction of the oestrogen receptor protein in rat neonatal cardiac fibroblasts after stimulation with angiotensin II, 17β-oestradiol, oestrone and coincubation with moexiprilat or the pure anti-oestrogen 7α-[9-(4,4,5,5,5-pentafluoropentylsulphinyl) nonyl] estra-1,3,5, (10)-triene-3,17β-diol (ICI 182780), to determine how the proliferative effects are mediated in cardiac fibroblasts.

Initially, immunoblotting was performed with an oestrogen receptor antibody. 17β-oestradiol and oestrone stimulated cells showed an induction of oestrogen receptor expression compared to unstimulated cells (ES: 12.8 fold ± 2.0; E_2: 14.7 fold ± 4.9; $n=3$, $P<0.05$). Coincubation with moexiprilat (10^{-7} M) led to a regression of 17β-oestradiol and oestrone-stimulated oestrogen receptor expression to the level of expression found in unstimulated cells. ICI 182780 (10^{-8} M) also inhibited the activation of the oestrogen receptor expression. In the presence of ICI 182780 and moexiprilat, cells revealed no signs of toxic effects, remained viable and attached (Figure 3).

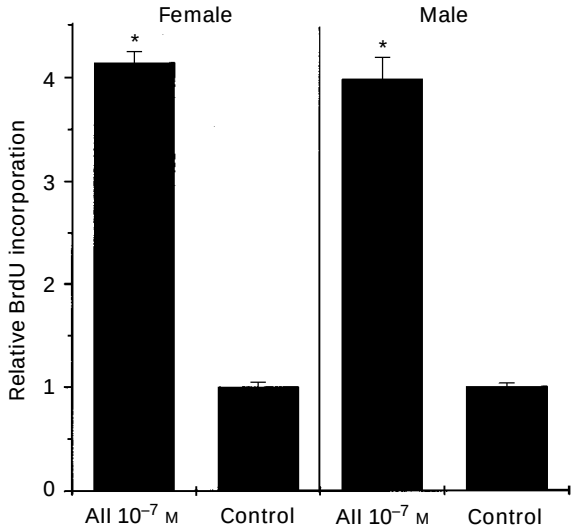

Figure 1 Effect of angiotensin II (AII) on rat cardiac fibroblast proliferation as measured by BrdU incorporation. Male and female cells were grown in the absence and presence of angiotensin II (10^{-7} M) and harvested after 24 h. Columns represent the mean DNA synthesis, as measured by BrdU cell proliferation ELISA, with s.e.mean ($n=9$). Activity is shown relative to control cells that were not exposed to hormones.

Next, we investigated the effect of angiotensin II (10^{-7} M) on the number of oestrogen receptors induced. Immunoblot analysis revealed a rapid induction of the oestrogen receptor (maximum 21.8 fold at 60 min) in a time-dependent fashion in the presence of AII (Figure 4).

To determine whether oestrogens can modulate the expression of growth related endogenous genes and the influence of moexiprilat on this expression, we investigated if the immediate early gene *egr-1* was present in cardiac fibroblasts and whether oestrogen exposure altered its expression in these cells. Immunoblot analysis of lysates from rat cardiac fibroblasts identified a band with a molecular weight of 82 kD corresponding to a known isoform of the *egr-1* protein. Total cellular lysates of cells incubated with 17β-oestradiol (E_2, 10^{-9} M), oestrone (ES, 10^{-9} M) and 2-methoxy-oestradiol (ME, 10^{-9} M), which served as a control, were analysed on SDS-PAGE. Immunoblot analysis showed a time-dependent activation of *egr-1* expression after oestrone stimulation, while stimulation with 17β-oestradiol lead to a minor increase of *egr-1* expression. 2-Methoxy-oestradiol did not modify *egr-1* expression (Figure 5).

To elucidate further the effects of moexiprilat on oestrogen induced cardiac fibroblast growth, we analysed *egr-1* expression after incubation with moexiprilat.

In the absence of 17β-oestradiol only a weak signal could be detected. Treatment of fibroblasts with 10^{-9} M 17β-oestradiol for 60 min resulted in a 3.8 fold (± 0.25) increase of *egr-1* expression. Treatment of fibroblasts with 10^{-9} M oestrone for 60 min lead to a 5.1 fold (± 0.24) increase of *egr-1* expression. After coincubation with moexiprilat (10^{-7} M) regression of the oestrone-induced stimulation could be observed. Coincubation with the oestrogen receptor antagonist ICI 182780 (10^{-8} M) lead to inhibition of *egr-1* induction by oestrone, demonstrat-

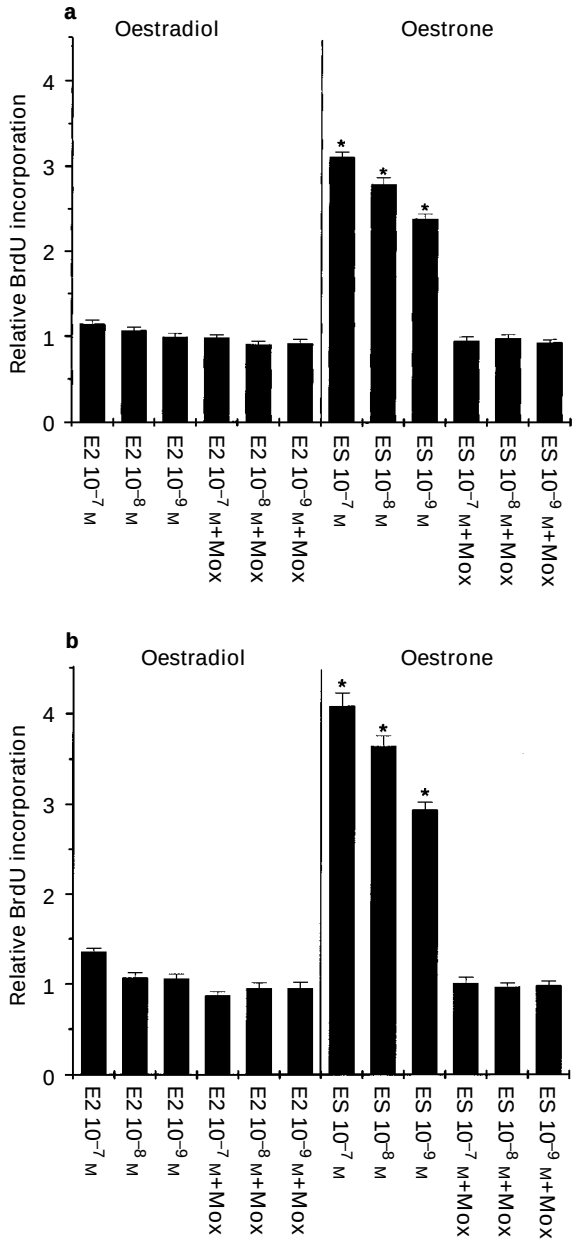

Figure 2 Effect of oestrogens on proliferation of male (a) and female (b) rat cardiac fibroblast as measured by BrdU incorporation. Cells were grown in the absence and presence of 17β-oestradiol (E2), oestrone (ES) (both 10^{-9} M) and moexiprilat (10^{-7} M) and harvested after 24 h. Columns represent the mean DNA synthesis, as measured by BrdU cell proliferation ELISA, with s.e.mean (n = 9). Activity is shown relative to control cells that were not exposed to hormones. Coincubation with the pure antioestrogen ICI 182780 (10^{-8} M) served as a control of oestrogen action.

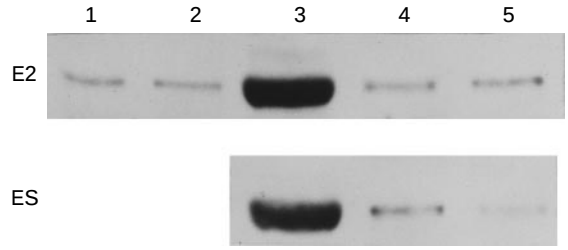

Figure 3 Induction of oestrogen receptor protein in rat neonatal cardiac fibroblasts after stimulation with 17β-oestradiol, oestrone and coincubation with moexiprilat or the pure anti-oestrogen ICI 182780. Total cellular lysates were analysed on SDS-PAGE and immunoblotted with an oestrogen receptor antibody. Cells were incubated without oestrogen or moexiprilat (lane 1), with moexiprilat alone (lane 2), 17β-oestradiol (E2) (lane 3) or oestrone (ES, both 10^{-9} M) (lane 3). Lane 4 represents lysates from cells coincubated with 17β-oestradiol or oestrone and moexiprilat. Lane 5 represents lysates from cells coincubated with 17β-oestradiol or oestrone (both 10^{-9} M) and the pure anti-oestrogen ICI 182780 (10^{-8} M). Oestrone lead to a significant increase of oestrogen receptor protein, this induction was inhibited by moexiprilat or the anti-oestrogenic compound ICI 182780.

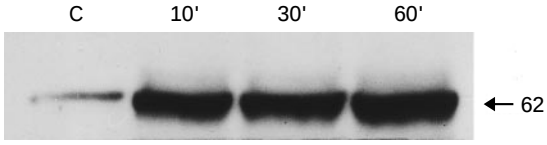

Figure 4 Induction of oestrogen receptor protein in rat neonatal cardiac fibroblasts after stimulation with angiotensin II. Total cellular lysates of cells were incubated with angiotensin II (10^{-7} M) for 10–60 min. Immunoblot analysis of oestrogen receptor protein revealed a rapid induction of the oestrogen receptor (maximum 21.8 fold after 60 min of incubation) in a time-dependent fashion.

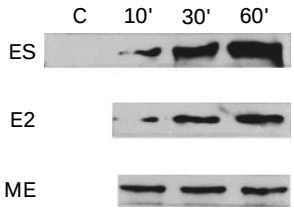

Figure 5 Immunoblot analysis of *egr-1* expression in cardiac fibroblasts after stimulation with oestrogen. Total cellular lysates of cells were incubated for 10–60 min with 17β-oestradiol (E2, 10^{-9} M), oestrone (ES, 10^{-9} M), and 2-methoxy-oestradiol (ME, 10^{-9} M), the latter served as a control. Protein samples were analysed by SDS-PAGE. Immunoblot analysis showed a significant, time-dependent activation of *egr-1* expression after oestrone stimulation, while stimulation with 17β-oestradiol lead to a minor increase of *egr-1* expression. Stimulation with 2-methoxy-oestradiol showed only basal expression of *egr-1*. One of three similar studies is shown.

ing the specificity of the oestrogen-mediated protein induction (Figure 6). In control experiments where the primary antibody was omitted, no signal was observed (data not shown).

Discussion

Left ventricular hypertrophy is attributed with a high incidence of sudden cardiac death and reveals significant gender-based differences. Treatment of individuals with hypertensive heart disease with ACE inhibitors has been shown to reduce left ventricular hypertrophy and to inhibit the progressive cardiac fibrosis (Brilla, 1996). Data from the Framingham Heart Study suggest that the change of oestrogen plasma levels in women before and after the menopause may play an important role in the morbidity and mortality of arterial hypertension (Schatzkin *et al*., 1984). While the role of oestrogens in atherosclerosis and coronary heart disease is currently under investigation, little is known about the role of oestrogens in the pathogenesis of cardiac hypertrophy and progressive cardiac fibrosis found in patients with arterial hypertension.

The present study shows that the ACE inhibitor moexiprilat may modulate cardiac fibrosis, found in postmenopausal women with hypertensive heart disease, by inhibiting of oestrogen receptor-mediated cardiac fibroblast growth.

Our data demonstrate that oestrogens differ in their proliferative capacity to induce cardiac fibroblast growth. 17β-

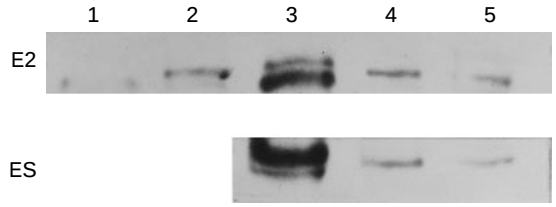

Figure 6 Induction of *egr-1* protein in rat neonatal cardiac fibroblasts after stimulation with 17β-oestradiol, oestrone and coincubation with moexiprilat. Total cellular lysates of cells were incubated with 17β-oestradiol (E2) and oestrone (ES, both 10^{-9} M) and analysed on SDS-PAGE gel electrophoresis and immunoblotted with a *egr-1* antibody. 17β-oestradiol stimulated cells (lane 3) compared to unstimulated cells (lane 1) showed an induction (3.8 fold, $\pm$0.25) of *egr-1* expression. Oestrone stimulated cells (lane 3) compared to unstimulated cells showed an induction of *egr-1* expression. Coincubation with moexiprilat (10^{-7} M) (lane 4) and ICI 182780 (10^{-8} M; lane 5) lead to a regression of oestrone stimulated *egr-1* expression. Moexiprilat alone (lane 2) did not alter *egr-1* expression. Data from one of three similar studies are shown.

oestradiol, which is synthesized in gonadal tissues and has a decreased plasma level after the menopause, does not significantly induce cardiac fibroblast growth. However, oestrone, which shows a significant increase in plasma levels after the menopause and is predominantly synthesized in extragonadal tissues, is a strong proliferative stimulus of fibroblasts obtained from female and male animals. These observations suggest that 17β-oestradiol and oestrone differ in their potential to activate cellular growth.

The effects of oestrogens on growth are mediated through binding to its nuclear receptor. The mechanisms of oestrogen-mediated effects in cardiac fibroblasts may be explained by our recent observation that cardiac fibroblasts of either sex contain functional oestrogen receptors (Grohé *et al*., 1994). The induction of the oestrogen receptor by 17β-oestradiol and oestrone was specific and reversible, as coincubation experiments with the pure anti-oestrogen ICI 182780 show. These data demonstrate that the oestrogen receptor is functionally intact and is induced differentially by oestrogens in cardiac fibroblasts.

Recent observations by Kalenga *et al*. (1995) show that angiotensin II can stimulate oestrogen synthesis in target tissues. However, the mechanisms by which angiotensin II and angiotensin converting enzyme inhibition interact with oestrogen in the pathogenesis of cardiac fibrosis are poorly understood. In our hands, angiotensin II stimulated oestrogen receptor protein expression in a rapid, time-dependent fashion in cardiac fibroblasts. Therefore we investigated the effect of moexiprilat on the expression of the oestrogen receptor in cardiac fibroblasts stimulated with oestrogens. Moexiprilat inhibited the induction of the oestrogen receptor by oestrone.

To evaluate further the effects of moexiprilat on oestrone-induced proliferation, we examined the role of the immediate early gene *egr-1* in this process. The immediate early genes are critical in the control of proliferation and differentiation in a large array of different cell types. It is well established, that oestrogens can induce the expression of *c-fos* and *egr-1* (early growth response gene-1) (Cicatiello *et al*., 1993). Transcriptional activation of *c-fos* is mediated through trans-acting oestrogen-responsive elements in its promotor region and is induced by oestrogens in cardiac fibroblasts (Grohé *et al*., 1996). The *egr-1* gene has a half-palindromic site of an oestrogen-responsive element in its 5′ flanking region, which can be considered as a *cis*-acting element for transcriptional activation by the oestrogen receptor. In our study, 17β-oestradiol and oestrone differed in the activation of *egr-1*. While oestrone led to a significant increase of the *egr-1* protein, 17β-oestradiol showed a significantly smaller effect. Moexiprilat inhibited the oestrogen induced activation of *egr-1* expression in cardiac fibroblasts. This may be explained by the inhibition of oestrogen receptor-mediated transactivation in the presence of the ACE inhibtor. These data show that the effect of oestrone on cardiac fibroblast growth is mediated through activation of the oestrogen receptor and potential downstream target genes such as *egr-1*. This effect is blocked by moexiprilat.

Recent studies have underlined the significance of moexiprilat in the treatment of patients with arterial hypertension (White *et al*., 1994). The data presented here contribute to our understanding of how treatment with an ACE inhibitor may eventually lead to a regression of left ventricular hypertrophy found in postmenopausal women. Furthermore the changes in plasma profile of both 17β-oestradiol and oestrone may well play a critical role in the progressive cardiac fibrosis found in postmenopausal women with hypertensive heart disease. It is known that oestrone belongs to a class of short-acting oestrogens (such as 17α-oestradiol, estriol), which, in contrast to 17β-oestradiol, cause binding of the ligand-receptor complex in the nucleus only for a short period of time. However, the exact mechanisms by which oestrone affects cellular growth remain to be elucidated.

The proliferative capacity of oestrogen metabolites largely varies in target tissues, this observation (specific cell type differences) may contribute to the underlying basis for the difference between oestrone and 17β-oestradiol in their potential to activate fibroblast growth. Recent studies suggest that oestrogens, i.e. oestrone, may cause direct effects on cellular growth not only by direct binding to its classical receptor but also by activating alternative signal transduction pathways, i.e. the mitogen-activated protein kinase pathway (Migliacco *et al*., 1996). This model may contribute to our understanding of the complex regulation of the ligand-receptor interaction of oestrogen-stimulated cellular growth. Further investigations are required to understand the complex interaction of the renin-angiotensin-aldosterone system (RAAS) with oestrogens in hypertensive heart disease.

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