We have previously shown that the myocardium is a target tissue for estrogen. Here, we have identified rapid non-nuclear estrogen effects on the expression of the early growth response gene-1 (Egr-1) in cardiomyocytes. Egr-1 mRNA and protein were rapidly and strongly induced by estrogen in an estrogen receptor-dependent manner via the extracellular signal-regulated kinase, ERK1/2. A promoter analysis study of a 1.2-kilobase Egr-1 promoter fragment revealed that the serum response elements (SREs) but not the estrogen response elements or AP-1 sites are responsible for Egr-1 induction by estrogen, identifying a novel mechanism of estrogen receptor-dependent gene activation in the myocardium. Both estrogen receptor-a and -b induced the Egr-1 promoter via the SREs as well as an artificial promoter consisting of only five SREs in cardiomyocytes. Electrophoretic mobility shift assays showed that a protein complex containing serum response factor or an antigenically related protein was recruited to the SREs by estrogen treatment of primary cardiomyocytes. The recruitment of the protein complex was inhibited by the specific estrogen receptor antagonist ICI 182,780 as well as the MEK inhibitor PD 98059. Taken together, these results identify SREs as important promoter control elements for an estrogen receptor-dependent mechanism of gene activation in the myocardium.

Gender-specific differences in the occurrence of cardiac disease and the protective role of estrogens in the heart have been established in numerous clinical studies (1, 2). Hormone replacement therapy may prevent the primary development of coronary artery disease in postmenopausal women (1) but have not been effective in secondary prevention trials (3). These findings call for a better understanding of the role and function of estrogen and estrogen-like substances in the heart. The vast majority of studies have concentrated on vascular hormone effects (4, 5). However, cardioprotection by estrogen is not necessarily restricted to the vasculature. We have previously shown that functional estrogen receptors in the myocardium regulate the expression of relevant target genes such as connexin 43 (6) and α-myosin heavy chain, one of the major contractile proteins in the heart (7). In addition to the classical genomic regulation of target gene expression, a new role for estrogens as mediators of rapid non-genomic effects has recently been identified. These rapid effects include the stimulation of nitric oxide release in vascular cells (8, 9) and the activation of Ras and Raf-1 kinase (10), resulting in the activation of ERK1/2 and the phosphorylation of transcription factors in both non-myocytes (11–15) and cardiomyocytes (16). In the present work we have for the first time analyzed in detail one of the mechanisms of rapid gene induction by estrogen in the myocardium by performing a detailed promoter analysis of a bona fide estrogen target gene identified by us, the early growth response gene 1 (Egr-1).

Surprisingly, a cluster of serum response elements (SREs) turned out to be the determinants of transcriptional Egr-1 activation by estrogen in primary cardiomyocytes. Contrary to our original hypothesis neither the estrogen response elements (EREs) nor the AP-1 sites in the Egr-1 promoter fragment are relevant in this context.

MATERIALS AND METHODS

Cell Culture—Primary neonatal rat cardiomyocytes were isolated from 2-day-old Wistar rats as described previously (17). Cells were preplated for 1.5 h to remove non-myocytes, and the supernatant containing the cardiomyocytes was collected. Cells were counted in a Fuchs-Rosenthal chamber and seeded onto Petri dishes at a density of $8 \times 10^4$ cells/cm$^2$ in phenol-red free minimum Eagle’s medium (Sigma) supplemented with 292 mg/liter l-glutamine, 350 mg/liter sodium bicarbonate (Sigma), and 5% charcoal-stripped fetal calf serum (CC-Pro). Cells were cultured for 24 h at 37 °C with 1% CO$_2$ after which myocytes were washed with PBS and the medium was changed to phenol-red free minimum Eagle’s medium containing 2% controlled process serum replacement-1 (CPSR-1, Sigma). Cardiomyocyte cultures contained 95% myocytes as assessed by immunofluorescence staining with an antibody against the cardiomyocyte-specific troponin-T (Sigma).

Northern Blotting—Myocytes were treated with 10 nm water-soluble 17β-estradiol (E$_2$, Sigma) $\pm$ 1 μM specific estrogen receptor antagonist ICI 182,780 (Zeneca) for the indicated times. Cells were washed with 1× PBS, and total RNA was isolated using the Trizol reagent (Life Technologies, Inc.). 10 μg of total RNA was electrophoresed in 1% agarose gels containing formaldehyde and blotted onto nylon membranes (Hybond N+, Amersham Pharmacia Biotech). Hybridizations were performed at 68 °C in Quickhyb solution (Stratagene) following the manufacturer’s suggestions. cDNA probes for Egr-1 and GAPDH were labeled radioactively using a random primed labeling kit (Amer- 

1 The abbreviations used are: ERK1/2, extracellular signal-regulated kinase 1 and 2; E$_2$, estrogen; ERE and $\beta$, estrogen receptor $\alpha$ and $\beta$; SRE, serum response element; ER, estrogen response element; AP-1, activating protein-1; Egr-1, early growth response gene-1; SBF, serum response factor; PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift assay; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase.
Estrogen-Receptor-dependent Gene Activation via SREs

sham Pharmacia Biotech). Membranes were washed by several changes of washing buffer (2× SSC, 0.1% SDS, and 0.2× SSC, 0.1% SDS) at 68 °C, and radioactivity was detected by exposure to x-ray film or by phosphorimaging.

Immunoblotting—After E₂ treatment for the indicated number of times, cells were washed with 1× PBS and lysed in 6-well plates by adding 200 µl of Laemmli buffer (Bio-Rad). Samples were sonicated to break up DNA strands and boiled for 5 min, and proteins were separated on 10% SDS-polyacrylamide gels. Resolved proteins were transferred electrophoretically to nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech), and membranes were blocked in 5% nonfat milk powder, 0.05% Tween 20 in 1× PBS. Immunoblotting was performed with polyclonal antibodies directed against Egr-1 (1:1500, Santa Cruz Biotechnology), actin (1:500, Santa Cruz), phospho-specific polyclonal antibodies against activated ERK1/2 (1:500, New England Biolabs), polyclonal antibodies against SRF (1:1000, Santa Cruz), and monoclonal antibodies against ERK1/2 (1:500, New England Biolabs). Immunoreactive proteins were detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

Plasmids—The 1.2-kilobase Egr-1 promoter (GenBank™ accession number X12617) as well as the deletion constructs were cloned in the SalI site of the pX2 luciferase vector (Invitrogen) and have been described previously (18). The sizes and positions of the deletions are shown in Fig. 2B. Briefly, construct B consisted of the Egr-1 promoter fragment between positions −689 and −1200 plus nucleotides −1 to −31. Construct C consisted of 501 base pairs containing 5 SREs located between positions −199 and −700 of the Egr-1 promoter. Construct D consisted of 186 base pairs containing 3 SREs located between positions −514 and −700 of the Egr-1 promoter. The cis-reporter plasmid pSRE-luc contains a basic promoter element (TATA box) joined to five tandem repeats of the murine Egr-1 promoter. Oligonucleotide probes used were as follows: SRE 3, 5' -CACAGCAGTTTATGGGAGTGG-3'; SRE 4, 5' -ACAGCAGTTTATGGGAGTGG-3'; SRE 5, 5' -CAAGGCGCTATATAAGGAGGACG-3'. The reaction contained 1 µg of poly(dI-dC) (Roche Molecular Biochemicals)/3 µg of nuclear protein as nonspecific competitor. Samples were incubated at 4 °C for 10 min before loading onto 5% polyacrylamide gels that were pre-run for 2 h in 0.5× Tris borate-EDTA. Electrophoresis was continued at 220 V to fractionate the protein-DNA complexes. Gels were vacuum-dried in a gel dryer (Bio-Rad) and exposed to x-ray film. For supershift assays 1 µg of a polyclonal rabbit antibody against SRF (Santa Cruz) was added to the reaction mixture. Controls containing antibodies incubated with labeled oligo in the absence of protein were included to ensure that the antibodies did not bind to the DNA directly (not shown).

RESULTS

Egr-1 Expression Is Rapidly Induced by Estrogen in Cardiomyocytes—Treatment of isolated neonatal cardiomyocytes with 10 nM E₂ resulted in a rapid and transient induction of Egr-1 mRNA after 15 min of treatment (Fig. 1A), followed by induction of Egr-1 protein after 90 min (Fig. 1B). The rapid induction of Egr-1 was completely inhibited by the pure estrogen receptor antagonist ICI 182,780 (Fig. 1, A and B). Because Egr-1 is rapidly induced via ERK1/2 in other cell types, we tested whether this mitogen-activated protein kinase (MAPK) signal transduction pathway is relevant to Egr-1 induction by E₂ in cardiomyocytes. Cells were treated with the MEK inhibitor PD 98059 or the p38 MAPK inhibitor SB 202190 to differentiate between the involvement of both signaling pathways. PD 98059 completely blocked Egr-1 protein induction by estrogen. This was not the case when the p38 MAPK inhibitor was used by SB 202190 (Fig. 1B). The activation through phosphorylation of ERK1/2 by estrogen was tested. E₂ treatment resulted in a rapid activation of ERK1/2 within 5 min in a similar manner to
fetal calf serum and phorbol 12-myristate 13-acetate stimulation (Fig. 1C). Egr-1 was therefore rapidly activated by E2 in an estrogen receptor-dependent pathway comprising ERK1/2 but not p38 MAPK.

Estrogen Up-regulates the Egr-1 Promoter in Cardiomyocytes—To characterize the mechanisms underlying Egr-1 induction, we analyzed a 1.2-kilobase Egr-1 promoter fragment for potential estrogen response elements. The presence of two putative AP-1 sites in the upstream region of the Egr-1 promoter has been described by others (18, 21). Two ERE half-palindromic sites separated by 20 base pairs in close vicinity to the 2 AP-1 sites were identified. It has previously been shown that estrogen receptors can regulate the expression of genes via repetitive half-palindromic ERE sites (22) and through AP-1 sites (23). Further binding sites for regulatory factors in the Egr-1 promoter fragment include 5 SREs. The Egr-1 promoter fragment cloned in front of a luciferase reporter (Egr-1-luc) was induced 6-fold by E2 in primary rat cardiomyocytes (n = 11, p ≤ 0.001), which was significantly inhibited by 1 μM ICI 182,780 (n = 11, p ≤ 0.001). A comparison of the activities of the individual deletion constructs as a percentage of Egr-1-luc without stimulus is shown. Egr-1-luc or deletion constructs (Constructs B–D) were transiently transfected, and cardiomyocytes were treated with 100 nM E2. E2 treatment resulted in a 6-fold induction of Egr-1-luc when compared with base-line activity of this construct. Construct B, which contained only the ERE and AP-1 sites, had 7% of the base-line activity of Egr-1-luc and was not significantly induced by estrogen. Construct C, containing all 5 SREs, had 80% of the base-line activity of Egr-1-luc and was induced 6-fold by estrogen when compared with the base-line activity of the construct (n = 7, p ≤ 0.05). Construct D, containing only the 3 most upstream SREs, had 50% of the base-line activity of Egr-1-luc and was induced 4-fold by estrogen when compared with the base-line activity of this construct (n = 7, p ≤ 0.05). Statistical analyses were performed using the Student’s t test.

FIG. 2. 17β-Estradiol stimulates Egr-1 promoter activity. A, primary neonatal cardiomyocytes were transiently transfected with a 1.2-kilobase Egr-1 promoter fragment cloned in front of a luciferase reporter gene (Egr-1-luc) or with the vector alone (pXp2-luc). E2-treatment (100 nM; a dose-finding study showed no remarkable difference in transfection experiments using 10 and 100 nM E2) resulted in a 6-fold induction of Egr-1 promoter activity (n = 11, p ≤ 0.001), which was significantly inhibited by 1 μM ICI 182,780 (n = 11, p ≤ 0.001). B, a comparison of the activities of the individual deletion constructs as a percentage of Egr-1-luc without stimulus is shown. Egr-1-luc or deletion constructs (Constructs B–D) were transiently transfected, and cardiomyocytes were treated with 100 nM E2. E2 treatment resulted in a 6-fold induction of Egr-1-luc when compared with base-line activity of this construct. Construct B, which contained only the ERE and AP-1 sites, had 7% of the base-line activity of Egr-1-luc and was not significantly induced by estrogen. Construct C, containing all 5 SREs, had 80% of the base-line activity of Egr-1-luc and was induced 6-fold by estrogen when compared with the base-line activity of the construct (n = 7, p ≤ 0.05). Construct D, containing only the 3 most upstream SREs, had 50% of the base-line activity of Egr-1-luc and was induced 4-fold by estrogen when compared with the base-line activity of this construct (n = 7, p ≤ 0.05). Statistical analyses were performed using the Student’s t test.

receptors, as it was significantly inhibited by the estrogen receptor antagonist ICI 182,780 (Fig. 2A, n = 11, p ≤ 0.001).

To identify control elements in the Egr-1 promoter through which estrogen induces Egr-1, a deletion construct containing two ERE half-sites as well as two AP1 sites (Construct B; see Fig. 2B) was analyzed first, because it contains known target sites for estrogen receptors. The basal activity of this construct was 7% compared with the entire promoter fragment, and E2 treatment resulted in only a minor induction (Fig. 2B). This suggested that ERE half-sites and AP1 sites are not major contributors to the estrogen effect on Egr-1 in this cell system. Therefore, other deletion constructs containing the cluster of 5 SREs (Construct C) as well as a deletion construct containing only the 3 most upstream SREs (Construct D) were analyzed. Constructs C and D were induced 6- and 4-fold, respectively, by E2 (Fig. 2B, n = 7, p ≤ 0.001). These results showed that E2-dependent regulation of Egr-1 in myocardial cells resides in the SRE-containing promoter region. This finding has not been described before.
ERα and ERβ Induce Egr-1 Gene Activation via SREs—

Because we have recently identified both estrogen receptor α and β in cardiac muscle cells (6), co-transfection experiments were performed to delineate the respective roles of these two receptors in the rapid induction of an estrogen target gene in the heart. Co-transfection of ERα or ERβ with Egr-1-luc resulted in a 21- and 19-fold induction, respectively, of the promoter fragment in the presence of E2 (Fig. 3A). This showed that both estrogen receptors are able to activate Egr-1 expression to an approximately similar extent.

Because constructs containing SREs but no estrogen receptor DNA binding sites (constructs C and D) reacted to E2
stimulation in a manner similar to the entire promoter fragment, it was investigated as to whether ERα and/or ERβ could induce the transcriptional activity of these constructs. Overexpression of ERα resulted in a 13- and 9-fold induction of constructs C and D, respectively, whereas ERβ overexpression resulted in a 20- and 12-fold induction, respectively (Fig. 3, B and C). Taken together these results showed that both estrogen receptors are able to activate Egr-1 expression. Furthermore, this demonstrated that the SRE-containing promoter region and not the ERE/AP-1-containing region is the main control region for both ERα- and ERβ-mediated induction of Egr-1 induction in cardiomyocytes.

An Artificial Promoter Containing Only SREs Is Induced by Estrogen Treatment—To test whether the SREs in the Egr-1 promoter are responsible for the induction by estrogen, an artificial promoter containing 5 SREs cloned in front of a luciferase reporter gene was transfected into primary neonatal cardiomyocytes (Fig. 4A). Estrogen treatment resulted in a 3.3-fold induction of this artificial promoter construct (n = 7; p < 0.05). The specific estrogen receptor antagonist ICI 182,780 inhibited the induction by estrogen, showing a specific estrogen-receptor mediated induction via SREs. Overexpression of ERα as well as ERβ resulted in a 9- and 6.5-fold induction of this artificial SRE-containing promoter construct (Fig. 4B). These data therefore demonstrates that SRE motifs alone can confer an estrogen response to a heterologous promoter and that this response is mediated specifically via both ERα and ERβ.

**Estrogen Recruits Serum Response Factor-like Proteins to the SREs—**EMSAs were performed utilizing nuclear extracts prepared from E2-treated and untreated cardiomyocytes to investigate whether estrogen treatment induced binding of transcription factors to the SREs in the Egr-1 promoter. Oligonucleotide probes matching the 3 upstream SREs (the SREs contained in Construct D; see Fig. 2B) were used in EMSAs. These SREs were chosen because construct D (containing only these SREs) was still significantly induced by estrogen (see Fig. 2B). The formation of specific protein-DNA complexes was induced by E2 treatment when oligonucleotides representing either SRE 3, 4, or 5 were used (Fig. 5A, representative data for SRE 5 are shown), indicating that hormone treatment altered the transcription factor recruitment in an estrogen-dependent manner. Analysis of the effect of inhibitors of the different signaling pathways on complex formation showed that ICI 182,780 as well as the MEK inhibitor PD 98059, but not the p38 MAPK inhibitors SB 202190 and SB 203580, inhibited the formation of specific protein-DNA complexes (Fig. 5B). This finding is in accordance with the induction pattern of Egr-1 observed at the endogenous protein level (see Fig. 1B). Supershift assays with polyclonal antibodies against the serum response factor (SRF) identified a protein with the antigenic characteristics of SRF as one of the proteins in the protein-DNA complex (Fig. 5A). Taken together these results identify a novel mechanism of gene activation by estrogen receptors: in the myocardium estrogen results in the phosphorylation of ERK1/2. Activation of the MAPK pathway ultimately leads to the recruitment of transcription factors that are antigenically related to SRF, to the SREs of the Egr-1 promoter, and to subsequent gene induction.

**DISCUSSION**

In this study we show the following. 1) The early growth response gene-1 (Egr-1), a zinc finger transcription factor (24), is rapidly induced by physiological concentrations of 17β-estradiol in cardiomyocytes. 2) This induction is estrogen receptor-dependent and mediated via activation of the ERK1/2 pathway. 3) The serum response elements and not the estrogen response elements or AP-1 sites in the Egr-1 promoter are responsible for the activation by estrogen. 4) SRE motifs alone are able to confer an estrogen receptor-dependent estrogen response to a heterologous promoter. 5) Estrogen treatment leads to the recruitment of a transcription factor(s), which includes SRF or a SRF-like protein, to the SREs. 6) Together, these results identify a novel rapid mechanism of gene activation by estrogen in the myocardium.

Egr-1 is a transcription factor with an important function in various tissues including the cardiovascular system. In atherosclerotic lesions of mice and humans, elevated levels of Egr-1 mRNA as well as elevated levels of Egr-1-inducible genes such as intercellular adhesion molecule-1 (ICAM-1), platelet-derived growth factor-α (PDGF-A), tumor necrosis factor-α (TNFα),...
transforming growth factor-β1 and -β3 (TGFβ1 and -β3), and superoxide dismutase-1 (SOD1) were detected implying a function for Egr-1 in repair of vascular lesions after injury (25). Furthermore, a function for Egr-1 in the myocardium has been indicated by studies showing that Egr-1 is highly induced in hypertrophied hearts (26). Our group has shown that Egr-1 can also be induced by hypertrophic stimuli such as endothelin, angiotensin II, adrenoreceptor stimulation, or stretch treatment of cardiomyocytes (27, 28). A pathophysiologically relevant function for Egr-1 in the myocardium is also suggested by the results from Saadane et al. (29) who showed that the lack of Egr-1 in mice results in altered expression of pathophysiologically relevant myocardial genes. It has also been shown that the overexpression of Egr-1 in neonatal cardiomyocytes resulted in the induction of α-myosin heavy chain, indicating a role of Egr-1 as a mediator of myocardial contractile gene expression (30). The induction of Egr-1 by estrogen in the myocardium provides evidence that estrogen directly acts on cardiomyocytes and represents a possible mechanism by which estrogen could modulate the expression of important myocardial genes downstream of Egr-1 such as the major contractile protein α-myosin heavy chain. In another study we have in fact shown that estrogen does modulate the expression of α-myosin heavy chain in spontaneously hypertensive rats, an in vivo model of cardiac hypertrophy (7). Although this was a long-term animal study, the induction of Egr-1 by estrogen might represent a possible mechanism for the alteration of α-myosin heavy chain expression by estrogen.

The focus of the present work was on the molecular mechanisms of estrogen action in the myocardium. The results revealed a novel way of estrogen receptor-dependent gene induction in the heart. Promoter analysis showed that transcriptional activation of myocardial genes by estrogen receptors can be mediated via serum response elements. The Egr-1 promoter contains two estrogen response element half-sites, two AP-1 sites in the distal region, and a proximal region with five functional serum response elements. In transfection experiments we could show that, contrary to our original hypothesis, the ERE/AP-1 sites do not play a role in the induction of the Egr-1 promoter by estrogen. This result was unexpected because it has been shown that estrogen receptors are able to bind to repetitive ERE half-sites or half-sites separated by several base pairs, resulting in gene activation (22). Furthermore, it has been shown that differential effects of the two known estrogen receptors on gene activation may be mediated via AP-1 sites (23). This is, however, not the case for the Egr-1 promoter in cardiomyocytes, where both receptors have an activation function. The serum response elements in the Egr-1 promoter region were instead essential for estrogen inducibility. A construct containing all 5 SREs as well as a construct

![Fig. 5. 17β-Estradiol treatment recruited transcription factors to the SREs in the Egr-1 promoter. A, nuclear extracts of cardiomyocytes treated with 10 nM E2, resulted in the binding of protein complexes to the SREs. Labeled oligonucleotide probes matching SREs 3, 4, and 5 of the Egr-1 promoter were used. Representative experiments using probes for SRE 5 are shown. When SRE 3, 4, and 5 probes were used, specific protein-DNA complexes were formed that were competed away with an excess of unlabeled specific oligonucleotide probes but not by a nonspecific oligonucleotide probe. An anti-SRF antibody shifted the SRE complex (Anti-SRF Supershift), identifying SRF or an antigenically related protein in the complex. Here a representative experiment for SRE 5 is shown, but the same results were also obtained for SREs 3 and 4 (not shown). B, the recruitment of transcription factors containing SRF to the SREs were inhibited by the estrogen receptor antagonist ICI 182,780 (1 μM) as well as by PD 98059 (10 μM). SB 202190 (10 μM) and SB 203580 (10 μM) had no effect on the formation of the protein-DNA complex at the SREs. A representative experiment using a SRE 5 probe is shown. Similar results were observed when probes for SREs 3 and 4 were used (not shown). FCS, fetal calf serum.](http://www.jbc.org/)
Estrogen Receptor-dependent Gene Activation via SREs

containing only the three most upstream sites were both induced by estrogen, showing that serum response elements play important roles in gene activation by estrogen. A study has provided indirect evidence for the involvement of SREs and AP-1 sites in the activation of c-Fos by estrogen via MAPK in neuroblastoma cells (14), but no direct experiments for the induction via SREs were shown. While this manuscript was in the revision process, a paper was accepted for publication in this journal showing that estrogen induced the c-fos promoter via the SRE (the c-fos promoter contains only one SRE and no ERαs) in MCF-7 cells (31). The authors also showed that the induction was mediated in an estrogen receptor-dependent manner via ERK1/2. This study in mammary carcinoma cells is therefore in accordance with our work in cardiomyocytes, showing that the estrogen receptor-mediated gene induction via SREs may be a rather general phenomenon that can be extrapolated to other target genes and cell types.

Our analysis of the signaling pathways involved in Egr-1 activation in cardiomyocytes demonstrated that it is specifically mediated via ERK1/2 but not the p38 MAPK pathway, because Egr-1 activation was abolished by the MEK inhibitor PD 98059 but not by the p38 MAPK inhibitor SB 202190. This finding is in accordance with observations in other cell systems showing that Egr-1 activation is mediated by an ERK1/2-dependent mechanism (32–34). p38 MAPK has also been implicated in the activation of Egr-1 (35), but this is clearly not the case in cardiomyocytes. Analysis of the protein complex bound to the SREs upon E2 treatment revealed that a protein complex containing a factor antigenically similar to SRF is recruited to the SREs in response to physiological estrogen levels. An anti-SRF antibody resulted in a supershift of this complex. Because transcription factors of the TCF (ternary complex factor) family are phosphorylated specifically by ERK1/2 and have also been shown to interact directly with SRF resulting in the activation of target genes via serum response elements (36), the possibility cannot be completely excluded that a TCF member, in addition to SRF, is involved in the induction of Egr-1 by estrogen.

Bernal-Mizrachi et al. (37) showed that Egr-1 is induced upon glucose treatment of pancreatic β-cells via the proximal 2 SREs in the Egr-1 promoter and that this induction is, at least in part, mediated via the protein kinase A and Ca2+/CaM pathways and not via the ERK1/2 pathways. Interestingly, this induction is mediated via the proximal SREs, whereas the distal SREs are relevant for induction by estrogen, suggesting differential functions for these elements. Nevertheless, these data, together with our data suggest that the SREs in the Egr-1 promoter might act as a point of convergence of different signaling pathways activated upon various stimuli in the cell. However, because different cell types were used in the experiments, no definite conclusion can be drawn.

Evidence for activation of MAPK by estrogen is also provided by a study showing that estrogen receptors are capable of activating c-Src kinase, putatively by direct interaction, leading to activation of the downstream c-src effectors in MCF-7 breast cancer cells (38, 39), as well as by a more recent study showing that estrogen induced the prolactin gene via MAPK activation in rat pituitary cells (40). In both of these mechanisms of MAPK activation, extranuclear estrogen receptors are involved, and in the latter study the involvement of a membrane-bound estrogen receptor-α is postulated. It has been shown in primary chicken hamster ovary cells that overexpression of ERα and ERβ leads to expression of intracellular receptors as well membrane-associated receptors (41). It is therefore not possible to draw any conclusions on the involvement of potentially membrane-bound estrogen receptors in the rapid effects described here.

Investigation of the role of the two estrogen receptor subtypes revealed that both ERα and ERβ were able to induce transcriptional activation of the Egr-1 promoter in co-transfection experiments. No statistically significant differential effects of the two receptors on Egr-1 promoter induction were observed. These effects were also due to specific action of the transfected receptors, because the estrogen receptor antagonist ICI 182,780 inhibited induction by both ERα and ERβ. Analysis of the induced promoter region as well as a heterologous promoter containing only SREs also showed that both ERα and ERβ, or eventually heterodimers formed between transfected and endogenous receptors, were able to induce the Egr-1 promoter via the SREs, indicating that each receptor individually may be involved in rapid non-nuclear estrogen effects. It will be interesting to investigate whether ERα and ERβ behave differently with respect to rapid activation of the pathway we describe when different estrogenic ligands and/or different cellular contexts are used.

Taken together, this study shows that rapid non-nuclear action of estrogen receptors is relevant to myocardial gene activation and can lead to a genomic nuclear response. The rapid induction of Egr-1 by both ERα and ERβ may in theory represent a mechanism by which estrogen, via extranuclear estrogen receptors, exerts an array of effects through modulation of Egr-1 target gene expression. Conceptually it is possible that modulation of gene expression by estrogen or estrogen-like substances through the pathway we describe here might have a therapeutic potential in the treatment of myocardial disease.

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Tertia de Jager, Theo Pelzer, Stephan Müller-Botz, Asiya Imam, Jenny Muck and Ludwig Neyses

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