Biochemical and Biophysical Research Communications **268,** 192–200 (2000)
doi:10.1006/bbrc.2000.2073, available online at http://www.idealibrary.com on IDEAL®

17β-Estradiol Prevents Programmed Cell Death in Cardiac Myocytes

Theo Pelzer,*[,1] Michael Schumann,*[,1] Manfred Neumann,† Tertia deJager,* Michael Stimpel,* E. Serfling,† and Ludwig Neyses*[,2]
**Department of Medicine and †Department of Pathology, University of Würzburg, D-97080 Würzburg, Germany*

Received November 28, 1999

The cardioprotective effects of estrogens are clearly established. However, the underlying mechanisms are poorly understood. Because programmed cell death (apoptosis) probably contributes to the loss of cardiac myocytes in heart failure and because estrogens prevent apoptosis in breast cancer cells, we investigated whether the loss of cardiac myocytes by programmed cell death could be prevented by physiological doses of 17β-estradiol. Apoptosis of cultured cardiac myocytes was induced by staurosporine. 17β-estradiol (10 nM) had an antiapoptotic effect as determined by morphological analysis, vital staining using the Hoechst dye 33342 and terminal transferase dUTP nick-end labeling (TUNEL). As a potential mechanism for the antiapoptotic effect of 17β-estradiol we found a reduced activity of the ICE-like protease caspase-3 in hormone-treated myocytes. Furthermore, inhibition of apoptosis by estradiol was associated with a reduced activity of NF-κB transcription factors, particularly p65/RelA and p50. To our knowledge, these data provide the first indication that 17β-estradiol in physiological concentrations inhibits apoptosis in cardiac myocytes. The antiapoptotic effect of estrogens might contribute to the known cardioprotective effect of estrogens and provides a starting point for the development of future treatment options.

Key Words: **apoptosis; estrogen; cardiac muscle; caspase-3; NF-κB.**

The protective role of estrogens in the cardiovascular system has been established in numerous clinical trials (1). The assumption, that female sex hormones are largely responsible for the virtual absence of cardiovascular disease in premenopausal women compared to males with otherwise identical risk factors, is based on two findings. First, cardioprotection is lost in parallel with declining estrogen serum levels after menopause (2). Second, hormone replacement therapy (HRT) efficiently prevents coronary artery disease in postmenopausal women (3). The occurrence of programmed cell death in the myocardium has been demonstrated recently in several different models of heart failure including ischemic heart disease as well as non-ischemic heart failure such as myocarditis or dilative cardiomyopathy (4). Because estrogens inhibit apoptosis in non-myocyte cell types (5) and because we have recently shown that estrogens exert direct effects on cardiac myocytes, (6) we have here tested the hypothesis that estrogens are able to inhibit apoptosis in cardiac myocytes. The assessment of apoptotic myocyte death based on a single technique alone is known to be difficult. In this study, care was taken to analyze apoptotic myocyte death by three independent methods including morphological analysis, Hoechst dye 33342 staining and TUNEL assay. Inhibition of caspase-3 activity and apoptosis related NF-κB activation was identified as a potential mechanism for the anti-apoptotic action of estrogens in cardiac myocytes treated with staurosporine.

METHODS

Cell culture. Neonatal rat ventricular myocytes were cultured from 2-day-old Wistar rats as previously described (7). At day 1 medium was changed to MEM/5 ± 17β-estradiol (10 nM, Sigma). Beginning with day 3, myocytes were treated with staurosporine (0.1 μM, Sigma).

DNA laddering. Myocytes were grown on 10-cm culture plates under estrogen-free conditions for 72 h ± 0.1 μM staurosporine and washed twice with PBS before scraping off the dishes. Genomic DNA was extracted using a commercial kit (DNA Extraction Kit, Stratagene), separated on 1.8% agarose gels stained with ethidium bromide and examined under UV light (8).

Vital staining with Hoechst dye 33342. The Hoechst dye 33342 was used to assess nuclear morphology by fluorescence microscopy under ultraviolet light (9). The Hoechst dye was added to cells growing on glass slides during the last hour of the experiment in a final concentration of 10 μg/ml. Cells were washed twice with PBS,

[1] T.P. and M.S. contributed equally to this work.
[2] To whom correspondence should be addressed at Department of Medicine, Bau 4, University of Würzburg, Josef-Schneider Strasse 2, D-97080 Würzburg, Germany. Fax: +49-931-201-3151. E-mail: L.Neyses@medizin.uni-wuerzburg.de.

0006-291X/00 $35.00

fixed with 1% paraformaldehyde, mounted with Mowiol and coverslipped.

In situ TUNEL assay. *In situ* TUNEL assays (10) were performed using a kit obtained from Boehringer Mannheim (In Situ Cell Death Detection Kit) according to the manufacturer's instructions. Cardiac myocytes were treated with 10 nM 17β-estradiol and 0.1 μM staurosporine for 72 h, washed with PBS fixed with paraformaldehyde (4%, 30 min, RT). Endogenous peroxidase was blocked with 0.3% H_2O_2/MeOH (5 min, RT) and cells treated with 0.5% Triton X-100 (10 min, 4°C). Cells were blocked and washed in PBS before incubation with td-transferase and nucleotide mixture labeled with fluorescein (1 h, 37°C). After washing, an anti-fluorescein antibody horseradish peroxidase conjugate was added (30 min, 37°C) followed by addition of the colorigenic substrate 3,3′-diaminobenzidine (DAB). The cells were counterstained with methyl-green (Sigma), mounted with Mowiol and coverslipped. 12,000 myocytes were evaluated by conventional light microscopy and brown versus blue nuclei were counted.

Caspase-3 activity assay. Activity of the ICE-like protease Caspase-3 was determined using a commercial kit (Apoalert assay, Clontech) according to the manufacturer's instruction with minor modifications for adherent cell cultures (11). Briefly, cultured cardiac myocytes washed and scraped off the dishes before centrifugation (1700 rpm, 6 min, 4°C). The resulting pellet was resuspended in lysis buffer (10 min, 4°C), the homogenate was centrifuged (12000 rpm, 3 min, 4°C) and substrate for caspase-3 (Ac-DEVD-AMC) was added to the final supernatant (1 h, 37°C). Levels of released 7-amino-4-methylcoumarin were measured with a luminescence spectrometer LS 50B (Perkin-Elmer) at an excitation wavelength of 390 nm and emission wavelength of 480 nm.

Immunoblotting. Cardiac myocyte cultures were rinsed in 1 × PBS, scraped off the tissue culture plates, solubilized in lysis buffer containing 50 mmol/L Tris (pH 7.40), 120 mmol/L NaCl, 5 mmol/L EDTA (pH 8.0), 1 mmol/L PMSF (Sigma) and 1% Triton X-100 (4°C). The homogenate was centrifuged (12,000 rpm, 5 min, 4°C). Protein concentration in the supernatant was determined by Bradford assay and equal amounts of protein were separated on 12% SDS-PAGE gels. The resolved proteins were transferred on PVDF membranes by electroblotting and filters and blocked in BLOTTO (1 × PBS, 5% non-fat milk powder, 1% Tween 20), washed and incubated with anti-Bcl-2 or anti-troponin-T antibodies overnight (4°C). Blots were washed and immunoreactive proteins detected by enhanced chemiluminescence.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts of cardiac myocytes were prepared as described elsewhere (12). For each EMSA 5 μg of nuclear extract were incubated with 4000 to 8000 cpm (equivalent to 0.25 ng) of a ^{32}P-labeled NF-κB oligonucleotide probe derived from the κB enhancer element of the IL-2 promoter: 3′-GACCAAGAGGGATTTCACCCCTAAATC-5′ (13). The reaction mix contained 1 μg poly-dI/dC per 4 μg nuclear protein as nonspecific competitor. Samples were incubated (30 min, 4°C) and fractionated on a nondenaturing 6% PAA gel at 200V/15 cm. Gels were dried before autoradiography and/or phosphoimager analysis. In supershift EMSA, 1 μl of each NF-κB antibody was added and the incubation before electrophoresis was prolonged (1 h, 4°C).

RESULTS

Effect of Staurosporine on Nucleosome Fragmentation in Cardiac Myocytes

Apoptosis, in contrast to nonapoptotic cell death, leads to the appearance of a characteristic laddering pattern of genomic DNA subjected to gel electrophoresis, which results from internucleosomal cleavage of genomic DNA (14). Figure 1 indicates that genomic DNA from cardiac myocytes treated with 0.1 μM staurosporine for 24 h but not DNA from control myocytes kept in medium alone revealed the oligonuclesomal DNA pattern typical of apoptotic cell death.

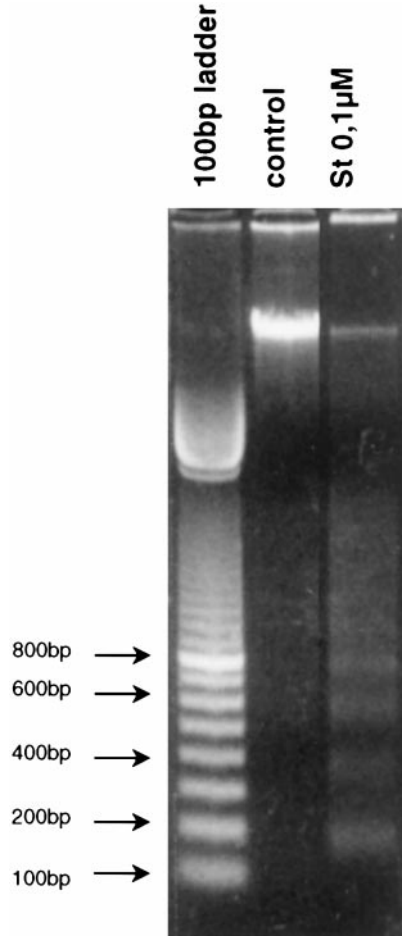

FIG. 1. Oligo-nucleosomal cleavage of genomic DNA from staurosporine-treated cardiac myocytes. Neonatal cardiac myocytes were cultured in either medium alone (control) or medium plus 0.1 μM staurosporine (St 0.1 μM) for 24 h. Only genomic DNA from staurosporine treated cardiac myocytes revealed the typical ladder-like pattern with oligo- and multimeric bands approximately 200 bp in size when stained with ethidium bromide. In parallel, the amount of high-molecular-weight genomic DNA decreased with staurosporine treatment.

Phase Contrast Microscopy and Hoechst Dye Staining of Apoptotic Cardiac Myocytes

Cell shrinkage, another indicative of apoptosis, (15) occurred in cardiac myocyte cultures treated with staurosporine but not in untreated control myocytes as assessed by conventional phase contrast microscopy (Figs. 2E and 2A, respectively). 17β-Estradiol prevented myocyte shrinkage as well as cytosol condensation in staurosporine treated myocytes (compare Figs.

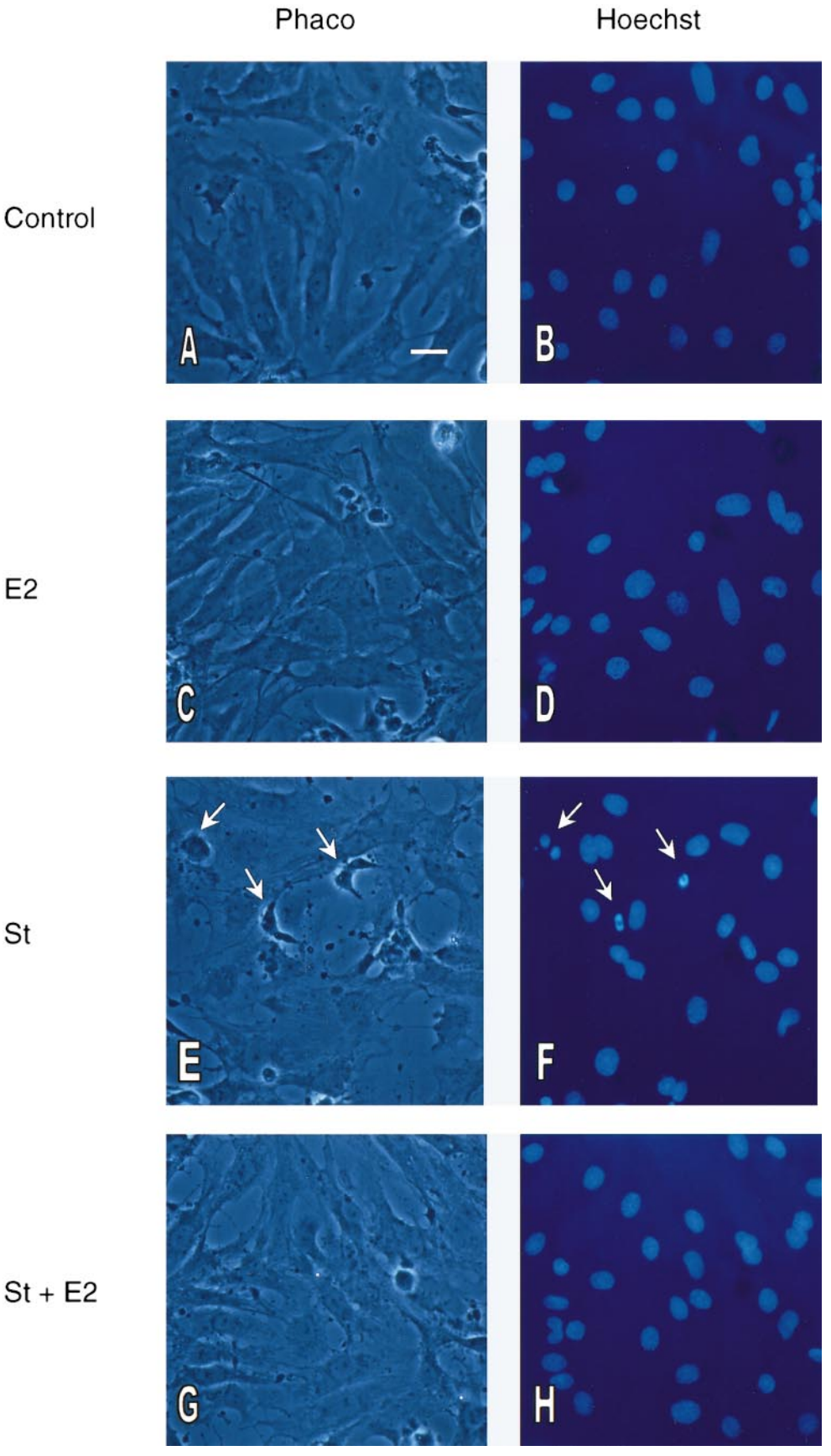

FIG. 2. 17β-Estradiol prevents nuclear fragmentation and formation of apoptotic bodies in staurosporine-treated cardiac myocytes. The morphology of cardiac myocytes treated with medium alone (Control) or either 10 nM 17β-estradiol (E2), staurosporine (St), or staurosporine plus estradiol (St + E2) was analyzed by phase-contrast microscopy (Phaco). Apoptotic cardiac myocytes (indicated by arrows) were observed frequently in staurosporine-treated myocytes but were virtually absent with the addition of 10 nM 17β-estradiol to staurosporine-treated myocytes. Nuclear fragmentation and condensation, as assessed by Hoechst dye 33342 staining (Hoechst) was also prevented in staurosporine-treated cardiac myocytes by adding 17β-estradiol. The identity of cardiac myocytes was verified by immunofluorescence using a troponin-T-specific antibody (not shown). Size bar is 25 μM.

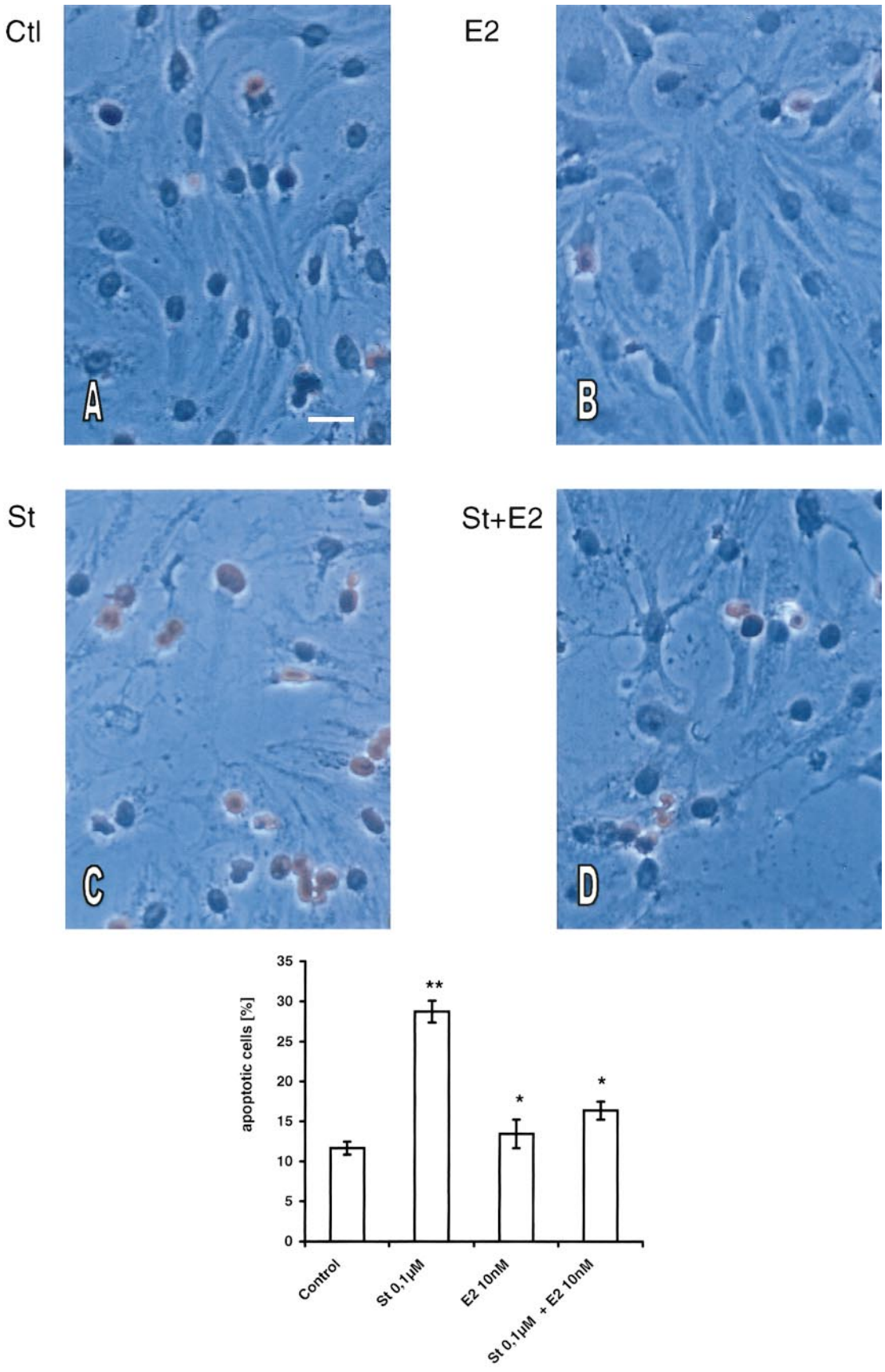

FIG. 3. *In situ* TUNEL assay. Upper panel: *In situ* TUNEL assay of neonatal cardiac myocytes treated with 0.1 μM staurosporine (St, C) revealed increased nuclear deposition of DAB (brown dye) compared to untreated control (A). Coincubation of cardiac myocytes in staurosporine plus 10 nM 17β-estradiol (St + E2) significantly reduced the number of TUNEL-positive nuclei (D). Myocytes treated with 10 nM estradiol (E2) alone showed no significant difference in TUNEL staining compared to control (C). Size bar is 25 μM. Lower panel: Staurosporine increased the apoptotic index by 247% over control. Coincubation with estradiol (St + E2) lowered the apoptotic index of staurosporine-treated cardiac myocytes (St) by 106.8% relative to control. Estradiol alone (E2) had no effect on the percentage of TUNEL-positive myocytes. Bars indicate percentage of TUNEL-positive cardiac myocytes ± SEM from 8 independent experiments, statistical significance was calculated by two-sided Student's *t*-test ($P < 0.1$; *$P < 0.05$; **$P < 0.01$).

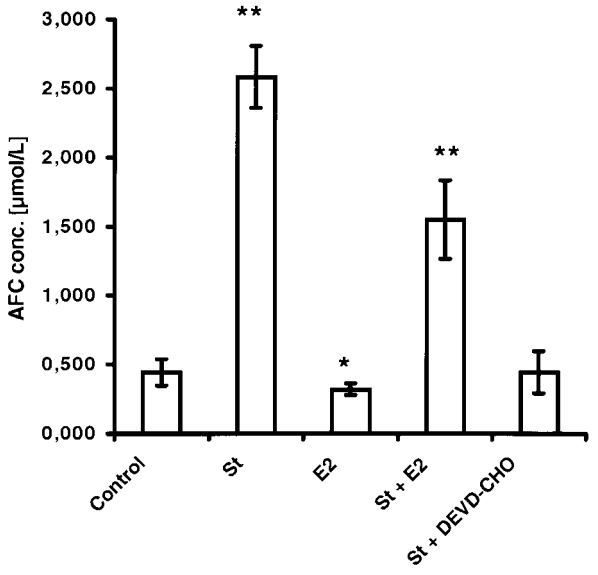

FIG. 4. Caspase-3 activity in cardiac myocytes is inhibited by 17β-estradiol. Caspase-3 activity was measured in lysates prepared from cardiac myocytes treated with medium alone (control), 0.1 μM staurosporine (St), 10 nM 17β-estradiol (E2), 0.1 μM staurosporine plus 10 nM 17β-estradiol (St + E2), or 0.1 μM staurosporine plus the caspase-3-specific inhibitor peptide DEVD-CHO (amino acid short code) for 6 h. Bars indicate absolute AFC concentration ± SEM from 10 independent experiments, each in triplicate measurements. Statistical significance was calculated by two-sided Student's *t*-test ($P < 0.1$; $^*P < 0.05$; $^{**}P < 0.01$).

2E and 2G). Highly condensed myocytes resembling apoptotic bodies appeared exclusively in myocyte not treated with estradiol (see arrows in Fig. 2E). 17β-Estradiol prevented staurosporine induced fragmentation of cardiac myocyte nuclei, another hallmark of apoptosis, (16) as visualized by Hoechst dye 33324 staining (compare Figs. 2F and 2H). Apoptotic nuclei revealed a bright fluorescence signal with appearance of multiple fragments in a single nucleus (Fig. 2F). Estradiol alone had no effect on nuclear fragmentation (Figs. 2C and 2D).

In Situ TUNEL Staining of Apoptotic Cardiac Myocytes

Staurosporine treatment of cardiac myocytes (Fig. 3C) resulted in a larger number of TUNEL-positive cardiac myocyte nuclei compared to control as shown in the upper panel of Fig. 3 (Fig. 3A). After addition of 17β-estradiol to staurosporine-treated myocytes we observed a reduction in TUNEL-positive nuclei (Fig. 3D). Estrogen treatment alone did not have an effect on the number of TUNEL-positive cardiac myocytes (Fig. 3B). Dye deposits were detected only in the nucleus; omission of either enzyme, dUTP, antibody–horseradish peroxidase conjugate or substrate revealed no staining, ruling out unspecific TUNEL staining. The absolute and the relative number of apoptotic myocytes was determined by counting TUNEL-positive myocytes out of a total of 12,000 myocytes in eight independent experiments (Fig. 3, lower panel). The relative number of apoptotic nuclei increased by 247% over untreated control with the addition of 0.1 μM staurosporine ($n = 10$, $P < 0.01$). Addition of 17β-estradiol to staurosporine-treated myocytes reduced the number of apoptotic nuclei by 106.8% relative to control ($n = 8$, $P < 0.05$). Estradiol alone had no effect on the apoptosis index.

Caspase-3 Assay

Caspase-3 activity in cardiac myocytes was induced by 0.1 μM staurosporine in a time- and dose-dependent fashion with a maximum of 586% over control (Fig. 4) at 6 h. Caspase-3 activity relative to control was 234% lower in cardiac myocytes treated with staurosporine plus 17β-estradiol than with staurosporine treatment alone as illustrated in Fig. 4. Estrogen-dependent reduction of caspase-3 activity was statistically significant ($n = 10$, $P < 0.01$). Of note, the observed magnitude of caspase activity reduction approximates the reduction in TUNEL-positive myocytes described above. Estradiol treatment alone did not alter caspase-3 activity significantly.

Activity of NF-κB Factors in Staurosporine- and Estradiol-Treated Cardiac Myocytes

In an attempt to unravel the mechanisms underlying the antiapoptotic effect of estrogen in cardiac myocytes, the expression of the pivotal antiapoptotic factor bcl-2 (17) as shown in Fig. 5. However, bcl-2 expression was not affected by either staurosporine or estrogen treatment. Next, we investigated the activity of NF-κB factors, (18) which become activated during apoptosis in non-myocyte cell lines (19). Nuclear extracts were

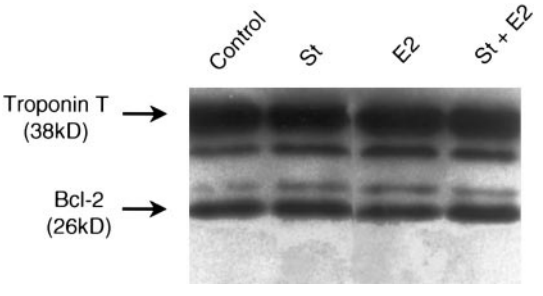

FIG. 5. Expression of Bcl-2 protein remains unchanged in cardiac myocytes treated with 17β-estradiol. The expression of Bcl-2 was analyzed by Western blotting of extracts from cardiac myocytes treated with medium alone (control), 0.1 μM staurosporine (St), 10 nM 17β-estradiol (E2), or 0.1 μM staurosporine plus 10 nM 17β-estradiol (St + E2). Equal loading between lanes was demonstrated by codetection of cardiac troponin T as an internal standard. No significant differences in Bcl-2 protein expression were detected.

Lane #	1	2	3	4	5		6	7	8	9	10	11	12
E_2 10nM	-	-	+	+	-		-	-	-	-	-	-	-
St 0,1μM	-	+	-	+	-		+	+	+	+	+	+	+
probe	+	+	+	+	+		+	+	+	+	+	+	+
myocyte extract	+	+	+	+	-		+	+	+	+	+	+	+
Anti-p65	-	-	-	-	-		-	+	-	-	-	-	-
Anti-c-Rel	-	-	-	-	-		-	-	+	-	-	-	-
Anti-RelB	-	-	-	-	-		-	-	-	+	-	-	-
Anti-p50	-	-	-	-	-		-	-	-	-	+	-	-
Anti-p52	-	-	-	-	-		-	-	-	-	-	+	-
NRS	-	-	-	-	-		-	-	-	-	-	-	+

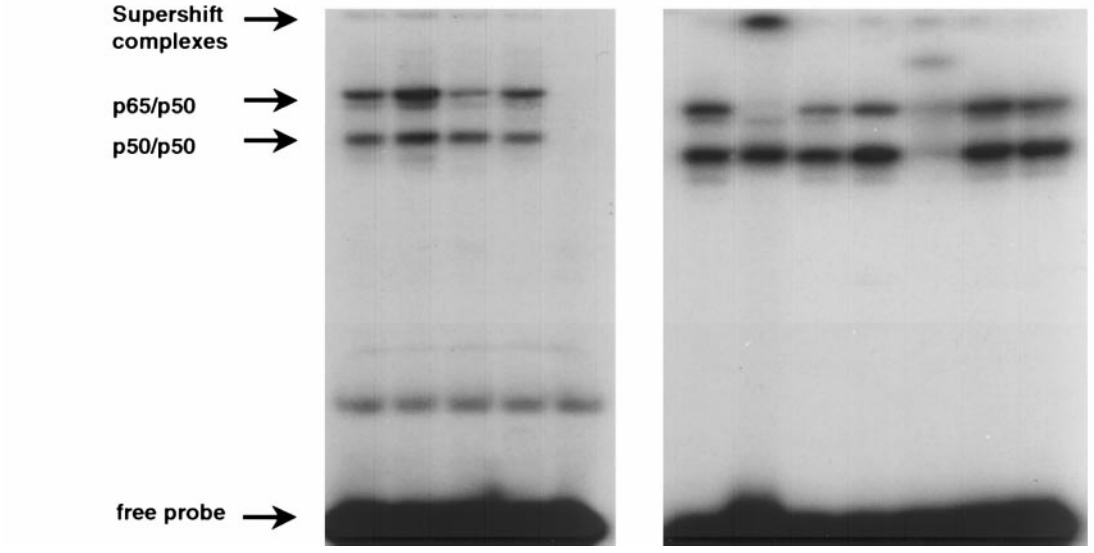

FIG. 6. The antiapoptotic effect of 17β-estradiol in cardiac myocytes is associated with a reduction in NF-κB gel shift activity. Nuclear extracts from cardiac myocytes treated with medium alone (lane 1), 0.1 μM staurosporine (lane 2), 10 nM 17β-estradiol (lane 3), or 0.1 μM staurosporine plus 10 nM 17β-estradiol (lane 4) for 24 h were subjected to electrophoretic mobility shift assay using a NF-κB probe. The band shift pattern of probe alone without myocyte extract is shown in lane 5. The composition of shifted NF-κB complexes was determined by supershift experiments using specific antibodies against NF-κB proteins as shown in lanes 6 to 12. The upper band represents p65/RelA-p50 heterodimers, the lower band p50 homodimers. All shifted NF-κB complexes could be titrated out using a 50-fold excess of unlabeled oligonucleotide (not shown).

prepared from cardiac myocytes treated with staurosporine or staurosporine plus 17β-estradiol and subjected to gel mobility shift analysis as illustrated in Fig. 6. When equal amounts of extract were loaded and shifted with an NF-κB oligonucleotide, 2 specific bands representing p65/p50 heterodimers or p50 homodimers were observed. With addition of staurosporine (lane 2) the intensity of both protein/DNA complexes was strongly increased compared to untreated control extracts (lane 1). However when estradiol was added to staurosporine treated myocytes, both bands became significantly less intense (compare lane 2 and lane 4). Quantification of band intensities by phosphoimager indicated a 6-fold reduction for the p65/p50 complex and a 5-fold reduction in band intensity for the p50 homodimer complex in extracts from cardiac myocytes treated with staurosporine plus estradiol versus cells treated only with staurosporine. Interestingly, myocytes treated with estradiol alone (lane 3) showed a reduction NF-κB gel shift activity when compared to untreated cells (lane 1). The identity of NF-κB was determined by a series of supershift experiments (lanes 6–12). A supershift was observed with an antibody against p65/RelA (lane 7). A second NF-κB factor involved appears to be p50 (lane 10) because the p50 antibody decreases the intensity of both bands in lane 10. This finding is a well known phenomenon, probably because the p50 antibody alters the tertiary structure of p50 protein, contained in the upper (p65/p50 heterodimers) *and* lower (p50 homodimers) band and thereby decreases the DNA affinity of both gel shift complexes. Therefore, the prevention of apoptosis in cardiac myocytes was associated with the inhibition of NF-κB (p65-Rel-A/p50) activity.

DISCUSSION

The results of this study indicate for the first time that apoptosis in cardiac myocytes can be prevented by physiological levels of 17β-estradiol. The mechanism likely involves inhibition of the apoptosis related activation of NF-κB, particularly its p65/RelA and p50 subunits.

The development of heart failure is characterized by progressive worsening of LV function. The concept of programmed cell death (apoptosis) of cardiac myocytes is currently discussed as a mechanism that reduces the contractile cell mass of the heart and thereby leads to impaired LV performance. As reviewed recently, (4) apoptotic loss of cardiac myocytes does occur in ischemic cardiomyopathy, myocarditis, dilative cardiomyopathy as well as acute myocardial infarction. The inhibition of apoptosis in cardiac myocytes might conserve the number of contractile myocytes and thus delay or even prevent LV dysfunction (and hence the development of heart failure). The results of this study suggest that estrogens, which are already in clinical use for a variety of disorders, may offer a practicable approach to the reduction of apoptosis cardiac myocytes may therefore merit further investigation.

Because the biological effects of estrogens generally require the presence of the estrogen receptor, a ligand dependent transcription factor that regulates the activity of hormone responsive target genes, (20, 21) we have previously demonstrated the presence and functionality of the estrogen receptor-α and -β in cardiac myocytes (6). Interestingly, estrogens are known to prevent apoptosis in hormone receptor positive breast cancers (5, 22). Furthermore, estrogens are able to protect against contractile dysfunction irrespective of coronary blood flow by mechanism which are currently not understood (23). We have therefore investigated, whether the same antiapoptotic effect, which promotes tumor growth, might be beneficial in the myocardium by preventing the apoptotic loss of cardiac myocytes.

At first, the results of our study show that staurosporine caused apoptotic rather than non-apoptotic cardiac myocyte death. This finding is consistent with previous studies, in which staurosporine acts as a efficient inducer of apoptosis in cardiac myocytes (24). Moreover, if estradiol treatment would inhibit a very potent pro-apoptotic agent such as staurosporine, this would potentially indicate a therapeutic potential of the hormone in a relatively large array of clinical conditions. Other pro-apoptotic stimuli (i.e., TNFα) were also tested but led to less efficient induction of apoptosis. This finding is consistent with other studies in which TNFα was a more potent pro-apoptotic factor in adult than in neonatal cardiac myocytes (25).

Since a single assay for apoptosis may be difficult to interprete, care was taken in the present study to assess myocyte death by four independent assays. First, apoptotic shrinkage of cardiac myocytes was encountered less frequently in cardiac myocytes treated with estradiol plus staurosporine than in cells treated with staurosporine alone. Second, we observed a significant reduction of condensation and fragmentation of myocyte nuclei with the addition of estrogen to staurosporine treated cardiac myocytes. Third, a statistically significant reduction of TUNEL positive cardiac myocytes was observed when estradiol was added to staurosporine treated cells. Apoptosis is mediated by the activation of caspases, a cascade of proteases that cleave a variety of pivotal cellular proteins and ultimately leads to degradation of cellular DNA (4). We determined whether the antiapoptotic effect of estradiol is associated with inhibition of caspase activity in staurosporine treated cardiac myocytes by measuring caspase-3 activity, which is known to be activated by staurosporine in cardiac myocytes (24). As shown in Fig. 4, physiological concentrations of 17β-estradiol greatly reduced the staurosporine dependent activation of caspase-3 in cultured cardiac myocytes. This result is consistent with the data obtained by morphological analysis, Hoechst dye 33342 and TUNEL staining (Figs. 2 and 3). Specificity of the assay for caspase-3 was demonstrated by addition of a synthetic peptide which selectively inhibited caspase-3 activity in our cardiac myocyte extracts. The inhibition of caspase-3 activity by estrogens is likely to be a phenomenon with relevance under *in vivo* conditions, since caspase-3 becomes activated also *in vivo* during myocardial infarction in cardiac myocytes undergoing apoptotic cell death (26). Furthermore, inhibition of caspases by a synthetic peptide in a rat model of acute myocardial infarction attenuated ischemia/reperfusion injury of the myocardium (27).

An important factor when studying estrogen effects is the employed hormone level. All data presented in this study were therefore obtained using 17β-estradiol (E2) only in strictly physiological concentrations, which is between 1 nM and 10 nM final concentration. Thus, the observed antiapoptotic estrogen effect on cardiac myocytes are not due to supraphysiological hormone levels.

To determine the molecular mechanism of the reduction of apoptotic cell death by estrogens we first analyzed the expression of the antiapoptotic factor Bcl-2. Bcl-2 has been shown to protect cardiac myocytes from apoptosis induced by p53 overexpression (17). Interestingly, we found expression of Bcl-2 unchanged in cardiac myocytes treated with staurosporine or estradiol. We concluded, that bcl-2 is not involved in the antiapoptotic effect of estrogens in our apoptosis model. Next we focused on estradiol effects on the activity of NF-κB, since activation of NF-κB is associated with apoptotic cell death in a broad variety of cell types as reviewed recently (28). In our experiments, staurosporine consistently induced apoptotic cell death and simul-

taneously increased NF-κB activity in cardiac myocytes as determined by EMSA analysis. 17β-estradiol effectively prevented apoptotic cell death as well as activation of NF-κB in staurosporine treated myocytes. Functional evidence for NF-κB activation in myocardial injury comes from a previous study which revealed a protective effect of oligonucleotide decoy elements directed against NFκBs during myocardial reperfusion injury (29, 30). A role for NF-κB in the development of human heart failure is also suggested by the finding that NF-κB activity is up-regulated in end-stage heart failure (31). However the exact function of NF-κB in the myocardium has not yet been established. NF-κB proteins function as transcription factors which are in the inactive form retained in the cytosol by interaction with specific inhibitors of the IκB family. Activation of NF-κB occurs via degradation of IκB's, resulting in nuclear translocation and transcriptional activation of NF-κB regulated genes such as interleukin-2 and interleukin-6 (28). At present we do not know exactly by which pathway estrogens inhibit NFκBs in cardiac myocytes.

At present we favor the hypothesis, that 17β-estradiol reduces NF-κB activity by inhibiting the DNA binding of NF-κB heterodimers. Addition of estradiol will activate the estrogen receptors in cardiac myocytes, which translocate to the nucleus and may repress NFκB transcriptional activity by direct or indirect protein/protein interaction. This interpretation is consistent with the presence of estrogen receptors in cardiac myocytes (6) and with the interaction of estrogen receptors with the NF-κB factor p65, (32) which is also a major component of gel shift complexes observed in this study. Furthermore, the functional relevance of the interaction of the estrogen receptor with NF-κB has recently been shown in the regulation of the IL-6 promoter (33). A second explanation for estradiol/NF-κB interaction could be inhibition NF-κB activity by activation of IκBs, which would retain NF-κB within the cytosol (18). However it is currently not known whether IκB expression is regulated by estrogens. Third, inhibition of caspase-3 activity by estrogens as shown in the current study might prevent degradation of IκBs, which are a substrate for caspase-3 (34). In this model, p65/RelA and p50 would be retained in the cytosol by IκBs resulting in decreased apoptosis. At last, another possible mechanism could be the retention of NF-κB within the cytosol by protein–protein interaction with the estrogen receptor. This hypothesis is consistent with the finding of direct interaction between estrogen receptors and NFκBs (32).

Because the present study was aimed at the identification of the cellular mechanism(s) by which estrogens protect the myocardium, further investigations are needed to address these mechanistic hypotheses on a subcellular/molecular level. Another intriguing route of investigation will be the screening of further estrogenic compounds, including substances with a tissue-specific activity profile like raloxifene.

REFERENCES

1. Grodstein, F., and Stampfer, M. J. (1995) The epidemiology of coronary heart disease and estrogen replacement in postmenopausal women. *Prog. Cardiovasc. Dis.* **38,** 199–210.
2. Gordon, T., Kannel, W. B., Hjortland, M. C., and McNamara, P. M. (1978) Menopause and coronary heart disease: The Framingham study. *Ann. Intern. Med.* **89,** 157–161.
3. Stampfer, M. J., Colditz, G. A., Willett, W. C., Manson, J. E., Rosner, B., Speizer, F. E., and Hennekens, C. H. (1991) Postmenopausal estrogen therapy and cardiovascular disease. *N. Engl. J. Med.* **325,** 756–762.
4. Haunstetter, A., and Izumo, S. (1998) Apoptosis, basic mechanisms and implications for cardiovascular disease. *Circ. Res.* **82,** 1111–1129.
5. Wang, T. T., and Phang, J. M. (1995) Effects of estrogen on apoptotic pathways in human breast cancer cell line MCF-7. *Cancer Res.* **55,** 2487–2489.
6. Grohe´, C., Kahlert, S., Löbbert, K., Stimpel, M., Karas, H., Vetter, H., and Neyses, L. (1997) Cardiac myocytes contain functional estrogen receptors. *FEBS Lett.* **416,** 107–112.
7. Simpson, P., and Savion, S. (1982) Differentiation of rat myocytes in single cell cultures with and without proliferating nonmyocardial cells. Cross-striations, ultrastructure, and chronotropic response to isoproterenol. *Circ. Res.* **50,** 101–116.
8. Tieger, E., Dam, T., Richjard, L., Wisnewsky, C., Tea, B., Gaboury, L., Tremblay, J., Schwartz, K., and Hamet, P. (1996) Apoptosis in pressure overload-induced heart hypertrophy in the rat. *J. Clin. Invest.* **97,** 2891–2897.
9. Lizard, G., Deckert, V., Dubrez, L., Moisant, M., Gambert, P., and Lagrost, L. (1996) Induction of apoptosis in endothelial cells treated with cholesterol oxides. *Am. J. Pathol.* **148,** 1625–1638.
10. Gavrieli, Y., Sherman, Y., and Ben-Sasson, S. A. (1992) Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* **119,** 493–501.
11. Wyllie, A. H. (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* **284,** 555–556.
12. Lassar, A. B., Buskin, J. N., Lockshon, D., David, R. L., Apone, S., Hauschka, S. D., and Weintraub, H. (1989) MyoD is a sequence specific DNA binding protein requiring a myc homology to bind to the muscle creatine kinase enhancer. *Cell* **58,** 823–831.
13. Briegel, K., Hentsch, B., Pfeuffer, I., and Serfling, E. (1991) One base pair change abolishes the T cell-restricted activity of a κB-like proto-enhancer element from the interleukin 2 promoter. *Nucleic Acids Res.* **19,** 5929–5936.
14. Brown, D. G., Sun, X. M., and Cohen, G. M. (1993) Dexamethasone-induced apoptosis involves cleavage of DNA to large fragments prior to internucleosomal fragmentation. *J. Biol. Chem.* **268,** 3037–3039.
15. Elsasser, A., Schlepper, M., Klovekorn, W. B., Cai, W. J., Zimmermann, R., Muller, K. D., Strasser, R., Kostin, S., Gagel, C., Munkel, B., Schaper, W., and Schaper, J. (1997) Hibernating myocardium: An incomplete adaptation to ischemia. *Circulation* **96,** 2920–2931.
16. Majno, G., and Joris, I. (1995) Apoptosis, oncosis and necrosis: On overview of cell death. *Am. J. Pathol.* **146,** 3–15.
17. Kirshenbaum, L. A., and de Moissac, D. (1997) The bcl-2 gene product prevents programmed cell death of ventricular myocytes. *Circulation* **96,** 1580–1585.

18. Baeuerle, P., and Baltimore, D. (1996) NF-κB: Ten years after. *Cell* **87,** 13–20.
19. Grimm, S., Bauer, M., Baeuerle, P. A., and Schulze-Osthoff, K. (1996) Bcl-2 down-regulates the activity of transcription factor NF-κB induced upon apoptosis. *J. Cell Biol.* **134,** 13–23.
20. Walter, P., Green, S., Krust, A., Bornert, J. M., Jeltsch, J. M., Staub, A., Jensen, E., Scrace, G., Waterfield, M., and Chambon, P. (1985) Cloning of the human estrogen receptor cDNA. *Proc. Natl. Acad. Sci. USA* **82,** 7889–7893.
21. Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S., and Gustafsson, J. A. (1996) Cloning of a novel estrogen receptor expressed in the rat prostate and ovary. *Proc. Natl. Acad. Sci. USA* **93,** 5925–5930.
22. Wilson, J. W., Wakeling, A. E., Morris, I. D., Hickman, J. A., and Dive, C. (1995) MCF-7 human mammary adenocarcinoma cell death *in vitro* in response to hormone-withdrawal and DNA damage. *Int. J. Cancer* **61,** 502–508.
23. Kolodgie, F. D., Farb, A., Litovsky, S. H., Narula, J., Jeffers, L. A., Lee, S. J., and Virmani, R. (1997) Myocardial protection of contractile function after global ischemia by physiologic estrogen replacement in the ovarectomized rat. *J. Mol. Cell Cardiol.* **29,** 2403–2414.
24. Yue, T. L., Wang, C., Romanic, A. M., Kikly, K., Keller, P., DeWolf, W. E., Hart, T. K., Thomas, H. C., Storer, B., Gu, J. L., Wang, X., and Feuerstein, G. Z. (1998) Staurosporine-induced apoptosis in cardiomyocytes: A potential role of caspase-3. *Mol. Cell Cardiol.* **30,** 495–507.
25. Krown, K. A., Page, M. T., Nguyen, C., Zechner, D., Gutierrez, V., Comstock, K. L., Glembotski, C. C., Quintana, P. J., and Sabbadini, R. A. (1996) Tumor necrosis factor alpha-induced apoptosis in cardiac myocytes. Involvement of the sphingolipid signaling cascade in cardiac cell death. *J. Clin. Invest.* **98,** 2854–2865.
26. Black, S. C., Huang, J. Q., Rezaiefar, P., Radinovic, S., Eberhart, A., Nicholson, D. W., and Rodger, I. W. (1998) Co-localization of the cysteine protease caspase-3 with apoptotic myocytes after *in vivo. J. Mol. Cell Cardiol.* **4,** 733–742.
27. Yaoita, H., Ogawa, K., Maehara, K., and Maruyama, Y. (1998) Attenuation of ischemia/reperfusion injury in rats by a caspase inhibitor. *Circulation* **97,** 276–281.
28. May, M., and Ghosh, S. (1998) Signal transduction through NF-κB. *Immunol. Today* **19,** 80–88.
29. Sawa, Y., Morishita, R., Suzuki, K., Kagisaki, K., Kaneda, Y., Maeda, K., Kadoba, K., and Matsuda, H. (1997) A novel strategy for myocardial protection using *in vivo* transfection of *cis* element "decoy" against NFkappaB binding site: Evidence for a role of NFkappaB in ischemia–reperfusion injury. *Circulation* **96**(Suppl. 9), II–II2804.
30. Morishita, R., Sugimoto, T., Aoki, M., Kida, I., Tomita, N., Moriguchi, A., Maeda, K., Sawa, Y., Kaneda, Y., Higaki, J., and Ogihara, T. (1997) *In vivo* transfection of *cis* element "decoy" against nuclear factor-κB binding site prevents myocardial infarction. *Nature Med.* **8,** 894–899.
31. Wong, S. C., Fukuchi, M., Melnyk, P., Rodger, I., and Giaid, A. (1998) Induction of cyclooxygenase-2 and activation of nuclear factor-kappaB in myocardium of patients with congestive heart failure. *Circulation* **98,** 100–103.
32. McKay, L. I., and Cidlowski, J. A. (1998) Cross-talk between nuclear factor-κB and the steroid hormone receptors: Mechanism of mutual antagonism. *Mol. Endo.* **12,** 45–56.
33. Ray, P., Ghosh, S., Zhang, D. H., and Ray, A. (1997) Repression of interleukin-6 gene expression by 17-β estradiol: Inhibition of the DNA-binding activity of the transcription factors NF-IL6 and NF-κB by the estrogen receptor. *FEBS Lett.* **409,** 79–85.
34. Barkett, M., Xue, D., Horvitz, H. R., and Gilmore, T. D. (1997) Phosphorylation of IkappaB-alpha inhibits its cleavage by caspase CPP32 *in vitro. J. Biol. Chem.* **272,** 29419–29422.