Suppression of Nuclear Factor-κB by Glucocorticoid Receptor Blocks Estrogen-Induced Apoptosis in Estrogen-Deprived Breast Cancer Cells

Ping Fan1, Doris R. Siwak2, Balkees Abderrahman1, Fadeke A. Agboke3, Smitha Yerrum1, and V. Craig Jordan1

Abstract

Our clinically relevant finding is that glucocorticoids block estrogen (E2)-induced apoptosis in long-term E2-deprived (LTED) breast cancer cells. However, the mechanism remains unclear. Here, we demonstrated that E2 widely activated adipose inflammatory factors such as fatty acid desaturase 1 (FADS1), IL6, and TNFα in LTED breast cancer cells. Activation of glucocorticoid receptor (GR) by the synthetic glucocorticoid dexamethasone upregulated FADS1 and IL6, but downregulated TNFα expression. Furthermore, dexamethasone was synergistic or additive with E2 in upregulating FADS1 and IL6 expression, whereas it selectively and constantly suppressed TNFα expression induced by E2 in LTED breast cancer cells. Regarding regulation of endoplasmic reticulum stress, dexamethasone effectively blocked activation of protein kinase RNA-like endoplasmic reticulum kinase (PERK) by E2, but it had no inhibitory effects on inositol-requiring protein 1 alpha (IRE1α) expression increased by E2. Consistently, results from reverse-phase protein array (RPPA) analysis demonstrated that dexamethasone could not reverse IRE1α-mediated degradation of PI3K/Akt-associated signal pathways activated by E2. Unexpectedly, activated GR preferentially repressed nuclear factor-κB (NF-κB) DNA-binding activity and expression of NF-κB-dependent gene TNFα by E2, leading to the blockade of E2-induced apoptosis. Together, these data suggest that trans-suppression of NF-κB by GR in the nucleus is a fundamental mechanism thereby blocking E2-induced apoptosis in LTED breast cancer cells. This study provided an important rationale for restricting the clinical use of glucocorticoids, which will undermine the beneficial effects of E2-induced apoptosis in patients with aromatase inhibitor–resistant breast cancer.

Introduction

Antihormone therapy is a standard treatment of estrogen receptor (ER)-positive breast cancer (1). However, resistance to this therapy is an inevitable challenge in the clinic. Paradoxically, estrogen (E2) has the capacity to induce apoptosis in antihormone therapy–resistant breast cancer models in vitro and in vivo (2–5). In fact, E2-induced apoptosis has clinical significance for the treatment of aromatase inhibitor–resistant breast cancer (6) and reduction of breast cancer incidence in estrogen replacement therapy (ERT) for postmenopausal women (7). Further clinically relevant laboratory findings suggest that the anti-inflammatory agent, dexamethasone, and the synthetic progestin medroxyprogesterone acetate (MPA), which has glucocorticoid activity, can block E2-induced apoptosis in long-term E2-deprived (LTED) breast cancer cells (8). However, antiapoptotic mechanisms of glucocorticoids are unknown.

Long-term E2 deprivation is a selective pressure on breast cancer cell lines (9), as well as for patients during antihormone therapy (10), which results in stress responses for adaptation to the E2 deficiency (10, 11). In addition to elevation of ERα expression (4, 5), many signaling pathways, including metabolism, stress, and inflammatory responses, are modulated after E2 deprivation (10, 11). Notably, all of these alterations result in apoptosis in response to E2, instead of proliferation (4, 5). It is confirmed that nuclear ERα is an initial site for E2 to induce apoptosis in LTED breast cancer cells, which can be completely blocked by the tamoxifen (12). Our further observations demonstrate that accumulation of stress responses, including endoplasmic reticulum stress, oxidative stress, and inflammatory stress, is a major mechanism by which E2 induces apoptosis (12, 13). Particularly, endoplasmic reticulum is a critical regulatory site for conveying signals between the nucleus and cytoplasm to decide the cell fate (12, 14, 15). The endoplasmic reticulum stress sensor, protein kinase RNA-like endoplasmic reticulum kinase (PERK) is responsible for homeostasis of unfolded proteins and is also a key driver of E2-induced apoptosis (12, 14, 15). Specifically, PERK links endoplasmic reticulum stress with oxidative stress and increases transcription factor
NF-κB DNA-binding activity to induce TNFα in E2-deprived breast cancer cells (12, 15, 16). Our recent findings have demonstrated that the PERK/NF-κB/TNFα axis plays a critical role in E2-induced apoptosis (15, 16). In parallel, two other endoplasmic reticulum stress sensors, inositol-requiring protein 1 alpha (IRE1α) and ATF-6, mainly mediate endoplasmic reticulum-associated degradation of PI3K/Akt-associated signaling pathways (14). These different functions of endoplasmic reticulum stress sensors suggest that abnormal protein folding and lipid metabolism occur after treatment with E2.

Furthermore, stress responses widely activate inflammatory factors, such as IL6, FADS1, and TNFα, in LTED breast cancer cells after treatment with E2 (12, 13). Glucocorticoids have clinical implications with potent anti-inflammatory action and they control stress responses (17). Their binding receptor GR is a multitasking transcription factor that exerts its biological functions via trans-activation or trans-repression of various nuclear transcription factors depending on the cellular context (18, 19). In addition to interaction between ERα and GR (20), additional studies have demonstrated that GR trans-represses stress-responsive transcription factors, such as activator protein-1 (AP-1) and NF-κB (21, 22). Our results confirmed that NF-κB is a critical transcription factor for linking stress response and apoptosis in MCF-7:5C cells after treatment with E2, which is mediated by another transcription factor, STAT3 (15). Nevertheless, how GR interacts with these transcriptional factors to affect E2-induced apoptosis in LTED breast cancer cells remains unclear.

We sought here to further understand how glucocorticoids modulate E2-induced stress responses and affect E2-induced apoptosis via GR in two LTED breast cancer cells, MCF-7:5C and MCF-7:2A. These two cell lines have different levels of antioxidative systems: MCF-7:2A cells are more resistant than MCF-7:5C cells, which leads to a delayed and moderate apoptosis in MCF-7:2A cells after E2 treatment (11). In this study, our results demonstrate that E2 and dexamethasone have similar regulatory effects on activation of the adipose inflammatory factors FADS1 and IL6/IL6 receptor (IL6R). Furthermore, they are synergistic or additive in increasing FADS1 and IL6/IL6R expression in these cells after combination treatment. However, dexamethasone selectively suppresses induction of TNFα expression by E2 in LTED breast cancer cells. With respect to endoplasmic reticulum stress, dexamethasone selectively modulates two unfolded protein response (UPR) sensors activated by E2. This difference demonstrates that dexamethasone effectively blocks activation of PERK by E2 but has no inhibitory effects on the lipid metabolism–associated sensor IRE1α. Consistently, results from RPPA analysis demonstrate that dexamethasone cannot reverse degradation of PI3K/Akt–associated pathways mediated by IRE1α in LTED breast cancer cells. A mechanistic finding is that dexamethasone preferentially suppresses NF-κB DNA-binding activity, which prevents activation of NF-κB–dependent TNFα and ultimately results in complete blockade of E2-induced apoptosis. Together, these findings have important clinical implications for the conservative therapeutic application of glucocorticoids in treatment of advanced aromatase inhibitor–resistant breast cancer.

Materials and Methods

Materials

 Estradiol (E2) and dexamethasone were purchased from Sigma-Aldrich. The caspase-8 inhibitor Z-IETD-FMK was bought from BioVision. Recombinant human TNFα was obtained from Bioyox. For Western blotting, antibodies against total eIF2α, phosphorylated eIF2α, IRE1α, total Akt, phosphorylated Akt, total PDK1, phosphorylated PDK1, total STAT3, phosphorylated STAT3, NF-κB p65, NF-κB p105, cleaved PARP, and caspase-7 were obtained from Cell Signaling Technology. ERα (sc-544) and GR (sc-8992) were purchased from Santa Cruz Biotechnology.

Cell culture conditions and cell proliferation assays

Wild-type MCF-7 cells and E2-deprived MCF-7:5C and MCF-7:2A cells were cultured as described previously (12). The DNA fingerprinting pattern of these cell lines is consistent with that reported by the ATCC. All cell lines were validated according to their short tandem repeat (STR) profiles at The University of Texas MD Anderson Cancer Center Characterized Cell Line Core (CCLC). The STR patterns of all cell lines were consistent with those from the CCLC standard cells (Supplementary Table S1). The DNA content of the cells, a measure of proliferation, was determined as described previously (12) using a DNA fluorescence Quantitation kit (Bio-Rad Laboratories).

Annexin V binding assay to detect apoptosis

A FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen) was used to quantify apoptosis of MCF-7:5C and MCF-7:2A cells through flow cytometry according to the manufacturer’s instructions. In brief, MCF-7:5C and MCF-7:2A cells were seeded in 10-cm dishes. The next day, the cells were treated with different compounds for different periods. Cells were suspended in 1× binding buffer, and 1 × 10^5 cells were stained simultaneously with FITC-labeled Annexin V and propidium iodide (PI) for 15 minutes at room temperature. The cells were analyzed using a BD Accuri C6 plus flow cytometer.

NF-κB (p65) transcription factor DNA-binding assay

MCF-7:5C and MCF-7:2A cells were treated with a vehicle control (0.1% DMSO), E2 (1 nmol/L), dexamethasone (0.1 μmol/L), or a combination of them for different times. Nuclear protein was extracted from the cells according to the manufacturer’s instructions (Cayman Chemical). NF-κB (p65) DNA-binding activity in the cells was detected using an NF-κB (p65) Transcription Factor Assay Kit (Cayman Chemical).

RPPA

MCF-7:5C cells were seeded in 6-well plates. Twenty-four hours later, cells were treated with a vehicle control (0.1% DMSO), E2 (1 nmol/L), dexamethasone (0.1 μmol/L), MPA (1 μmol/L), or a combination of dexamethasone or MPA with E2 for different times. Cells were then harvested in lysis buffer (1% Triton X-100, 50 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 1.5 mmol/L MgCl2, 1 mmol/L EGTA, 100 mmol/L NaF, 10 mmol/L sodium pyrophosphate, 1 mmol/L Na3VO4, 10% glycerol containing freshly added protease and phosphatase inhibitors). Protein samples obtained from these cells were analyzed in the Functional Proteomics Reverse Phase Protein Array (RPPA) Core at UT MD Anderson Cancer Center (Houston, TX).

Immunoblotting

MCF-7:5C and MCF-7:2A cells were harvested in cell lysis buffer (Cell Signaling Technology) supplemented with Protease Inhibitor Cocktail Set I and Phosphatase Inhibitor Cocktail Set II.
Combination treatment with E2 and dexamethasone gradually inhibited induction of pS2 and c-Fos expression by E2 when the treatment time was prolonged (Fig. 1F and G; Supplementary Fig. S1B and S1C).

Remarkably, combination treatment synergistically increased the expression of GILZ after 24 hours (Supplementary Fig. S1E). In contrast, combination treatment with E2 and dexamethasone downregulated the expression of GILZ in wild-type MCF-7 cells despite that E2 and dexamethasone alone had similar regulatory tendency to that in LTED breast cancer cells (Supplementary Fig. S1F). Together, these results suggested that GR and ERα cross-talk regulates the expression of their respective target genes, depending on the context of cells.

Dexamethasone selectively blocks induction of TNFα expression after E2 treatment

E2 widely activates inflammatory genes, including TNFα, IL6, and FADS1, in LTED breast cancer cells (12, 13). To determine the anti-inflammatory effects of dexamethasone on LTED breast cancer cells after treatment with E2, we treated MCF-7:5C with E2, dexamethasone, or a combination of them for different times. Our results demonstrated that both E2 and dexamethasone suppressed TNFα mRNA expression after 24 hours of treatment. Dexamethasone was more potent than E2 in inhibiting TNFα expression (Fig. 2A). After 48 hours of treatment, E2 began to increase TNFα expression, whereas dexamethasone consistently inhibited it. Combination treatment with E2 and dexamethasone completely inhibited TNFα expression (Fig. 2B). With extension of the treatment time to 72 hours, induction of TNFα expression by E2 peaked. In contrast, dexamethasone remarkably inhibited TNFα expression and blocked E2-induced TNFα expression (Fig. 2C). We observed a similar pattern in the regulation of another TNF family member, lymphotixin beta (LTβ), by E2 and dexamethasone in MCF-7:5C cells (Fig. 2D; Supplementary Fig. S2A and S2B). MPA has glucocorticoid activity (8), which also significantly blocked the increased expression of TNFα induced by E2 (Fig. 2E). Different from induction of TNFα and LTβ expression, treatment with E2 quickly increased FADS1 and IL6R expression in MCF-7:5C cells after 24 hours of treatment (Supplementary Fig. S2C and S2D). Furthermore, E2 increased FADS1 expression much greater in MCF-7:5C cells than in wild-type MCF-7 cells (Fig. 2F), indicating that fatty acid metabolism was more active in LTED breast cancer cells after E2 treatment than in wild-type MCF-7 cells. Similar to E2, dexamethasone increased FADS1, IL6, and IL6R expression after 24 hours of treatment. E2 and dexamethasone were synergistic or additive in upregulating FADS1, IL6, and IL6R expression after combination treatment (Fig. 2G–I). As anticipated, the effect of MPA was weaker than that...
of dexamethasone in increasing FADS1 expression at the same concentration (Supplementary Fig. S2E), and MPA further upregulated FADS1 and IL6 expression after combination treatment with E2 (Supplementary Fig. S2F and S2G). These results indicated that glucocorticoids regulate the expression of these inflammatory factors with distinct mechanisms in LTED breast cancer cells.

Differential modulation of the endoplasmic reticulum stress sensors IRE-1α and PERK by dexamethasone

In addition to regulating inflammation, dexamethasone is implicated to be a stress controller in the clinic (17). Accumulation of endoplasmic reticulum stress is a key event in induction of apoptosis by E2 in LTED breast cancer cells (12, 14, 15). To determine how dexamethasone modulates endoplasmic reticulum stress, we examined alteration of three sensors (IRE1, PERK, and ATF6) in MCF-7:5C cells after treatment with E2, dexamethasone, or a combination of them. Our results demonstrated that IRE1 mRNA expression levels were quickly elevated after 2 hours of exposure to E2 (Fig. 3A). In comparison, we found no significant changes in PERK mRNA expression after E2 treatment except for transient upregulation of it by E2 during 4–6 hours of treatment (Fig. 3B). With respect to ATF6, treatment with E2 barely altered its mRNA expression (Supplementary Fig. S2H). Dexamethasone slightly reduced basal levels of IRE1 but was unable to reduce IRE1 mRNA expression elevated by E2 (Fig. 3C). As for PERK, both E2 and dexamethasone weakly reduced mRNA expression, but the combination of them increased it (Fig. 3D). In contrast, both E2 and dexamethasone remarkably reduced PERK mRNA expression in wild-type MCF-7 cells. After combination treatment, PERK mRNA expression levels were similar to E2 alone treated MCF-7 cells (Supplementary Fig. S2I). ATF6 mRNA expression levels did not change remarkably after the combination
MCF-7:5C cells were treated with E2 (1 nmol/L), or a combination of them for 24 hours. TNFα mRNA expression was quantitated by RT-PCR. E2 increased continuously after 12 hours of treatment with E2 (Fig. 3G). Attenuation of protein expression by E2 was effectively blocked by dexamethasone, even though dexamethasone itself increased the phosphorylation of eIF2α after 24 hours of treatment (Fig. 3H). All of these results suggested that IRE1α is activated by E2 prior to PERK in LTED breast cancer cells. Also, dexamethasone differentially regulates these sensors in response to lipid and protein metabolism in the endoplasmic reticulum.

Activated GR does not affect degradation of PI3K-associated pathways mediated by IRE1α. To investigate the functional regulation of various proteins related to lipid metabolism or stress responses by ERα and GR, we performed RPPA analysis of MCF-7:5C cells after treatment with E2, dexamethasone, or a combination of them for different times. We screened more than four hundred proteins and...
demonstrated that E2 was more potent than dexamethasone in upregulating and downregulating the expression of many proteins that widely localize in the nucleus, cytosol, and plasma membrane (Fig. 4A). As expected, E2 decreased ERα protein expression, but increased the phosphorylation of ERα. In contrast, dexamethasone did not affect ERα protein expression or phosphorylation (Fig. 4A). Expression of the ERα-dependent gene insulin-like growth factor receptor-1 (IGF-1R; ref. 25) was quickly upregulated by E2, but dexamethasone did not affect its expression. Similarly, PI3K/Akt was rapidly activated by E2 and remained activated for 48 hours, but dexamethasone did not affect the phosphorylation of Akt (Fig. 4A). Subsequently, we observed clear degradation of Akt-associated proteins (such as mTOR, p70S6K, and 4E-BP1, etc) in MCF-7:5C cells after 72 hours of E2 treatment. Treatment with dexamethasone maintained the phosphorylation of Akt at levels similar to those in control cells, but did not prevent the degradation of these Akt-associated proteins by E2. In addition, PDK1 and Akt are parallel signals, and both of them are downstream signals of PI3K (26). We found that E2 weakly increased the phosphorylation of PDK1 at an early stage of treatment and decreased it at 72 hours. Dexamethasone had almost no effect on phosphorylation of PDK1 (Fig. 4A). The regulatory pattern for PDK1 was similar to that for Akt in that E2 began to remarkably downregulate its expression after 72 hours. Dexamethasone could not block the degradation of PDK1. In addition, treatment with E2 activated the stress response pathways p38 and STAT3. Dexamethasone effectively blocked the phosphorylation of p38 and moderately blocked the phosphorylation of STAT3 (Fig. 4A). We validated these alterations detected by RPPA via Western blotting (Fig. 4B–D). MPA had a regulatory pattern similar to that for dexamethasone in signaling pathways detected by RPPA (Supplementary Fig. S3). Together, these
findings suggested that GR mainly exerts its biological functions on nuclear transcription factors but not in the cytosol of LTED breast cancer cells.

Dexamethasone suppresses NF-κB DNA-binding activity and associated apoptosis induced by E2 in LTED breast cancer cells

Recently, we have found that induction of TNFα expression by E2 is strictly dependent on NF-κB DNA-binding activity in LTED breast cancer cells (15). Selective suppression of TNFα expression (Fig. 2A–C) implies that the function of NF-κB is repressed by dexamethasone. Treatment with E2 or dexamethasone reduced NF-κB mRNA expression after 24 hours, and combination treatment decreased it to a greater extent (Supplementary Fig. S4A–S4C). We did not see a reduction in NF-κB p65 protein expression, but NF-κB p105 protein expression decreased after 72 hours of combination treatment with E2 and dexamethasone (Supplementary Fig. S4D). Notably, NF-κB DNA-binding activity was increased by E2 after 48 hours of treatment in MCF-7:5C cells (Fig. 5A; Supplementary Fig. S4E) versus 6 days in MCF-7:2A cells (Supplementary Fig. S4F), whereas this activity was completely blocked by dexamethasone (Fig. 5A; Supplementary Fig. S4F). Our recent study demonstrated that NF-κB is an oxidative stress inducer after E2 treatment in MCF-7:5C cells (27). Therefore, dexamethasone effectively inhibited expression of the oxidative stress indicator HMOX1 that was increased by E2 after suppression of NF-κB activity (Supplementary Fig. S4G). In addition, E2 increased expression of the stress-responsive transcription factors nuclear factor erythroid-derived 2-like 2 (Nrf2) and hypoxia-inducible factor 1-alpha (HIF-1α), whereas dexamethasone reduced the basal levels of their expression. Furthermore, dexamethasone effectively blocked the induction of their expression by E2 (Supplementary Fig. S4H and S4I). With respect to apoptotic biomarkers, E2 increased cleaved PARP and caspase-7 after 72 hours of treatment in MCF-7:5C cells, but not at 24 hours. Dexamethasone alone did not change the expression of these two biomarkers but completely blocked the cleavage of PARP and caspase-7 induced by E2 (Fig. 5B). We observed similar effects on another LTED breast cancer cell line, MCF-7:2A cells (Supplementary Fig. S5A), which exhibit delayed apoptosis after E2 treatment (11). Importantly, treatment with dexamethasone completely blocked E2-induced apoptosis in two LTED breast cancer cells (Fig. 5C and D; Supplementary Fig. S5B and S5C). The GR antagonist RU486 alone could not block E2-induced apoptosis, but it remarkably reversed inhibitory
effects of dexamethasone on the blockade of E2-induced apoptosis (Fig. 5C and D). Furthermore, low dose of TNFα remarkably induced apoptosis after 48 hours of treatment in MCF-7:5C cells, which could be effectively blocked by dexamethasone (Fig. 5E; Supplementary Fig. S5D). Consistently, the inhibitor of caspase-8, a downstream signal of TNFα, completely blocked E2-induced apoptosis in LTED breast cancer cells (Supplementary Fig. S5E and S5F). All of these results suggested that NF-κB/TNFα axis is crucial for E2 to induce apoptosis and activated GR is potent to suppress its induction by E2, thereby blocking E2-induced apoptosis.

Dexamethasone modulates E2-induced inflammation and apoptosis via GR

To confirm that dexamethasone modulates E2-induced inflammation and apoptosis via GR, we effectively knocked down GR expression in MCF-7:5C cells using a specific siRNA (Fig. 6A). We then treated GR siRNA-transfected cells with E2, dexamethasone, and a combination of them for 72 hours, using scrambled siRNA-transfected cells as controls. The results demonstrated that depletion of GR alone increased the percentage of Annexin V binding in the cells, indicating that GR has the potential to regulate apoptosis in MCF-7:5C cells (Fig. 6B). Downregulation of GR expression did

Figure 5. Suppression of NF-κB DNA-binding activity and associated apoptosis in LTED breast cancer cells by dexamethasone (Dex). A, Suppression of NF-κB DNA-binding activity by dexamethasone. MCF-7:5C cells were treated with E2 (1 nmol/L), dexamethasone (0.1 μmol/L), or a combination of them for 72 hours. Cells were then harvested for extraction of nuclear protein. The NF-κB DNA-binding activity in the cells was measured using an NF-κB (p65) transcription factor assay kit. *, P < 0.05. B, Inhibition of apoptotic biomarkers by dexamethasone. MCF-7:5C cells were treated as described in A for 24 hours and 72 hours. Cleaved PARP and caspase-7 were measured by Western blot analysis. C and D, Blockade of E2-induced apoptosis by dexamethasone. MCF-7:5C cells were treated with E2 (1 nmol/L), dexamethasone (0.1 μmol/L), RU486 (1 μmol/L), or in different combinations as indicated for 72 hours. Apoptosis was measured by an annexin V binding assay through flow cytometry. *, P < 0.05; **, P < 0.001. E, Inhibition of TNFα-induced apoptosis by dexamethasone. MCF-7:5C cells were treated with TNFα (2.5 ng/mL), dexamethasone (0.1 μmol/L), or in a combination of them for 48 hours. Apoptosis was measured by an annexin V binding assay through flow cytometry. **, P < 0.001.
not affect the ability of E\textsubscript{2} to induce apoptosis, but dexamethasone could not prevent E\textsubscript{2}-induced apoptosis under these conditions (Fig. 6B).

Consistently, expression of the E\textsubscript{2}-induced apoptosis-associated inflammatory factors TNF\textalpha, LTB, HMOX1, and Bim was not affected after knockdown of GR, whereas dexamethasone could not effectively block their expression induced by E\textsubscript{2} after depletion of GR. As for regulation of FADS1 and IL6 expression, dexamethasone could barely upregulate their expression after knockdown of GR. It could not further increase IL6 or FADS1 expression after combination

Figure 6.  
Dexamethasone (Dex) modulates E\textsubscript{2}-induced inflammation and apoptosis via GR.  

A, Knockdown of GR expression in MCF-7:5C cells. Cells were transfected with scrambled siRNA or GR siRNA for 72 hours. Cell lysates were then harvested. GR expression was measured by Western blot analysis.  

B, Regulation of E\textsubscript{2}-induced apoptosis after knockdown of GR. MCF-7:5C cells were transfected with scrambled siRNA or GR siRNA for 72 hours. Then, scrambled siRNA-transfected cells were treated with a vehicle (0.1\% EtOH) or E\textsubscript{2} (1 nmol/L). GR siRNA-transfected cells were treated with a vehicle (0.1\% EtOH), E\textsubscript{2} (1 nmol/L), dexamethasone (0.1 \mu mol/L), or a combination of them for 72 hours. Cells were then harvested for annexin V binding assay. **, \textit{P}<0.001.  

C–F, Regulation of inflammation and apoptosis-associated factors after depletion of GR. MCF-7:5C cells were knocked down of GR and treated with E\textsubscript{2} or dexamethasone as described in B for 72 hours. TNF\textalpha (C), LTB (D), HMOX1 (E), and FADS1 (F) mRNA expression was quantitated by RT-PCR. *, \textit{P}<0.05; **, \textit{P}<0.001.
with E2 when GR expression was downregulated (Fig. 6F; Supplementary Fig. S6B). Our results suggested that dexamethasone primarily targets GR to modulate E2-induced inflammatory responses and apoptosis. Both E2 and dexamethasone commonly upregulate FADS1 and IL6/IL6R expression via respective receptors. However, they exerted distinct effects on NF-κB DNA-binding activity, which was increased by E2 but suppressed by dexamethasone. This is a fundamental mechanism underlying dexamethasone blockade of E2-induced apoptosis (Fig. 7).

**Discussion**

The discovery of E2-induced apoptosis not only has clinical relevance to treat aromatase inhibitor-resistant breast cancer and reduce breast cancer incidence in postmenopausal women (6, 7), but also a general principal has emerged to understand sex steroid–induced apoptosis in long-term androgen-deprived prostate cancer (28). Our further findings demonstrate that dexamethasone and MPA with glucocorticoid activity block E2-induced apoptosis in LTED breast cancer cells (8). This observation has clinical significance for the interpretation of breast cancer incidence in postmenopausal women receiving different hormone replacement therapy (HRT) in the Women’s Health Initiative (7, 29). However, the mechanisms remain unclear. It is known that E2 initially overactivates nuclear ERα that leads to the accumulation of stress responses including endoplasmic reticulum stress, oxidative stress, and inflammatory stress in LTED breast cancer cells (12, 13). These unresolved stress responses cause E2-induced apoptosis in these cells (12–16). Among these stress-associated factors, the PERK/NF-κB/TNFα axis plays a critical role in mediating E2-induced apoptosis (15, 16). Herein, we demonstrate that dexamethasone preferentially and consistently represses NF-κB DNA binding, which results in selective inhibitory effects on the induction of TNFα expression. Consequently, dexamethasone completely blocks E2-induced apoptosis in LTED breast cancer cells.

NF-κB is a key transcription factor in mediating inflammatory and stress responses (30, 31). Furthermore, its transcriptional activity is suppressed by the lipogenesis transcription factors CCAAT/enhancer binding protein β (C/EBPβ) and PPARγ in LTED breast cancer cells (15, 27), indicating that NF-κB has a close relationship with lipid metabolism. In this study, NF-κB is regulated by both E2 and dexamethasone but in an opposite direction in LTED breast cancer cells. We have observed that E2 increases NF-κB DNA-binding activity via activation of PERK, but not through the canonical pathway in MCF-7/5C cells (15, 16). Unlike E2, dexamethasone decreases NF-κB DNA-binding activity and consistently represses the expression of the NF-κB–dependent gene TNFα. These data suggest that dexamethasone preferentially inhibits NF-κB DNA binding in LTED breast cancer cells. Our result is consistent with a report that activated GR potently prevents NF-κB from chromatin occupancy (32). In addition, the GR target gene GILZ can bind to NF-κB directly and inhibit its DNA-binding activity (33). Therefore, suppression of the NF-κB/TNFα axis by dexamethasone is one of the major mechanisms that blocks E2-induced apoptosis.

Glucocorticoids are prescribed as anti-inflammatory and anti-stress agents in the clinic (34, 35), and they exhibit proapoptotic or antiapoptotic effects in a tissue- or cell type–dependent manner (36). In MCF-7–derived cell lines, treatment with dexamethasone inhibits cell proliferation but does not induce apoptosis (8). As for the regulation of inflammatory responses, dexamethasone is not a complete inhibitor of them in LTED breast cancer cells, which increases the expression of adipose inflammatory factors FADS1 and IL6/IL6R and upregulates more upon adding dexamethasone to E2. This may be related to the activation of the lipogenesis transcription factor C/EBPβ by treatment with dexamethasone (37). Unexpectedly, dexamethasone differentially regulates the function of endoplasmic reticulum stress sensors, which prevents E2 from activation of PERK, but it has no inhibitory effects on IRE1α expression increased by E2. This distinctive regulation of PERK and IRE1α also indicates that dexamethasone differentially regulates protein and lipid metabolism in the endoplasmic reticulum (12, 14–16). Although how dexamethasone differentially regulates these two sensors remains unclear, treatment with dexamethasone alone increases phosphorylation of the PERK downstream signal eIF2α (Fig. 3H), implying that dexamethasone does not directly inhibit PERK function. Most likely, E2 increases the expression of some short half-life nuclear proteins, leading to accumulation of unfolded proteins in the endoplasmic reticulum. In contrast, dexamethasone has the potential to inhibit expression of these nuclear proteins, thereby reducing
the unfolded protein burden in the endoplasmic reticulum. The AP-1 family member c-Fos is one of the candidates as a short-lived transcription factor, which is activated by E_2 (Fig. 1G; ref. 38). In support of our view, other studies have demonstrated that activated GR preferentially suppresses the function of AP-1 family members (39, 40). In addition, synergistic upregulation of SKG1 expression by E_2 and dexamethasone facilitates maintenance of the homeostasis in the endoplasmic reticulum to antiapoptosis (41, 42). All of these findings suggest that activated GR is a potent regulator of multiple stress-responsive transcription factors, thereby modulating stress and inflammatory responses.

Finally, it is important to note that our results mainly focused on MCF-7-derived cells due to the limitation of cell lines (43). MCF-7 and T47D are two representative ERE- and GR-positive breast cancer cell lines. After long-term E_2 deprivation, there is no change in GR expression in MCF-7 and T47D. As for the expression of EREs, MCF-7-derived cell lines continue to express EREs. However, T47D-derived cell line loses EREs, thereby having no response to E_2 (44). This is also the reason why E_2-induced apoptosis is observed in different sources of MCF-7-derived LTBD cell lines (4, 5).

In summary, E_2 and dexamethasone regulate E_2-induced stress and apoptosis through their respective receptors. Cross-talk between ERα and GR exists (45, 46), but ERα is more potent than GR in widely activating signaling pathways in LTBD breast cancer cells. In this study, activation of GR by dexamethasone or MPA blocks E_2-induced apoptosis via trans-suppression of NF-κB DNA-binding activity. We found a similar mechanism underlying blockage of E_2-induced apoptosis by the agonist of PPARγ in MCF-7/Sc cells (72). These results demonstrate that different ligands activate respective transcription factors to commonly suppress the function of AP-1 family member c-Fos (27). These results demonstrate that different ligands activate respective transcription factors to commonly suppress the DNA-binding activity of NF-κB, a molecule critical to mediate stress, inflammation, and apoptosis in LTBD breast cancer cells (15, 16, 27). They also imply that the biological function of ERs is affected by several other transcription factors depending on the ligands present in the nucleus, thereby dynamically remodeling the chromatin occupancy (47–49).

Transcriptional activator FoxA1 has been identified as a pioneer factor to be involved in the dynamic chromatin transitions after activation of GR and ERα in breast cancer cells (50). These findings have built up a strong basis for us to further investigate how GR and ERα interact with each other on the chromatin occupancy in the estrogen-resistant breast cancer cells, compared with the wild-type breast cancer cells. Our data further provide a novel understanding of E_2-induced apoptosis through extensive interactions of EREs with inflammation- and stress-associated transcription factors, such as NF-κB and PPARγ (15, 16, 27), which facilitates apoptosis-associated inflammation and stress after long-term E_2 deficiency.

Importantly, these transcription factors are also therapeutic targets for different diseases (51–53). Together, this study not only supports the deployment of antilipocorticoids to enhance sex steroid–induced apoptosis in cancer therapy, but also provides an insight into the mechanism of MPA with glucocorticoid activity to increase breast cancer incidence in the WHI. Ongoing strategic studies in our laboratory are addressing the wide application of glucocorticoid action in regulating sex steroid–induced apoptosis in a range of models of normal and cancer cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: P. Fan, B. Abderrahman, V.C. Jordan
Development of methodology: P. Fan
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P. Fan, D.R. Siwak, F.A. Agboke
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P. Fan
Writing, review, and/or revision of the manuscript: P. Fan, D.R. Siwak, B. Abderrahman, V.C. Jordan
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P. Fan, D.R. Siwak, S. Yerrum
Study supervision: P. Fan, B. Abderrahman, V.C. Jordan

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Suppression of Nuclear Factor-κB by Glucocorticoid Receptor Blocks Estrogen-Induced Apoptosis in Estrogen-Deprived Breast Cancer Cells

Ping Fan, Doris R. Siwak, Balkees Abderrahman, et al.