Proinflammatory Cytokines Enhance Estrogen-dependent Expression of the Multidrug Transporter Gene ABCG2 through Estrogen Receptor and NFκB Cooperativity at Adjacent Response Elements*5*

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Constitutive activation of NFκB in estrogen receptor (ER)-positive breast cancer is associated with tumor recurrence and development of anti-estrogen resistance. Furthermore, a gene expression signature containing common targets for ER and NFκB has been identified and found to be associated with the more aggressive luminal B intrinsic subtype of ER-positive breast tumors. Here, we describe a novel mechanism by which ER and NFκB cooperate to up-regulate expression of one important gene from this signature, ABCG2, which encodes a transporter protein associated with the development of drug-resistant breast cancer. We and others have confirmed that this gene is regulated primarily by estrogen in an ER- and estrogen response element (ERE)-dependent manner. We found that whereas proinflammatory cytokines have little effect on this response element (ERE)-dependent manner. We found that proinflammatory cytokines have little effect on this gene in the absence of 17β-estradiol, they can potentiate ER activity in an NFκB-dependent manner. ER allows the NFκB family member p65 to access a latent NFκB response element located near the ERE in the gene promoter. NFκB recruitment to the gene is, in turn, required to stabilize ER occupancy at the functional ERE. The result of this cooperative binding of ER and p65 at adjacent response elements leads to a major increase in both ABCG2 mRNA and protein expression. These findings indicate that estrogen and inflammatory factors can modify each other’s activity through modulation of transcription factor accessibility and/or occupancy at adjacent response elements. This novel transcriptional mechanism could have important implications in breast cancer, where both inflammation and estrogen can promote cancer progression.

The estrogen 17β-estradiol (E2)2 is a steroid that plays an important role in reproductive tissues, as well as in the skeletal, cardiovascular, immune, and central nervous systems, by regulating a number of cellular processes, such as proliferation, differentiation, and survival. In the classical mechanism of estrogen action, E2 binds to the estrogen receptor (ER) to promote receptor homodimerization. The ligand-bound receptor binds to cognate DNA sequences, called estrogen response elements (EREs), which leads to coregulator recruitment and target gene transcription. In addition to direct DNA binding, ER can also regulate gene transcription via protein-protein interaction with other DNA-binding transcription factors, such as the proteins of the AP-1 complex and Sp1 (1, 2).

Estrogen can also modulate gene transcription by the proinflammatory transcription factor NFκB, which, like ER, influences numerous cellular processes. In the classical NFκB pathway, binding of proinflammatory cytokines to their receptors activates the IκB kinase (IKK) complex, which phosphorylates the inhibitory protein IκB, leading to its subsequent ubiquitination and proteasomal degradation. NFκB family members p65 and p50 can then translocate to the nucleus and regulate transcription of a cohort of genes by binding to specific DNA elements called NFκB response elements (NFκBREs). Numerous studies in a variety of physiological systems and models have demonstrated that mutual transrepression occurs between ER and NFκB, with ER repressing transcription by NFκB (3) and NFκB repressing transcription by ER (4, 5).

However, in breast cancer cells, positive cross-talk between ER and NFκB may also be particularly important. Inhibition of NFκB has been shown to restore responsiveness to anti-estrogens in cell lines that were originally resistant to endocrine therapy (6, 7). Recent studies have also found that hormone-dependent tumors with a high risk for recurrence have constitutive activation of NFκB (8). Moreover, recent work from our laboratory highlights a high degree of positive cross-talk between ER and NFκB in the synergistic up-regulation of a number of common target genes. This synergistic gene signature is associated with the more aggressive luminal B subtype of ER-positive breast tumors and delineates the responsiveness of ER-positive breast tumors to tamoxifen therapy (9).

One gene from this signature that is highly important in breast cancer response to therapeutic drugs is ABCG2 (ATP-binding cassette transporter G2) (9). The ABCG2 gene encodes an ABC transporter that is capable of pumping a number of endogenous and exogenous agents out of cells (10). ABCG2, also known as BCRP (breast cancer resistance protein), causes the efflux of a spectrum of anticancer drugs out of breast cancer.
cells, including mitoxantrone, camptothecin-derived and indolocarbazole topoisomerase I inhibitors, methotrexate, flavopiridol, and quinazoline ErbB1 inhibitors, and can thereby contribute to drug-resistant breast cancers (10). Furthermore, overexpression of ABCG2 has been shown to confer anticancer drug resistance in the breast cancer cell line MCF-7 (11).

Because of its known role in promoting drug-resistant breast tumors, we sought to understand the mechanism by which the combination of E2 and proinflammatory cytokines leads to highly elevated levels of ABCG2 expression in breast cancer cells. Although previous studies have shown that ABCG2 can be either up- or down-regulated by E2, or proinflammatory cytokines acting independently (12–15), here, we establish that cooperativity between ER and p65 occurs at two adjacent response elements, leading to a synergistic increase in both ABCG2 mRNA and protein expression in breast cancer cells.

**EXPERIMENTAL PROCEDURES**

**Reagents, Antibodies, and Cell Culture**—E2 was obtained from Sigma. The cytokines TNFα, IL-1β, and IL-6 were obtained from R&D Systems (Minneapolis, MN). ICI 182,780 was purchased from Tocris (Ellisville, MO). The small molecule inhibitor 8-[(benzylthio)methyl]theophylline was a generous gift from Dr. David Shapiro (University of Illinois at Urbana-Champaign). Adenoviral vectors for GFP and dominant-negative IkBα (IkBα-DN) were kindly provided by Drs. Michael O’Donnell and Dr. Ruxana Sadikot (University of Illinois at Chicago), respectively, and expressed as described previously (16). The small molecule inhibitor IKK VII, which inhibits both IKKa and IKKB, was obtained from EMD Chemicals, Inc. (Hawthorne, NY). Antibodies for ChIP assays, anti-ERα (sc-543) and anti-p65 (sc-372), were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and the rabbit IgG polyclonal antibody (12-370) was obtained from Millipore (Temecula, CA). Antibodies used for Western blotting were as follows: anti-ERα (RT-1641-P0, Thermo Scientific), anti-ABCG2 (MAB4146, Millipore), anti-p65 (sc-372, Santa Cruz Biotechnology; and 4764, Cell Signaling), anti-phospho-p65 (3033, Cell Signaling), anti-IkBα (4814, Cell Signaling), anti-phospho-IkBα (2859, Cell Signaling), anti-H3 (05-928, Millipore), and anti-β-actin (A5441, Sigma). MCF-7 cells were kindly provided by Dr. Benita Katzenellenbogen (University of Illinois at Urbana-Champaign) and cultured as described previously (16).

**mRNA Analysis by Quantitative PCR (qPCR)**—RNA isolation, reverse transcription, and qPCR were carried out as described previously (16). 36B4 was used as an internal control. Primer sequences for qPCR are listed in supplemental Table 1.

**Plasmids and Mutagenesis**—Fragments of the ABCG2 promoter (−243 to +362 and −115 to +362) subcloned into the pGL3-basic reporter plasmid were kindly provided by Drs. D. Ross (University of Maryland) and W. Beck (University of Illinois at Chicago). The ABCG2 (−243 to +362) plasmid was used to mutate the CRE and either one or both NFκBRE sequences using the QuikChange Lightning site-directed mutagenesis kit (Stratagene, La Jolla, CA). Wild-type and mutant sequences are given in supplemental Table 1. Mutagenesis was confirmed by sequencing using a 3730 DNA analyzer (Applied Biosystems, Foster City, CA). MCF-7 cells were transiently transfected with reporter constructs, and Dual-Luciferase assays were carried out as described previously (16).

**ChIP Assay**—ChIP assays were carried out as described previously with certain modifications (16, 17). MCF-7 cells were seeded in 10-cm plates and grown in phenol red-free medium containing 5% charcoal/dextran-stripped calf serum. On reaching 90% confluency, cells were treated as described in the figure legends. Cross-linking was carried out with 1.5% formaldehyde for 15 min at room temperature. Cells were lysed with SDS lysis buffer (1% SDS, 10 mM EDTA, pH 8.0, and 50 mM Tris·HCl, pH 8.1), and chromatin was sonicated three times for 10 s using a Fisher Model 100 Sonic Dismembrator. Protein A-coated magnetic beads (100-02D, Dynabeads, Invitrogen) washed three times with 5% BSA in PBS were incubated overnight at 4 °C with 1 μg of ERα or 4 μg of p65 antibody. Antibody-bound beads were washed two times with 5% BSA in PBS and incubated overnight at 4 °C with sonicated chromatin diluted in dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, pH 8.0, 16.7 mM Tris·HCl, pH 8.1, and 167 mM NaCl). Beads were serially washed with low salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 8.0, 20 mM Tris·HCl, pH 8.1, and 150 mM NaCl), high salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, pH 8.0, 20 mM Tris·HCl, pH 8.1, and 500 mM NaCl), and LiCl immune complex wash buffer (0.25 m LiCl, 1% IGEPA-L CA-630, 1% deoxycholic acid (sodium salt), 1 mM EDTA, pH 8.0, and 10 mM Tris·HCl, pH 8.1) and two times with Tris/EDTA (10 mM Tris·HCl, pH 8.0, and 1 mM EDTA). DNA was eluted from the beads in elution buffer containing 0.1 m sodium bicarbonate and 1% SDS. Decross-linking was carried out for 16 h at 65 °C. DNA was purified using QIAquick columns (Qiagen, Valencia, CA) and eluted in prewarmed water. Approximately 5% of the cell lysate was removed as input prior to immunoprecipitation. Inputs were diluted at 1:20 or 1:200. The percent maximum occupancy was calculated as follows: the -fold change for each treatment was determined relative to the input for each sample and the untreated control for each experiment using the ΔΔCt method. The percent maximum occupancy for each assay was then calculated relative to the treatment with the highest -fold change, which was set to 100%. Data were plotted as the mean percent maximum occupancy ± S.E. for at least three independent assays. The primers for ChIP-qPCR are listed in supplemental Table 1.

**Western Blotting**—Whole-cell and nuclear extracts were prepared from MCF-7 cells using M-Per and NE-Per reagents, respectively (Pierce), containing protease and phosphatase inhibitors (Roche Applied Science). SDS-PAGE was carried out, and proteins were detected by Western blotting using the antibodies indicated, with β-actin or H3 serving as an internal loading control.

**DNA Affinity Pulldown Assay (DAPA)**—DNA probes were prepared from ABCG2 reporter constructs using a biotinylated forward primer. The resulting 434-bp probes with either an intact or mutant CRE or NFκBRE were hybridized to streptavidin-coated magnetic beads (Invitrogen). Nuclear extracts were prepared from MCF-7 cells using a nuclear extraction kit (AY2002, Panomics, Fremont, CA) following the manufac-
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FIGURE 1. TNFα potentiates the up-regulation of ABCG2 mRNA and protein by E2. A, MCF-7 cells were treated with E2 (10 nM), TNFα (10 ng/ml), or both for up to 4 h. RNA was isolated, and ABCG2 mRNA levels were examined by qPCR using the ΔΔCt method. 36B4 was used as an internal control. *, p < 0.05 compared with E2 alone at the same time point. B, MCF-7 cells were treated for 2 h with or without TNFα (10 ng/ml) in the presence of increasing doses of E2. *, p < 0.05 compared with TNFα alone at the same dose. C, MCF-7 cells were treated with E2 (10 nM), TNFα (10 ng/ml), or both for 6 h. ABCG2 and β-actin protein levels were examined by Western blotting as described under “Experimental Procedures.” Treatment with vehicle (V) served as a control.

FIGURE 2. TNFα potentiates ABCG2 expression by enhancing ER occupancy at an essential ERE. A and B, ABCG2 mRNA levels were examined by qPCR in MCF-7 cells pretreated for 2 h with or without the ER antagonist ICI 182,780 (ICI; 1 μM) or a small molecule inhibitor of ERα binding to ERE, 8-[(benzylthio)methyl]theophylline (TPBM; 20 μM), followed by treatment with E2 (10 nM), TNFα (10 ng/ml), or both for an additional 2 h. *, p < 0.05 compared with E2 alone. DMSO, dimethyl sulfoxide. C, MCF-7 cells were transfected with 1 μg of an ABCG2-luciferase reporter plasmid in which the ERE located at −180 was intact (−243), mutated (mutERE), or deleted (−115), along with 200 ng of the Renilla luciferase control plasmid pGL4.70. Dual-Luciferase assays were carried out following treatment with E2, TNFα, or both for 4 h as described under “Experimental Procedures.” *, p < 0.05 compared with E2 alone. D, MCF-7 cells were treated with E2, TNFα, or both for up to 60 min. ChIP assay was performed, and ER recruitment to the ABCG2 regulatory region containing the ERE was examined by qPCR. *, p < 0.05 compared with E2 alone. ER occupancy in response to different treatments is expressed as the percentage of maximum ER occupancy, which was observed at 45 min of E2 + TNFα treatment. E, ChIP assay was performed with an antibody specific for ERα or a nonspecific IgG antibody as a negative control. qPCR was performed for the ABCG2 regulatory region containing the ERE (Enhancer) or for a far upstream region (Upstream) as a negative control. *, p < 0.05 compared with E2 alone; nd, non-detectable. F, nuclear extracts were prepared from MCF-7 cells treated with E2 and TNFα for 45 min and incubated with a DNA probe from the ABCG2 gene in which the ERE was either intact (ERE-WT) or mutated (ERE-mut). DAPA was performed as described under “Experimental Procedures,” and ER binding to the probes was examined by Western blotting.

Statistical Analysis—qPCR and reporter data were analyzed by two-way analysis of variance, followed by a post hoc Bonferroni test. Significance for all statistical tests was set at p < 0.05. The data shown are the mean ± S.E. from at least three independent determinations.

RESULTS

TNFα Potentiates E2-mediated Regulation of ABCG2 in an NFκB-dependent Manner—To confirm the finding from our microarray study that showed synergistic up-regulation of ABCG2 by the combination of E2 and TNFα compared with either E2 or TNFα alone (9), we examined regulation of ABCG2 mRNA in ER-positive MCF-7 breast cancer cells over a period of 4 h. We observed that a rapid and robust up-regulation of ABCG2 expression occurred in response to E2 + TNFα compared with a modest increase in response to E2 and minimum regulation in response to TNFα (Fig. 1A). Dose-response studies showed that ABCG2 mRNA was regulated by E2 in a typical dose-dependent manner, an effect that was significantly increased by the presence of TNFα (Fig. 1B). In contrast, TNFα had a minimum effect on ABCG2 expression at all doses tested in the absence of E2 but showed a typical dose response in the presence of E2 (Fig. 1C). Examination of ABCG2 protein levels showed a pattern of regulation similar to mRNA levels, with E2 stimulation of ABCG2 expression greatly enhanced by E2 in combination with TNFα (Fig. 1D). These findings indicate that TNFα can significantly potentiate the effect of E2 on ABCG2 expression at both the mRNA and protein levels but has little effect on the gene in the absence of E2.

To understand the mechanism by which TNFα enhances E2-mediated expression of ABCG2, we initially examined the role of ER. We found that the pure ER antagonist ICI 182,780 completely blocked ABCG2 regulation both by E2 alone and by E2 + TNFα (Fig. 2A). Furthermore, use of a small molecule inhibitor that prevents ER binding to DNA, 8-[(benzylthio)methyl]theophylline (19), had an effect similar to ICI...
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To examine whether ER binds to the ERE of the ABCG2 promoter, DAPA was carried out. Nuclear extracts from MCF-7 cells treated with E2 + TNFα were incubated with DNA probes containing the regulatory region of ABCG2, with the ERE intact or mutated. As determined by Western blotting, ER binding to the probes was greatly reduced when the ERE was mutated (Fig. 2F), indicating specific ER binding to this site in the ABCG2 promoter. These studies indicate that the ability of TNFα to increase the transcriptional regulation of ABCG2 by E2 is mediated by enhanced ER occupancy and activity specifically at a functional ERE in the ABCG2 promoter.

Our previous studies on other common E2 and TNFα target genes suggested that much of the cross-talk between these factors requires theNFκB pathway (9, 16). NFκB was further implicated in the regulation of ABCG2 by our finding that IL-1β, another proinflammatory cytokine that activates NFκB, could enhance E2-regulated expression of ABCG2. On the other hand, IL-6, which does not activate NFκB, could not potentiate E2 action (Fig. 3A).

To further examine the role of NFκB, IκBα-DN, which blocks NFκB activation because it cannot be phosphorylated or subsequently degraded in response to cytokine treatment, was used. The enhanced regulation of ABCG2 by E2 + TNFα over that seen with E2 alone was completely abrogated by IκBα-DN (Fig. 3B). Furthermore, the enhanced occupancy of ER at the ABCG2 ERE with E2 + TNFα was substantially reduced, nearly to the level of E2 alone, by IκBα-DN (Fig. 3C) or by a small molecule inhibitor of IKK (Fig. 3D). These findings indicate a clear role for NFκB in the ability of TNFα to potentiate E2 action through increased ER occupancy at the ABCG2 gene. Taken together, our results indicate that ER is the primary factor required to drive transcription of ABCG2, whereas TNFα, acting through the NFκB pathway, mainly operates to augment ER action on this gene.

E2 Allows NFκB Action through a Latent NFκBRE—To determine the mechanism by which NFκB can potentiate ER action, we examined the regulatory region of the ABCG2 promoter for possible response elements through which NFκB may exert its effect. Interestingly, we found two putative NFκBREs (N1 and N2) located downstream of the ABCG2 ERE (Fig. 4A). Because TNFα cannot regulate expression of ABCG2 on its own, these NFκBREs appear not to be functional. However, mutational analysis revealed that the NFκBRE closer to the ERE, N1, but not the one farther downstream, N2, was required for TNFα to potentiate E2 action (Fig. 4B). The effect of mutating both NFκBREs was similar to that of mutating N1 alone, indicating that this response element is sufficient to mediate the effects of NFκB on this gene. However, these findings also indicate that N1 is a latent response element that requires the presence of E2 along with TNFα to become functional.
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To examine whether the NFκB family member p65 is recruited to the N1 site of the ABCG2 promoter in an ER-dependent manner, A, MCF-7 cells were pretreated for 2 h with or without the ER antagonist ICI 182,780 (1 μM), followed by treatment with E2, TNFa, or both for 45 min. ChIP assay was carried out, and p65 recruitment to the ABCG2 promoter was examined by qPCR.

B, nuclear extracts were prepared from MCF-7 cells with E2 and TNFa for 45 min and incubated with a DNA probe from the ABCG2 gene in which N1 was either intact (N1-WT) or mutated (N1-mut). DAPA was performed as described under “Experimental Procedures,” and p65 binding to the probes was examined by Western blotting.

C, MCF-7 cells were treated with E2 (E), TNFa (T), or both for 5 or 15 min, and whole-cell (WCE) and nuclear (NE) extracts were prepared. Phosphorylated and total IκBα and β-actin protein levels were examined in whole-cell extracts, and phosphorylated and total p65 and histone H3 protein levels were examined in nuclear extracts by Western blotting.

DISCUSSION

ABCG2 is a transmembrane transporter that carries out the important biological function of effluxing multiple endogenous and exogenous substances out of cells (10). On account of its ability to efflux multiple drugs, overexpression of this protein has been associated with drug-resistant cancers, including breast cancer (10, 11). Also, a recent microarray study from our laboratory identified ABCG2 to be part of a gene signature that is associated with the more aggressive luminal B subtype of ER-positive breast tumors, which emphasizes the clinical relevance of this gene (9). Because of this noteworthy biological and clinical significance of ABCG2, the regulation of this gene has been extensively studied. Others have found that E2 can up- or down-regulate ABCG2 depending on the cellular background, with differences potentially due to altered ratios of ERα to ERβ expression in the cell (10, 12–14, 21, 22). Similarly, ABCG2 expression has also been shown to be either up- or down-regulated by numerous cytokines, including TNFa and IL-1β, in a cell-specific manner (14, 15). In addition, a number of other
transcription factors, signaling pathways, and growth factors can also regulate ABCG2 expression (23–26). Moreover, the ABCG2 gene can be regulated through epigenetic mechanisms, including histone deacetylase inhibition and DNA methylation (27, 28), as well as by post-transcriptional microRNA-dependent mechanisms (29, 30). This high degree of regulation suggests that ABCG2 expression is tightly controlled and exquisitely sensitive to external stimuli. Furthermore, combinations of transcription factors, such as we find with ER and NFκB, may work together in a combinatorial and cell-specific manner to fine-tune expression of ABCG2.

Our studies indicate that the combination of estrogen and proinflammatory cytokines can activate a novel transcriptional mechanism by which ER and NFκB cooperate to up-regulate expression of ABCG2 in breast cancer cells. We have demonstrated that E₂ up-regulates ABCG2 expression in an ER- and ERE-dependent manner, whereas TNFα appears to have no effect on this gene in the absence of E₂ (Fig. 7). However, TNFα is capable of enhancing ER activity through a latent NFκB response element that displays no activity in the absence of E₂, but is required for TNFα potentiation of E₂ activity. Once recruited, p65 is capable of stabilizing ER occupancy on the gene. This reciprocal binding between ER and NFκB at adjacent response elements ultimately leads to enhanced regulation of ABCG2.

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FIGURE 7. Proposed model for TNFα potentiation of E₂-regulated ABCG2 expression involves cooperativity between ER and p65 at adjacent response elements. E₂ is capable of up-regulating ABCG2 expression through ER binding to an ERE in the ABCG2 promoter, whereas in the absence of E₂, TNFα has no effect on ABCG2 transcription. However, in the presence of both E₂ and TNFα, we found that ER is required to allow recruitment of the NFκB subunit p65 to a latent NFκBRE that displays no activity in the absence of E₂ but is required for TNFα potentiation of E₂ activity. Once recruited, p65 is capable of stabilizing ER occupancy on the gene. This reciprocity between ER and NFκB at adjacent response elements ultimately leads to enhanced regulation of ABCG2.

Although cooperativity between transcription factors at adjacent response elements is fairly common, we have demonstrated here for the first time that such a reciprocal interaction can occur between ER and NFκB at adjacent response elements and leads to the synergistic up-regulation of ABCG2.

The mechanism by which cooperativity between ER and NFκB occurs is likely to involve a number of factors, including the kinetics of transcription factor-DNA interaction. Transcription factors have been shown to exhibit a rapid and dynamic interaction with DNA, termed “hit and run” (47). This model suggests that a transcription factor interacts with the promoter briefly (hit) and then is rapidly displaced from its binding site (run). The observed cooperativity in ER and NFκB occupancy at the ABCG2 gene may be the result of increased frequency of transcription factor-DNA interactions, a decreased rate of transcription factor displacement, or both. How ER and p65 may alter each other’s interaction with DNA is not known but could be explained by protein-protein interactions, either directly between ER and p65 or indirectly through formation of a coregulator complex (32, 36, 48–53).

In conclusion, our findings indicate that expression of ABCG2 can be synergistically up-regulated by a novel mechanism of cross-talk between ER and NFκB at adjacent response elements. This finding could have important implications for patients with ER-positive breast tumors, in which both estradiol and proinflammatory cytokines are produced locally within the breast tumor microenvironment. Furthermore, our finding that ER and NFκB are capable of working together to increase ABCG2 expression is consistent with reports that both of these transcription factors are associated with drug resistance in breast cancer (54, 55) and that ER-positive breast tumors with constitutive activation of NFκB may be more aggressive and resistant to tamoxifen (6–8).
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