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ACTIVATION OF DIOXIN RESPONSE ELEMENT (DRE)- ASSOCIATED GENES BY BENZO(A)PYRENE 3,6-QUINONE AND BENZO(A)PYRENE 1,6-QUINONE IN MCF-10A HUMAN MAMMARY EPITHELIAL CELLS

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Abstract

Benzo(a)pyrene (BaP) is a known human carcinogen and a suspected breast cancer complete carcinogen. BaP is metabolized by several metabolic pathways, some having bioactivation and others detoxification properties. BaP-quinones (BPQs) are formed via cytochrome P450 and peroxidase dependent pathways. Previous studies by our laboratory have shown that BPQs have significant growth promoting and anti-apoptotic activities in human MCF-10A mammary epithelial cells examined *in vitro*. Previous results suggest that BPQs act via redox-cycling and oxidative stress. However, because two specific BPQs (1,6-BPQ and 3,6-BPQ) differed in their ability to produce reactive oxygen species (ROS) and yet both had strong proliferative and EGF receptor activating activity, we utilized mRNA expression arrays and qRT-PCR to determine potential pathways and mechanisms of gene activation. The results of the present studies demonstrated that 1,6-BPQ and 3,6-BPQ activate dioxin response elements (DRE, also known as xenobiotic response elements, XRE) and anti-oxidant response elements (ARE, also known as electrophile response elements, EpRE). 3,6-BPQ had greater DRE activity than 1,6-BPQ, whereas the opposite was true for the activation of ARE. Both 3,6-BPQ and 1,6-BPQ induced oxidative stress associated genes (HMOX1, GCLC, GCLM, and SLC7A11), phase 2 enzyme genes (NQO1, NQO2, ALDH3A1) PAH metabolizing genes (CYP1B1, EPHX1, AKR1C1), and certain EGF receptor associated genes (EGFR, IER3, ING1, SQSTM1 and TRIM16). The results of these studies demonstrate that BPQs activate numerous pathways in human mammary epithelial cells associated with increased cell growth and survival that may play important roles in tumor promotion.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs), such as benzo(a)pyrene (BaP) and 7,12-dimethylbenz(a)anthracene (DMBA), are well known complete carcinogens that produce skin cancer, lung cancer, and lymphoma in rodents and other species (Pelkonen and Nebert, 1982). In rodents, mammary cancer is produced by 7,12-dimethylbenz(a)anthracene (DMBA) (Ethier and Ullrich, 1982; Russo and Russo, 1996). BaP has recently been categorized by the International Agency for Research on Cancer (IARC) as a Group 1 carcinogen indicating that it is a known human carcinogen (Straif et al., 2005; IARC 2006). Environmental PAHs are associated with cancers and alterations in biomarkers of exposure (Farmer et al., 2003). The

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role of environmental PAHs in human breast cancer is uncertain. BaP has been found to transform human mammary cell lines *in vitro* (Balogh et al, 2006). While bulky PAH adducts can be detected in mammary tissues of exposed individuals, there has not an established epidemiologic connection between PAH exposure and human breast cancer (Gammon et al., 2002). Cigarette smoke, which contains PAHs and other substances, may present a slight increased risk for breast cancer (Egan et al., 2002). Combined PAH and solvent exposures have also been associated with an increased risk for human breast cancer (Brody and Rudel, 2003).

As a complete carcinogen, BaP is capable of both tumor initiation via genotoxic actions on DNA as well as tumor promotion through non-genotoxic signaling pathways (Luch et al, 2005). Mechanistic studies demonstrate that BaP metabolism is important for its tumor-initiating properties. Benzo(a)pyrene-7,8-diol-9,10-epoxide (BPDE I or [+] anti-BPDE) is an important metabolite formed by the metabolism of BaP by cytochrome P450s (CYP1A1, CYP1A2, and CYP1B1) and epoxide hydrolase (EPHX1). BPDE binds to DNA leading to mutations (G → T transversions) and tumor initiation (Conney et al, 2001). DMBA initiates ovarian cancer, skin cancer, and lymphoma via a CYP1B1-dependent process (Buters et al., 2003). Because we know that CYP1B1 and CYP1A1 are also expressed in mammary epithelial cells (Spink et al., 1998;Keshava et al., 2005), and *in vitro* experiments with normal human mammary epithelial cells and cell lines have shown that PAHs are bioactivated *in vitro* (Stampfer et al., 1981;Bartley et al., 1982;Bartley and Stampfer, 1985;Pruess-Schwartz et al., 1986), this bioactivation could also occur in the breast. There are however important species differences between rodents and humans for the bioactivation and metabolism of PAHs (Moore et al., 1986;Christou et al., 1987) and it is therefore important to examine PAH effects in relevant human systems.

An important series of metabolites of BaP that have been somewhat understudied for biologic effects are the BaP-quinones (BPQs). BPQs (1,6-BPQ, 3,6-BPQ, and 6,12-BPQ) are formed by CYPs of the 1A and 1B family (Shimada et al, 2001;Shimada and Guengerich, 2006) as well as by peroxidases via radical cations (Cavaleri et al, 1998), and by UV light (Reed et al., 2003). A 7,8-orthoquinone of BaP is formed from the 7,8-dihydrodiol of BaP by the AKR1C1 enzyme (Jin and Penning, 2006). The tumor initiating and DNA-binding activities of BPQs have previously been reported (Moorthy et al., 2002; Balu et al., 2004). Some BPQs activate signaling pathways in cells via binding to Ah receptors (Burczynski and Penning, 2000). Thus, BPQs have both genotoxic and non-genotoxic properties.

BPQs are also redox-cycling agents and have been shown to produce oxidative stress in cells and mitochondrial dysfunction (Zhu et al., 1995;Bolton et al., 2000). We have recently shown that BPQs produce reactive oxygen species (ROS), and more specifically superoxide anion, in human mammary epithelial cells (Burdick et al. 2003,Shi et al., 2005). The production of ROS was associated with activation of epidermal growth factor receptor (EGFR) signaling pathways leading to increased cell proliferation of human mammary epithelial cells (Burdick et al., 2003). In our studies, while both 1,6-BPQ and 3,6-BPQs activated EGFR and downstream signaling pathways, 1,6-BPQ produced significantly more ROS production than did 3,6-BPQ. If ROS production is not the critical mechanism associated with modulation of proliferative and anti-apoptotic pathways, we were interested in examining alternative pathways. Gene array analysis has recently proved useful in an assessment of PAH effects on target cells (Bartosiewicz et al., 2001;Keshava et al., 2005). Therefore the purpose of the present studies was to examine the mRNA induction profiles obtained with a MCF-10A human mammary epithelial cell model system of EGFR signaling using gene expression arrays and quantitative PCR. We compared the effects of 1,6-BPQ with 3,6-BPQ for the induction of approximately 22,000 human genes and found that the top induced genes (as measured by fold change) were associated with both dioxin response elements (DREs) and anti-oxidant response elements (AREs).

METHODS

Chemicals and reagents

All chemicals were purchased from Sigma (St Louis, MO), unless otherwise indicated. 1,6-BPQ and 3,6-BPQ were purchased from Midwest Research Institute (Kansas City, MO, USA) at >99% purity and maintained as a stock solution in anhydrous tissue culture grade dimethyl sulfoxide (DMSO) and stored at -20°C . Preparation of PAHs and cell culture treatments were carried out under low ambient lighting. The final concentration of DMSO in all experiments was 0.1%.

MCF-10A cell culture and treatments

MCF-10A cells are a spontaneously immortalized and growth factor-dependent mammary epithelial cell line that is grown on a Type I collagen - coated (PureCol™ Inamed Biomaterials, Fremont, CA) 100×20 mm dishes (Corning Glass, Corning, NY, USA) in serum-free, growth factor-defined media at 10% CO_2 and 37°C , as described elsewhere (Davis, JW 2000;Burdick et al., 2003). For treatments, MCF-10A cells, passage 42, were plated into nine 100 mm dishes at 3.5×10^5 cells per dish in 5 ml SFIHE (serum free media containing growth factors insulin, hydrocortisone epidermal growth factor) with 2% FBS, after 24 hours the media was removed and fresh SFIHE was added. On day 5 media was removed and cells were placed in serum free media containing hydrocortisone (SFH) and was without growth factors for approximately 24 hours, media was then replaced with treatment media in SFH. Treatments were run in triplicate at $1\mu\text{M}$ 1,6-BPQ and 3,6-BPQ and DMSO served as the vehicle control all treatments contained 0.1% DMSO. Cells remained in treatment media for 18 hours and were then harvested by trypsinization and like treatments were combined.

Transient Transfection Reporter Analysis

Transfection of MCF10A cells was performed in 12-well tissue culture plates using the FuGene 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN) according to manufacturer's instructions. Exposure to quinones was performed as specified, 24 hr after transfection. Normalization of transfection efficiency was performed using *Renilla* luciferase activity from the pRL-TK vector (Promega, Madison, WI), which was cotransfected with the response element reporter vectors. The *Renilla* Luciferase Assay Lysis Buffer (Promega) was used to acquire extracts for luciferase activity 72 hr after transfection. *Renilla* luciferase and firefly luciferase activities were determined using the *Renilla* Luciferase Assay System (Promega) and the Luciferase Assay System (Promega) according to assay instructions on a TD-20/20 Luminometer (Turner Designs Instruments, Sunnyvale, CA). To determine activation of the ARE, transfections were performed using pNQO1hARE vector (Moinova and Mulcahy, JBC, 1998). For backbone control, the pT81luc was used (Moinova and Mulcahy, JBC, 1998). To determine modulation of DRE activation, transfections were performed using pGudLuc6.1a; and the pGL3-basic vector (Promega) was used a backbone control (Phelan et al., 1998).

Isolation of total RNA

RNeasy Mini kits and QIA Shredder kits (Qiagen, Valencia, CA, USA) were used to isolate total RNA from treated cells. Trypsinized cells were collected into 15ml sterile, conical centrifuge tubes and centrifuged at $250 \times g$ for 10 minutes, the media was aspirated and following the provided protocol for Animal Cells, 600 μl of RLT buffer (supplied in kit) containing 2-Mercaptoethanol was placed onto each cell pellet, cells were vortexed and sample was then placed onto a QIA Shredder column. Columns were centrifuged at approximately $13,400 \times g$ for 2 minutes. The kit directions were then followed to complete the isolation process. RNA was eluted with 50 μl RNase free water and total RNA was calculated. RNA yields ranged from 45 μg to 53 μg . RNA integrity was assessed using the Agilent. After

confirmation of quality RNA, 18S and 28S peaks were clearly identified; cDNA synthesis was initiated using Affymetrix Human Genome U133A Array kit (Affymetrix, Santa Clara, CA)

Affymetrix gene arrays

Instructions provided by Affymetrix were followed in processing samples for assay on the Human Genome U133A Array (Assay ID 510681, Affymetrix Inc., Santa Clara, CA, USA). Briefly double stranded (ds) cDNA was synthesized from total RNA, the ds cDNA was cleaned up and ethanol precipitated. An *in vitro* translation was then performed and the resulting cRNA was quantified, and amounts were adjusted following the provided instructions. The cRNA was then fragmented and mixed into a hybridization cocktail which was applied to the U133A array and hybridized for 24 hours. The hybridization buffer was removed from the array. The array was then washed, stained and scanned. KUGR facility (UNM, Alb. NM, USA).

Real-time quantitative polymerase chain reaction (qRT-PCR)

Gene induction of seven genes, heme oxygenase 1 (HMOX-1; Assay ID HS00157965_m1), NAD(P)H dehydrogenase, quinone 1 (NQO1; Assay ID HS00168547_m1), epoxide hydrolase 1 (EPHX1; Assay ID HS00164458_m1), cytochrome P450, subfamily 1A1 (CYP1A1, Assay ID HS00153120_m1), cytochrome P450, subfamily 1B1 (CYP1B1, Assay ID HS00164383_m1), aldehyde dehydrogenase 3 family member A1 (ALDH3A1, Assay ID HS00167469_m1) and aldo-ketoreductase family 1 member C1 (AKR1C1, Assay ID HS00413886_m1) were measured by qRT-PCR. TaqMan® Gene Expression Assays for each of the above genes were purchased from Applied Biosystems (Foster City, CA). MCF-10A cells were treated in triplicate for 18 hours and MCF-10A total RNA was collected as described above. Reverse transcription reactions were completed using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Real-time polymerase reactions (PCR) were carried out using TaqMan® Universal PCR Master Mix and 8 ng (total RNA) template cDNA, for detection of CYP1A1 32ng (total RNA) of template cDNA was used. PCR thermal cycling parameters used were AmpErase UNG Activation 2 min at 50°C, polymerase activation 10 min 95°C, melt 15 sec at 95°C, anneal and extend 1 min at 60°C (melt and anneal/extend for 40 cycles). Comparative C_T (first amplification cycle exceeding threshold) method was used for relative quantification. GADPH was the endogenous reference and DMSO served as the calibrator. Real-time PCR was run on Applied Biosystem's 7900HT system with a standard 384-well block. To calculate the comparative C_T, briefly, we determined the difference in C_T (ΔC_T) values between target and endogenous control, then determined the fold difference in gene expression ($\Delta \Delta C_T$) the ΔC_T of the calibrator was subtracted from the ΔC_T of the target ($\Delta C_T \text{ target} - \Delta C_T \text{ calibrator}$). This calculation is described in detail by Applied Biosystems.

Analysis of mRNA data and statistics

The raw data from the Affymetrix chips were normalized using the Lowess procedure; data were further averaged for each 2-chip pair. All values for which at least one of the chip pairs had a normalized value below 100 was set to zero (minimum) in order to maximize the signal-to-noise ratio. On this basis, all columns were set at ZERO for 10,569 genes, for which the data were considered noise. The analysis presented further in this work was performed on the remaining 11,714 genes, which have at least one non-zero average column. 6. For these 11,714 genes, "Fold" ratios were computed per experiment pair, using the corresponding 2-chip averages of the BPQ exposure, compared to DMSO controls. The distribution of "Fold" ratios was then analyzed assuming a Gaussian distribution. Genes that had "Fold" changes equal to or above six times the standard deviation were individually examined for this study.

Computer-Based Promoter Analysis for Antioxidant- and Dioxin-Response Elements

The promoter region of genes with significant increases in gene expression after exposure to benzo(a)pyrene quinones were assessed for the presence of antioxidant and dioxin-response elements using computer-based methods. Ten-thousand basepairs of promoter sequence upstream of the transcriptional initiation sites (as specified in Entrez Gene) were acquired from the National Center for Biotechnology Information Map Viewer website (i.e., http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=9606). This sequence was analyzed for the presence of canonical antioxidant- and dioxin- response elements in promoter regions using MatInspector Software (Genomatix Software, Munich, Germany - <http://www.genomatix.de/>). Antioxidant response elements were identified as nuclear factor (erythroid-derived 2)-like 2, NRF2 – binding sites. Dioxin response elements were identified as aryl hydrocarbon/dioxin receptor, aryl hydrocarbon receptor/ARNT heterodimers, or aryl hydrocarbon/ARNT heterodimers, fixed core binding sites.

RESULTS

Affymetrix Discovery of Genes Induced by 3,6-BPQ and 1,6-BPQ

Three sets of Affymetrix gene chip experiments were conducted to determine the similarities and differences between mRNA expression profiles obtained following 3,6-BPQ and 1,6-BPQ treatments. Because we have previously shown that both 3,6-BPQ and 1,6-BPQ activate EGFR (Burdick et al, 2003) and also likely IGF-1R (Tannheimer et al., 1998) signaling pathways, we performed studies in serum-free media following an 18 hr removal of both EGF and insulin. Preliminary work demonstrated that the optimal exposure concentration for treatment of MCF-10A cells with BPQs was 1 μ M. Short term treatments (up to 24 hrs) produced no cytotoxicity. Previous experiments also suggested that many of the genes that we were interested in evaluating would require 6-18 hrs of exposure before an increase in mRNA would be detected. Therefore, our initial experiments examined the effects of 1 μ M 3,6-BPQ and 1 μ M 1,6-BPQ on mRNA expression following treatment of MCF-10A cells for 18 hrs.

Two experiments were conducted with 3,6-BPQ to determine the reproducibility of findings. In experiment 1, three 3,6-BPQ-treated cultures of MCF-10A cells were pooled and extracted for total RNA. Three control cultures of MCF-10A cells were treated with the control solvent (DMSO at 0.1%). Duplicate Affymetrix U-133G array chips were then used for analysis of mRNA expression. The raw data were normalized and processed, and the Gaussian distribution of fold induction changes was used to compare treated cells to control cells. This experiment was repeated a second time and the results of the mRNA fold induction are displayed statistically. In Figure 1, the combined results of the two experiments are shown. Data is plotted as statistical distribution (σ number or number of standard deviations from the mean fold change) of Affy probes versus the fold change in 3,6-BPQ-treated cells compared to controls. Approximately half (11,714 out of 22,000 gene probes) exceeded the twice background level of fluorescence required for analysis (Figure 1). As shown in Figure 1, there were 40 genes that demonstrated fold changes at 6σ ($p < .00001$) or greater. The identities of these genes are shown in Table I. The results shown in Table I are separated for Experiments 1 and 2, and are ranked by the 3,6-BPQ fold induction calculated from the fluorescence intensity values from the Affy chip. Although Affy chips are not used for mRNA quantitation, we found remarkably good agreement in the magnitude of fold changes produced by the 40 genes induced by 3,6-BPQ treatment. We used the average of the two experiments to plot the data shown in Figure 1. As a part of Experiment 2, we also added another test group of MCF-10A cells that were treated with 1 μ M 1,6-BPQ for 18 hrs. The statistical distribution of fold changes for probes is shown in Figure 2. In this experiment, we identified 63 genes that were induced 6σ or greater by 1,6-BPQ. The identities of these genes are shown in Table II.

The results obtained in the two experiments and reported in Tables I and II revealed a remarkable similarity in the gene lists that were induced by 3,6-BPQ and 1,6-BPQ with a notable exception that CYP1A1 was induced by 3,6-BPQ and not by 1,6-BPQ. A casual inspection of the other genes reveals many genes that are associated with the Ah receptor and activation of dioxin (or xenobiotic) response elements (DRE or XRE). Other genes identified with this method were found to be possibly associated with oxidative stress and activation of anti-oxidant (or electrophilic) response elements (ARE or EpRE) using the Biocarta[®] (<http://biocarta.com/genes/allpathways.asp>) mapping system (data not shown).

In Table III, we compared the results shown in Tables I and II and found that we could explain the genes most significantly induced by 3,6-BPQ and 1,6-BPQ as having either DREs/XREs or AREs/EpREs in their upstream (10 kB analysis) promoters. HMOX1 was the top gene induced by both 3,6-BPQ and 1,6-BPQ, presumably through both DREs and AREs. The majority of highly induced genes induced by 3,6-BPQ and 1,6-BPQ are associated with the activation of the AhR through the DRE (CYP1A1, CYP1B1, EPHX1, NQO1 and NQO2) or oxidative stress and/or electrophilic action through the ARE/EpRE (NQO1, ALDH3A1, UGT1A9). Interestingly a number of AKR genes were identified as activated via BPQs (AKR1C1, AKR1C2, and AKR1B10). A number of novel genes were identified as activated by BPQs through DREs or AREs/EpREs, including SLC7A11, GCLC, GCLM, PIR, ASPH, POGZ, EGFR, SQSTM1, ING1, IER3, and VIPR1.

Differential Activation of DRE and ARE/EpRE Constructs by BaP and BaP metabolites

To test the hypothesis that 3,6-BPQ and 1,6-BPQ activate MCF-10A genes through signaling pathways that activate DREs and AREs in MCF-10A cells, we performed transient transfection experiments in MCF-10A cells. In Figure 3, we found that BaP and a wide variety of its metabolites, including the 3,6- and 1,6-BPQs were found to activate the DRE-luciferase construct. These results are similar to those previously reported by Burczynski and Penning (2000). Interestingly, we found that 3,6-BPQ was a better activator of the DRE-luciferase than was 1,6-BPQ indicating that it may have more significant AhR effects than 1,6-BPQ. This observation may explain why 3,6-BPQ induced CYP1A1 mRNA, whereas 1,6-BPQ did not.

3,6-BPQ and 1,6-BPQ were also examined for their ability to activate an ARE sequence that was identified from the NQO1 promoter region and was cloned into a transient transfection system for luciferase detection (Phelan et al., 1998). As shown in Figure 4, we found that both 1,6-BPQ and 3,6-BPQ were capable of activating the ARE-luciferase construct in MCF-10A cells; however, 1,6-BPQ appeared to have better activity than did 3,6-BPQ. This result is not surprising, as we have shown that 1,6-BPQ generates more ROS than does 3,6-BPQ through redox-cycling (Burdick et al., 2003), confirming our previous observation that ROS may play a role in activating ARE.

qPCR Experiments for Gene Validation and Examination of Role of ARE and DRE in BPQ gene induction

In a final series of experiments, we tested the hypothesis that DRE and ARE are responsible for several key genes induced by 3,6-BPQ and 1,6-BPQ. In these experiments we compared the effects of these BPQs to well-established activators of the AhR/DRE pathway by TCDD (Pelkonen and Nebert, 1982) and the ARE/EpRE pathway by TBHQ (Nguyen et al., 2004). We utilized quantitative qRT-PCR and Taqman probes that were matched to the Affymetrix sequences used on arrays. Because we have previously shown that BPQs activate EGFR signaling pathways (Burdick et al., 2003), we also included EGF as a control for gene activation because we have previously shown that BPQs activate EGFR (Burdick et al., 2003). Results showed (Figures 7–9) that the response of the seven genes examined (HMOX-1, CYP1A1, CYP1B1, EPHX1, NQO1, AKR1C1, and ALDH3A1) could be categorized as either DRE >

ARE (CYP1A1 and CYP1B1 - Figure 6), ARE > DRE (HMOX1, AKR1C1, EPHX1 – Figure 7) or DRE ~ ARE (ALDH3A1 and CYP1B1 – Figure 8) responsive based on the induction of these genes to TCDD or TBHQ. CYP1A1 was most selectively induced by TCDD and by 3,6-BPQ. EGF did not activate any of the seven genes that we examined. These results support the conclusion of the previous finding that 3,6-BPQ has a more selective effect on AhR and DRE pathways than does 1,6-BPQ.

DISCUSSION

Our laboratory has shown that several BaP metabolites activate signaling pathways in mammary epithelial cells associated with increased intracellular Ca^{2+} (Tannheimer et al., 1997,1999), protein tyrosine kinase signaling (Tannheimer et al., 1998), cell proliferation and increased cell survival (Davis et al., 2001,2003;Burdick et al., 2003,2006). We have also shown that Ah receptor (AhR) agonists, such as TCDD, increase the production of TGF α leading to increased epidermal growth factor (EGF) signaling (Davis et al, 2001). Recently we showed that 1,6-BPQ and 3,6-BPQ activate EGFR signaling pathways in MCF-10A cells leading to activation of Erk and Akt leading to increased cell proliferation and survival (Burdick et al., 2003). Both 1,6- and 3–6 BPQ produced oxidative stress and EGFR signaling in MCF-10A cells, that could be prevented by overexpression of catalase or treatment with anti-oxidants. BP-quinones are well known redox-cycling agents that have been shown to exert oxidative stress in cells (Zhu et al., 1998).

The present studies were prompted by the observation that both 1,6-BPQ and 3,6-BPQ produced growth promoting effects in MCF-10A cells, yet 1,6-BPQ results in substantially more ROS formation, suggesting that other pathways might also be activated by these agents. We used gene expression array techniques to elucidate the pathways activated by BPQs. Our results showed that several classes of genes were induced by both BPQs, whereas there were several genes selectively induced by 1,6-BPQ and 3,6-BPQ. Based on the patterns of genes induced, promoter analyses of those genes, and the ability of BPQs to preferentially activate DRE compared to ARE luciferase constructs, we conclude that three main pathways are activated by BPQs: 1) AhR-dependent pathways associated with activation of DRE, signaling, and metabolism of PAHs and steroids; 2) oxidative stress pathways likely associated with Nrf2 and ARE activation; and 3) EGFR activated pathways. We also identified a series of novel gene targets (AQP3, BTN3A3, ME1, PIR and POGZ) that we cannot specifically link to known pathways based on current annotations. Because there is a clear overlap between many of the induced genes that have both DRE and ARE present in their promoter sequences, we will discuss the DRE and ARE pathways together.

We identified 23 genes that were induced by both 1,6-BPQ and 3,6-BPQ. The top gene in terms of fold induction by both agents was HMOX1. HMOX1 has been clearly associated with protection from oxidative stress in animals through the bilirubin pathway (Prawan et al., 2005). Its presence in mammary epithelial cells has not been reported, and its activation by BPQs has not been previously shown. Based on the finding that HMOX1 has both DRE and ARE present in its promoter region, it is likely that both AhR binding and oxidative stress (ROS) activity plays a role in HMOX induction. However, based upon our findings with TCDD and TBHQ, there does appear to be a simple explanation of how HMOX is regulated in MCF-10A cells. It is clear that HMOX is regulated via several direct and indirect mechanisms in many mammalian cells (Alam et al., 2004).

Other oxidative stress associated genes likely induced by a combination of DRE and ARE activation included NQO1, NQO2, and ALDH3A1. NQO1 and 2 are particularly important because they play a role in metabolism of quinones such as BPQs (Vasilidou et al, 2006). Although we found numerous DREs upstream in the promoter region of NQO1, the possibility

that TCDD induced increases in NQO1 is indirect and due to oxidative stress cannot be excluded (Radjendirane and Jaiswal, 1999). ALDH3A1 also plays a role in protecting cells from toxic aldehydes and is part of a battery of phase 2 enzymes induced by Nrf2 sensing of redox-active agents and electrophiles (Vasiliou et al., 2004). Another phase 2 gene induced by both BPQs was UGT1A9 (Wooster et al., 1991). PAH and steroid metabolism genes induced by BPQs included CYP1A1, CYP1B1, and EPHX1. It is interesting that CYP1B1 and EPHX1 were induced by both the 3,6-BPQ and 1,6-BPQ, whereas only 3,6-BPQ induced CYP1A1 in MCF-10A cells. This may have been due to the greater DRE activating properties of the 3,6-BPQ detected in the DRE luciferase assay. Other genes associated with oxidative stress included SLC7A11 which is associated with cystine and glutamate uptake for glutathione synthesis (Kaleeba and Berger, 2006), GCLC which is the rate limiting gene for glutathione synthesis, and the GCLM gene that regulates GCLC (Gipp et al., 1995; Gipp and Mulcahy, 2000.)

Several AKR family (AKR1C1, AKR1C2, and AKR1B10) genes were found to be induced by BPQs, presumably through the action of both DRE and ARE (Lou et al., 2006). AKR1C1 is particularly noteworthy because this enzyme converts BaP-7,8-dihydrodiol to the 7,8-ortho-BPQ which has been shown to be a tumor initiator (Jin and Penning, 2006; Balu et al., 2004) as well as a Ca^{2+} signaling agent (Gao et al., 2005). Other receptor signaling genes that were detected were EGFR, AhR, and VIPR1 (Couvineau et al., 1994), although it is unclear if this latter receptor is active in mammary epithelial cells. Other signaling genes included ASPH that has been proposed to catalyze aspartyl beta-hydroxylation of EGF domains and modulate Notch pathway signaling. Dinchuk et al. (2002), IER-3 Charles et al. (1993) cloned gly96, a mouse immediate-early gene inducible by serum growth factors that may play a role in reducing apoptosis, ING-1 Garkavtsev et al. (1996) reported that expression of high levels of transfected constructs of ING1 regulated cell growth, and SQSTM1 P62 which is a ligand for Src kinases (Park et al., 1995)

There were several genes that were selectively induced by 3,6-BPQ including CYP1A1 (discussed above), clusterin (CLU, a potential tissue remodeling protein, Jenne and Tschopp, 1992), TRIM16 (or EBBP which plays a role in estrogen or EGF activation of mammary epithelial cells, Beer, et al., 2002), CYP4F3 (a LTB4 hydroxylase, Kikuta et al., 1998), and the DNA repair gene TREX1 (Mazur and Perrino, 1999).

In summary, our studies demonstrate that DRE and ARE play a major role in the response of human mammary epithelial cells to BPQs. EGF receptor signaling may also contribute to genes induced by BPQs, although none of the seven genes that we chose for close examination (CYP1A1, CYP1B1, EPHX1, HMOX1, NQO1, AKR1C1, and ALDH3A1) were responsive to EGF treatment in MCF-10A cells. NQO1 is a major pathway responsible for protection of cells against quinone toxicity. This gene was highly induced in MCF-10A cells. Because of the known polymorphisms in NQO1, it will be of interest to determine whether the response of normal human mammary epithelial cells (HMEC) correlates with the differential expression or activity of this enzyme. We predict that people who express the NQO1*2 allele (Larson et al., 1999) will be supersensitive to quinones, which will be evidenced by extremely low concentrations (low nanomolar) causing oxidative stress. This hypothesis is currently being investigated as a mechanism for differential sensitivity to oxidative stress produced by quinones associated with breast cancer promotion.

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Figure 1.

Statistical distribution (σ = one standard deviation) of Affymetrix U133-A gene chip probes produced by 3,6-BPQ following an 18 hr incubation with MCF-10A cells. Results are shown for two combined experiments obtained following treatment of 3 cell cultures and pooling of RNA. Data is displayed for each Affymetrix probe as the fluorescence ratio of signals for all 3-6-BPQ treated compared to control solvent (0.01% DMSO) cell culture RNA samples that had fluorescence values equal or greater than twice the background fluorescence.

Figure 2.

As in Figure 1, statistical distribution of Affymetrix U133-A gene chip probes produced by 1,6-BPQ following an 18 hr incubation with MCF-10A cells.

Figure 3. DRE-luciferase results for MCF-10A transfected cells following 18 hr treatment with 1 µM 3,6-BPQ compared to 1,6-BPQ. Results are shown for a representative experiment with triplicate cultures of cells analyzed individually and expressed as the average $\pm$ SD of the mean. Both the 1,6-BPQ and 3,6-BPQ produced a statistical significant increase in DRE-luciferase activity ($p < .01$).

Figure 4.

ARE-luciferase results for MCF-10A transfected cells following 18 hr treatment with 1 μ M 3,6-BPQ compared to 1,6-BPQ. Results are shown for a representative experiment with triplicate cultures of cells analyzed individually and expressed as the average $\pm$ SD of the mean. Both the 1,6-BPQ and 3,6-BPQ produced a statistical significant increase in ARE-luciferase activity ($p < .01$).

DRE > ARE 18 Hr Activity in MCF-10A Cells

3,6-BPQ > 1,6-BPQ

Figure 5.

qRT-PCR results for CYP1A1 and CYP1B1 where the DRE activity appears to be greater than the ARE activity based on the activation of these genes by TCDD (DRE activator) and TBHQ (ARE activator). MCF-10A cells were treated with DMSO (solvent control), TCDD (10 nM), TBHQ (20 μ M), 10 μ g/ml EGF, 1 μ M 1,6-BPQ, or 1 μ M 3,6-BPQ for 18 hrs, followed by extraction of RNA and Taqman analysis. Results shown are the Mean $\pm$ SD for triplicate determination using Taqman probes ($p < .01$).

ARE > DRE Activity in MCF-10A Cells

Figure 6.

qRT-PCR results for HMOX1, AKR1C1, NQO1, and EPHX1 where the TBHQ ARE activity appears to be greater than the TCDD DRE activity. MCF-10A cells were treated with DMSO (solvent control), TCDD (10 nM), TBHQ (20 μ M), 10 μ g/ml EGF, 1 μ M 1,6-BPQ, or 1 μ M 3,6-BPQ for 18 hrs, followed by extraction of RNA and Taqman analysis. Results shown are the Mean $\pm$ SD for triplicate determination using Taqman probes ($p < .01$).

ARE ~ DRE 18 Hr Activity in MCF-10A Cells

3,6-BPQ > 1,6-BPQ

Figure 7.

qRT-PCR results for ALDH3A1 and CYP1B1 where the TBHQ ARE activity appears to be equivalent to the TCDD DRE activity. MCF-10A cells were treated with DMSO (solvent control), TCDD (10 nM), TBHQ (20 μ M), 10 μ g/ml EGF, 1 μ M 1,6-BPQ, or 1 μ M 3,6-BPQ for 18 hrs, followed by extraction of RNA and Taqman analysis. Results shown are the Mean $\pm$ SD for triplicate determination using Taqman probes ($p < .01$).

TABLE 1
Six Sigma Gene Changes for 3,6 BPQ sorted according to the AVERAGE FOLD

Probe	Gene	Description	FOLD 3,6- BPQ AVERAGE	FOLD 3,6- BPQ Exp 1	FOLD 3,6- BPQ Exp 2
203665_at	HMOX1	heme oxygenase (decycling) 1	32.3	33.4	31.3
205749_at	CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1	12.9	15.7	10.3
206561_s_at	AKR1B10	aldo-keto reductase family 1, member B10 (aldose reductase)	6.6	5.3	7.9
205623_at	ALDH3A1	aldehyde dehydrogenase 3 family, member A1	6.2	5.7	6.7
201467_s_at	NQO1	NAD(P)H dehydrogenase, quinone 1	5.5	3.4	7.7
209921_at	SLC7A11	solute carrier family 7, (cationic amino acid transporter, y+system) member 11	5.2	3.5	6.9
201468_s_at	NQO1	NAD(P)H dehydrogenase, quinone 1	4.9	3.8	6.1
202436_s_at	CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	4.1	3.3	5.0
210519_s_at	NQO1	NAD(P)H dehydrogenase, quinone 1	3.9	2.8	5.0
203925_at	GCLM	glutamate-cysteine ligase, modifier subunit	3.8	3.3	4.2
207469_s_at	PIR	pirin (iron-binding nuclear protein)	3.7	2.7	4.8
210896_s_at	ASPH	aspartate beta-hydroxylase	3.7	2.9	4.5
208791_at	CLU	clusterin	3.6	4.3	3.0
209641_s_at	ABCC3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	3.4	1.9	4.9
201631_s_at	IER3	immediate early response 3	3.4	3.3	3.4
217678_at	SLC7A11	solute carrier family 7, (cationic amino acid transporter, y+system) member 11	3.3	1.6	5.0
201195_s_at	SLC7A5	solute carrier family 7 (cationic amino acid transporter, y+system), member 5	3.2	2.7	3.8
202435_s_at	CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	3.3	1.8	4.6
202434_s_at	CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	3.3	3.2	3.2
209160_at	AKR1C3	aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II)	3.1	2.4	3.9
204341_at	TRIM16	tripartite motif-containing 16	3.1	3.1	3.1
204058_at	ME1	malic enzyme 1, NADP(+)-dependent, cytosolic	3.1	1.6	4.6
202923_s_at	GCLC	glutamate-cysteine ligase, catalytic subunit	2.8	2.1	3.5
217626_at	AKR1C1	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)	2.7	3.7	1.8
205019_s_at	VIPR1	vasoactive intestinal peptide receptor 1	2.7	2.3	3.1
39249_at	AQP3	aquaporin 3 (Gill blood group)	2.6	2.3	3.0
202922_at	GCLC	glutamate-cysteine ligase, catalytic subunit	2.6	1.8	3.5
204532_x_at	UGT1A10	UDP glucuronosyltransferase 1 family, polypeptide A10	2.6	3.2	2.0
209699_x_at	AKR1C2	aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)	2.6	1.8	3.3
209135_at	ASPH	aspartate beta-hydroxylase	2.6	1.7	3.4
216594_x_at	AKR1C1	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)	2.6	2.0	3.2
204820_s_at	BTN3A3	butyrophilin, subfamily 3, member A3	2.6	2.3	2.8
204151_x_at	AKR1C1	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)	2.5	1.8	3.1
206515_at	CYP4F3	cytochrome P450, family 4, subfamily F, polypeptide 3	2.5	2.2	2.7
202437_s_at	CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	2.4	1.7	3.2
205875_s_at	TREX1	three prime repair exonuclease 1	2.4	2.7	2.2
212153_at	POGZ	pogo transposable element with ZNF domain	2.4	2.1	2.7
211653_x_at	AKR1C2	aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)	2.4	1.8	2.9
202017_at	EPHX1	epoxide hydrolase 1, microsomal (xenobiotic)	2.4	2.0	2.8
218012_at	TSPYL2	TSPY-like 2	2.4	1.5	3.3

TABLE 2
Six Sigma Gene Changes for 1,6 BPQ sorted according to FOLD

Probe	Gene	Description	FOLD 1,6-BPQ
203665_at	<u>HMOX1</u>	heme oxygenase (decycling) 1	16.5
209921_at	<u>SLC7A11</u>	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	7.6
206561_s_at	<u>AKR1B10</u>	aldo-keto reductase family 1, member B10 (aldose reductase)	6.3
201467_s_at	<u>NQO1</u>	NAD(P)H dehydrogenase, quinone 1	5.8
210896_s_at	<u>ASPH</u>	aspartate beta-hydroxylase	5.7
217678_at	<u>SLC7A11</u>	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	5.1
204058_at	<u>ME1</u>	malic enzyme 1, NADP(+)-dependent, cytosolic	4.9
202923_s_at	<u>GCLC</u>	glutamate-cysteine ligase, catalytic subunit	4.4
201468_s_at	<u>NQO1</u>	NAD(P)H dehydrogenase, quinone 1	4.4
210519_s_at	<u>NQO1</u>	NAD(P)H dehydrogenase, quinone 1	4.4
209641_s_at	<u>ABCC3</u>	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	4.3
209160_at	<u>AKR1C3</u>	aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II)	4.2
207469_s_at	<u>PIR</u>	pirin (iron-binding nuclear protein)	4.2
209135_at	<u>ASPH</u>	aspartate beta-hydroxylase	4.1
202922_at	<u>GCLC</u>	glutamate-cysteine ligase, catalytic subunit	3.8
209699_x_at	<u>AKR1C2</u>	aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)	3.7
208415_x_at	<u>ING1</u>	inhibitor of growth family, member 1	3.6
216594_x_at	<u>AKR1C1</u>	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)	3.5
204151_x_at	<u>AKR1C1</u>	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)	3.4
213112_s_at	<u>SQSTM1</u>	sequestosome 1	3.4
211653_x_at	<u>AKR1C2</u>	aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)	3.4
221432_s_at	<u>SLC25A28</u>	solute carrier family 25, member 28	3.3
203925_at	<u>GCLM</u>	glutamate-cysteine ligase, modifier subunit	3.3
202435_s_at	<u>CYP1B1</u>	cytochrome P450, family 1, subfamily B, polypeptide 1	3.2
218446_s_at	<u>FAM18B</u>	family with sequence similarity 18, member B	3.2
202436_s_at	<u>CYP1B1</u>	cytochrome P450, family 1, subfamily B, polypeptide 1	3.2
212153_at	<u>POGZ</u>	pogo transposable element with ZNF domain	3.2
209121_x_at	<u>NR2F2</u>	nuclear receptor subfamily 2, group F, member 2	3.1
204341_at	<u>TRIM16</u>	tripartite motif-containing 16	3.1
212151_at	<u>PBX1</u>	pre-B-cell leukemia transcription factor 1	3.0
212767_at	<u>MTG1</u>	mitochondrial GTPase 1 homolog (S. cerevisiae)	3.0
202017_at	<u>EPHX1</u>	epoxide hydrolase 1, microsomal (xenobiotic)	3.0
205623_at	<u>ALDH3A1</u>	aldehyde dehydrogenase 3 family, member A1	2.9
209380_s_at	<u>ABCC5</u>	ATP-binding cassette, sub-family C (CFTR/MRP), member 5	2.9
204169_at	<u>IMPDH1</u>	IMP (inosine monophosphate) dehydrogenase 1	2.9
210266_at	<u>TXNRD1</u>	thioredoxin reductase 1	2.8
219034_at	<u>PARP16</u>	poly (ADP-ribose) polymerase family, member 16	2.8
203222_s_at	<u>TLE1</u>	transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)	2.8
208791_at	<u>CLU</u>	clusterin	2.8
39249_at	<u>AQP3</u>	aquaporin 3 (Gill blood group)	2.7
217626_at	<u>AKR1C1</u>	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)	2.7
221867_at	<u>LOC9683</u>	Nedd4 binding protein 1	2.7
203366_at	<u>POLG</u>	polymerase (DNA directed), gamma	2.6
214696_at	<u>LOC84981</u>	hypothetical protein MGC14376	2.6
202820_at	<u>AHR</u>	aryl hydrocarbon receptor	2.6
202210_x_at	<u>GSK3A</u>	glycogen synthase kinase 3 alpha	2.6
218639_s_at	<u>ZXDC</u>	ZXD family zinc finger C	2.6
205808_at	<u>ASPH</u>	aspartate beta-hydroxylase	2.6
211607_x_at	<u>EGFR</u>	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	2.6
219475_at	<u>LOC29948</u>	pregnancy-induced growth inhibitor	2.6
214196_s_at	<u>TPP1</u>	tripeptidyl peptidase I	2.6
203811_s_at	<u>DNAJB4</u>	DnaJ (Hsp40) homolog, subfamily B, member 4	2.5
209109_s_at	<u>TSPAN6</u>	tetraspanin 6	2.5
209592_s_at	<u>WDR68</u>	WD repeat domain 68	2.5
206515_at	<u>CYP4F3</u>	cytochrome P450, family 4, subfamily F, polypeptide 3	2.5
209005_at	<u>FBXL5</u>	F-box and leucine-rich repeat protein 5	2.5
218326_s_at	<u>LGR4</u>	leucine-rich repeat-containing G protein-coupled receptor 4	2.5
202884_s_at	<u>PPP2R1B</u>	protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), beta isoform	2.4
219534_x_at	<u>CDKN1C</u>	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	2.4
206829_x_at	<u>ZNF430</u>	zinc finger protein 430	2.4
212979_s_at	<u>LOC9747</u>	KIAA0738 gene product	2.4
215954_s_at	<u>C19orf29</u>	chromosome 19 open reading frame 29	2.4

Probe	Gene	Description	FOLD 1,6-BPQ
216237_s_at	<u>MCM5</u>	MCM5 minichromosome maintenance deficient 5, cell division cycle 46 (S. cerevisiae)	2.4

TABLE III

Analysis of Gene Promoters Induced by 1,6- and 3,6-B[a]P Quinones*

Gene Promoter	Number of Response Elements in Promoter		Response Element Location [§]	
	ARE/EpRE ¹	DRE/XRE ²	ARE/EpRE ¹	DRE/XRE ²
HMOX1 [#]	2	5	2411, 7119	2831, 4164, 6153, 7133, 7365
CYP1A1 [#]	1	13	7529	36, 379, 482, 873, 877, 965, 1038, 1364, 2099, 3058, 5594, 7497, 9171
CYP1B1 [#]	0	7	ni ³	825, 844, 980, 1015, 1483, 2304, 8531
AKR1C1 [#]	1	5	1054	1100, 2590, 2648, 3698, 8551
ALDH3A1 [#]	3	10	4930, 5174, 5660	74, 746, 1182, 2122, 2550, 4220, 4272, 5037, 7968, 8417
NQO1 [#]	1	7	381	306, 3119, 5031, 5556, 7330, 7644, 9516
EPHX1 [#]	0	8	ni	1525, 2410, 4379, 6141, 7598, 7685, 9900
AKR1B10	2	6	273, 7701	924, 1823, 5027, 5351, 6392, 9093
AKR1C2	3	0	2411, 5028, 7462	ni
SLC7A11	0	3	ni	4795, 7566, 7605
GCLC	0	5	ni	228, 2897, 3391, 3989, 5568
GCLM	3	1	2217, 3368, 9277	1966
PIR	0	6	ni	46, 78, 867, 2235, 4092, 9553
ASPH	1	4	3214	117, 405, 7716, 7838
POGZ	2	10	3868, 8575	541, 1669, 2024, 4578, 5155, 7101, 7393, 7587, 8859, 8954
AHR	1	1	4230	2598
EGFR	2	1	173, 7005	5095
SQSTM1	3	11	1901, 4087, 9936	1271, 1533, 1694, 1931, 2059, 2173, 3255, 3841, 5025, 6878, 9249
ING1	3	13	468, 1605, 7732	169, 1135, 1388, 2190, 2409, 2499, 3606, 3616, 3846, 5231, 6201, 9720, 9972
IER3	3	9	2878, 3637, 7204	431, 762, 1585, 2679, 4096, 7929, 8382, 9387, 9397
VIPR1	0	3	ni	796, 4453, 5503
UGT1A9	3	4	930, 4537, 9545	2205, 2381, 4283, 6051
NQO2	0	7	ni	701, 3047, 3724, 5329, 7439, 9588, 9600

* Promoter analysis was performed using MatInspector (Cartharius *et al.*, 2005) for 10Kb upstream of the gene transcriptional initiation site.[§] Listed as start of response element location upstream of transcriptional initiation site.[#] Expression level determined by both qPCR and microarray.¹ Antioxidant response element (ARE) = the nuclear factor (erythroid-derived 2)-like 2, NRF2 site.² Dioxin response element (DRE) or Xenobiotic response elements (XRE) = includes the aryl hydrocarbon/ dioxin receptor, aryl hydrocarbon receptor/ arnt heterodimers, and aryl hydrocarbon/ arnt heterodimers, fixed core sites.³ None identified.