Activation of the mTOR pathway by low levels of xenoestrogens in breast epithelial cells from high-risk women

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Breast cancer is an estrogen-driven disease. Consequently, hormone replacement therapy correlates with disease incidence. However, increasing male breast cancer rates over the past three decades implicate additional sources of estrogenic exposure including wide spread estrogen-mimicking chemicals or xenoestrogens (XEs), such as bisphenol-A (BPA). By exposing renewable, human, high-risk donor breast epithelial cells (HRBECs) to BPA at concentrations that are detectable in human blood, placenta and milk, we previously identified gene expression profile changes associated with activation of mammalian target of rapamycin (mTOR) pathway genesets likely to trigger prosurvival changes in human breast cells. We now provide functional validation of mTOR activation using pairwise comparisons of 16 independent HRBEC samples with and without BPA exposure. We demonstrate induction of key genes and proteins in the PI3K-mTOR pathway—AKT1, RPS6 and 4EBP1 and a concurrent reduction in the tumor suppressor, phosphatase and tensin homolog gene protein. Altered regulation of mTOR pathway proteins in BPA-treated HRBECs led to marked resistance to rapamycin, the defining mTOR inhibitor. Moreover, HRBECs pretreated with BPA, or the XE, methylparaben (MP), surmounted antiestrogenic effects of tamoxifen showing dose-dependent apoptosis evasion and induction of cell cycling. Overall, XEs, when tested in benign breast cells from multiple human subjects, consistently initiated specific functional changes of the kind that are attributed to malignant onset in breast tissue. Our observations demonstrate the feasibility of studying renewable human samples as surrogates and reinforce the concern that BPA and MP, at low concentrations detected in humans, can have adverse health consequences.

Abbreviations: AKT, v-Akt murine thymoma viral oncogene homolog 1; BPA, bisphenol-A; ER, estrogen receptor; HRBEC, high-risk donor breast epithelial cell; MFI, mean fluorescence intensity; MP, methylparaben; mTOR, mammalian target of rapamycin; OHT, 4-hydroxy tamoxifen; PI, propidium iodide; PTEN, phosphatase and tensin homolog gene; QPCR, quantitative polymerase chain reaction; ROS, reactive oxygen species; RPFNA, random periareolar fine needle aspirate; XE, xenoestrogens.

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Quantification of endogenous reactive oxygen species

Endogenous cellular reactive oxygen species (ROS) accrual was determined using live cell cultures loaded with the fluorogenic dye, carboxy-H2DCFDA (5-(and-6)-dichlorodihydrofluorescein diacetate), also known as C-400 (Invitrogen) in phenol red-free medium for 1 h followed by 30 min incubation in regular growth medium. After dye removal, cells were counterstained with PI, and intracellular oxidation of C-400 was measured by FACS-cytometry with the FL1 filter. Experiments were done in triplicate and 10,000 cells were acquired for each sample. ROS activity was expressed as mean fluorescence intensity (MFI) of the C400 dye. MFI of PI-negative (non-necrotic) cells was corrected for autofluorescence of unlabeled cells.

Results

Live HRBEC cultures display non-malignant breast epithelial attributes

In this study, HRBECs were derived from the unaffected contralateral breast tissue of 23 individual volunteers (mean age—58, range 40–81 years). Sixteen women had a personal history of breast cancer, 4 displayed high-risk histopathology, 20 had mammographically dense breasts and 19 had family history of breast cancer (supplementary Table S2 is available at Carcinogenesis Online). Routine cytology of the samples prior to cell culture showed multiple epithelial clusters (range 4 to >200). No atypia was seen (Figure 1A). RFPNA samples yielded short-term epithelial cultures with variable degrees of growth potential. Generally, the initial cell seeding produced robust growth within 2–3 weeks, enabling culture expansion for up to three passages and providing morphologically homogeneous populations of 10^6–10^7 epithelial cells. Due to the low serum concentration of the growth medium, stromal fibroblast expansion did not occur (Figure 1B). There were no selection criteria for RFPNA sampling except that the volunteer present a clinically defined increased risk of breast cancer. HRBECs were used as they grew out in sufficient numbers for one or more assays. Identification of the samples employed is included in the relevant data figures. Our overall cell culture experience with >130 RFPNA samples thus far yielded three spontaneously immortalized (IM) HRBEC cell lines, designated as IMM-PA024, IMM-PA025 and IMM-PA115, currently at passages 24–26.

Non-malignant epithelial characteristics of HRBEC cultures included:

a. Polarized growth in Matrigel—unlike breast cancer cell lines propagated in a three-dimensional matrix (which develop apolar colonies with random orientation of nuclei and the basement membrane protein, alpha-6 integrin), HRBEC colonies were distinctive. Polarized colonies, which displayed an acinar pattern of nuclear orientation and characteristic basal immunolocalization of alpha-6 integrin, were observed in all HRBEC cases tested (Figure 1C).

b. ER expression—distinct from ER-positive breast cancer cell lines, represented by T47D and MCF7, which express abundant ERα, and the ER-negative cancer cell line, SKBR3 in which ERα is undetectable, HRBEC lines (IMM-PA024, IMM-PA025 and IMM-PA115) displayed a low to moderate range of receptor protein (Figure 1D). On the other hand, levels of ERβ were relatively similar between HRBEC and cancer cell lines. Protein levels of both ER isoforms in six independent early passage HRBEC cultures (PA134, PA135, PA136, PA138, PA139 and PA140) were closely similar to those of spontaneously immortalized counterparts. We conclude that unlike many cancer cell lines, HRBECs represent an ERα-low/medium status. Levels of both ERα and ERβ proteins are consistent with the characteristics of non-malignant human breast cells (32,33).

Early passage HRBEC cultures (passage 2 or 3) of 23 independent cases were used to generate subsets of data described below. HRBECs from 16 cases were used for pairwise analysis in functional assays (supplementary Table S2 is available at Carcinogenesis Online). Like normal human epithelial cells, HRBECs display a finite life and senesce after three passages, providing a limited amount of starting experimental material. For this reason, it is usually possible to pursue only one or two whole cell-based assays from a single sample, such as determination of apoptotic fraction, ROS estimation or assays based...
on RNA amplification, for example by quantitative polymerase chain reaction (QPCR) or by microarrays (reported by us previously). Similarly, early passage HRBEC-derived protein lysates are often insufficient for quantitative sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blot analysis. Therefore, we have included immortalized-HRBEC lines in protein quantitation studies.

BPA exposure modulates the signal transduction cascade of the PI3K–mTOR pathway within non-malignant breast epithelial cells

By QPCR analysis of the subset of critical mTOR pathway genes, early passage HRBECs derived from six individuals demonstrated identifiable shifts in expression associated with BPA exposure (Figure 2A). Considerable inter-subject variability in relative transcript levels.
Fig. 2. BPA exposure modulates expression of mTOR pathway components and induces functional changes in breast epithelial cells. (A) QPCR measurements of relative transcript levels of mTOR pathway genes in early passage HRBEC cultures (PA024, PA025, PA072, PA075, PA081, and PA112) normalized to housekeeping genes. Data represent exposure to a low dose range of BPA or to a luteal serum estradiol (E2) level. Plotted values represent fold change for each gene in treated samples, relative to the corresponding untreated control sample. Each data point is an average of triplicate QPCRs. (B) BPA-induced alterations in mTOR pathway proteins in early passage (PA138 and PA140), immortalized HRBECs (IMM-PA024, IMM-PA025, and IMM-115) and breast cancer cells (T47D). Pretreatment with BPA reduces steady-state PTEN protein levels and promotes functional inactivation by increased phosphorylation (indicated by ‘P’) at sites S380/T382/T383 compared with untreated controls. Increased expression of total and phosphorylated AKT1 (at S473), RPS6 (at S235/236) and 4EBP1 (at multiple sites) is detectable in both non-malignant and malignant breast cells. Asterisks indicate shift in the molecular weight of phosphorylated 4EBP1. (C) Effects of BPA pretreatment in the induction of resistance to the mTOR inhibitor, rapamycin. Data plotted to represent Annexin V-positive apoptotic populations within breast cancer cell lines (T47D, SKBR3—left panel) and HRBEC lines (IMM-PA024, IMM-PA025, IMM-PA115—right panel). Each bar represents the mean and standard deviation of triplicate values. Increased apoptotic ratios were observed with increasing doses of rapamycin in all cultures without BPA pretreatment. Values demonstrating the effect of BPA in reducing rapamycin-induced apoptosis were statistically significant in all cell lines (\( P > 0.001 \)).
was associated with the cellular BPA response, due to which dose–response relationships were not detectable in this small sample size. However, normalized to housekeeping genes, a consistent decline was noted in transcript levels of the suppressor genes, PTEN (P = 0.02, two-tailed t-test against theoretical ratio = 1.0), TSC1 and TSC2 (P = 0.03). Conversely, downstream activators of the mTOR pathway, *eIF4B* and *eIF4E*, were upregulated in the presence of BPA (P = 0.003). Expression of *PIK3R1*, another mTOR activator, was elevated (P = 0.02), transcript levels of *RPS6*, a downstream effector of mTOR that is activated by phosphorylation, were unexpectedly lower (P = 0.03) and there was a trend (P = 0.07) toward increased mTOR transcripts.

Confirmation of the observed transcriptional alterations was subsequently pursued at the protein level comparing two early passage HRBEC cultures, three immortalized HRBEC lines and an ERα-positive breast cancer cell line, T47D (Figure 2B). As predicted by gene transcript quantitation, BPA exposure led to the following: a significant reduction in total PTEN protein levels; an increase in the inactive phosphorylated form of PTEN (PTEN<sup>Ser380/292/3</sup>) and increased total and phosphorylated (activated) forms of the mTOR activating kinase, AKT1 and two major downstream targets of mTOR: RPS6 and of 4EBP1 (at multiple sites, as shown by the increased molecular weight of the phosphorylated protein). Unlike RPS6 transcript data, pRPS6 levels were representative of mTOR pathway activation. Increased phosphorylation observed for upregulated proteins is strongly indicative of increased mTOR activity and consistent with the finding of higher mTOR transcript levels.

**XE exposure induces evasion of apoptotic cell death in HRBEC cultures**

In order to establish additional functional association with mTOR pathway regulation, we asked whether BPA pretreatment altered the sensitivity of IMM-HRBEC lines to the antitumor drug—rapamycin, a potent mTOR inhibitor. A significant reduction in rapamycin-induced apoptotic cell death was observed after BPA exposure of immortalized HRBECs and in ERα-positive as well as ERα-negative breast cancer cells (Figure 2C). This effect of BPA in circumventing cell death was most prominent at 100 nM rapamycin, a concentration at which the apoptotic ratio of drug-treated versus untreated control was >2-fold in the absence of prior BPA exposure.

To further assess a role for XEs in the evasion of apoptotic cell death, the antitesticogenic OHT was used to initiate cell death. Early passage HRBECs from eight subjects were compared with IMM-HRBEC lines and with breast cancer cell lines. Percent apoptotic cells were estimated in untreated control cells, those pretreated with 100 nM BPA or 1 μM MP prior to a 24 h OHT exposure or those exposed to OHT alone. Although the percentage of apoptotic cells was doubled in the presence of OHT alone in all cell cultures tested, the effect of OHT was almost undetectable in XE-treated cultures (Figure 3A). Representative FACS data are illustrated in Figure 3B.

In separate experiments, early passage HRBECs were evaluated for the potential to evade apoptosis after exposure to a wide range of BPA and MP concentrations (Figure 3C as averaged data and supplementary Figure S1 is available at Carcinogenesis Online for each sample). Maximum protection from apoptotic death was conferred by exposure to 100 nM BPA (58.39 ± 6.8%). An appreciable degree of apoptosis reduction was also observed at 10- to 100-fold lower BPA doses: 52.84 ± 6.8% at 10 nM, 41.24 ± 10.4% at 1 nM and 19.93 ± 7.9% at 100 pM (Figure 3C, left panel). A significant dose–response was observed when log BPA concentration was regressed on log percent reduction in apoptosis (P = 0.002, two-sided test for logistic regression). MP exposure of HRBECs also dramatically reduced the fraction of OHT-induced apoptotic cells at all three doses tested: 57.82 ± 6.77% at 1 μM (Figure 4A), 55.93 ± 10.54% at 100 nM and 28.14 ± 11.3% at 10 nM (Figure 3C, right panel). Similar to the BPA dose response, MP-induced changes in HRBECs were also concentration dependent (P = 0.001).

**XE exposure modulates cellular oxidative stress in HRBEC cultures**

To determine whether resistance to OHT-induced apoptosis in BPA or MP-pretreated cells was accompanied by the maintenance of proliferative potential, we measured the efficiency of bromodeoxyuridine incorporation during the S-phase of the cell cycle in IMM-HRBEC lines. Prior exposure to a dose range of either BPA or MP resulted in a dramatic, concentration-dependent complete to partial evasion from the G1-phase arrest induced by 10 μM OHT and a concurrent increase in the S-phase fraction (Figure 5A and B). In contrast, the growth inhibitory effects of OHT were not reversed by a simulated physiological concentration of 17β-estradiol and progesterone in the absence of BPA (data not shown). As with apoptosis evasion, maintenance of S-phase in OHT-treated cells was significantly correlated with increasing concentrations of BPA and MP (P < 0.001 for BPA; P < 0.001 for MP, two-sided test for mixed effects linear regression).

**Discussion**

We demonstrate that BPA activates the mTOR pathway in non-malignant HRBECs. Both transcript and protein quantitation analyses reflected changes in the most important representative elements of this signaling pathway. In live cells, BPA suppresses apoptosis, enhances S-phase and decreases ROS levels, a well-known prelude to apoptosis evasion. Live cells respond in a similar manner to the XE, MP, suggesting that mTOR activation might be a general effect of XEs. These functional assays serve to test and confirm the consequences of mTOR activation predicted by global expression profiling of a previous independent set of BPA-exposed HRBECs (24).

Chemicals tested here are so common in bodily fluids (3–8) as to make unexposed control subjects functionally, if not literally, unavailable. We provide a pragmatic approach to overcome this problem by using live, non-carcinogenic breast epithelial cell samples from high-risk donors (HRBECs) propagated in vitro with and without the chemicals of interest for pairwise comparisons of known end points of mTOR activation. HRBECs are samples from women who, based on extensive epidemiology, are identifiable as predisposed to malignant progression, even before overt cyt pathological alterations are present. The opportunity to demonstrate a concordant set of multiple end points portraying the status of the mTOR metabolic pathway—despite small sample size—underscores the suitability of this source of target human cells for recapitulating early functional changes induced by carcinogen exposure. There is, and always will be, a gap between human biology in vivo and its representation by surrogate in vitro models, but unlike the limited genotypic variation represented by common immortalized cell
Fig. 3. XE exposure promotes apoptosis evasion in HRBEC cultures. (A) Potential for XE-induced apoptosis evasion measured as percent reduction in Annexin V-positive cells by FACS analysis. Breast cancer cell lines (T47D and SKBR3), HRBEC cell lines (IMM-PA024, IMM-PA025 and IMM-PA115) and early passage HRBEC (PA094, PA099, PA103, PA106, PA107 and PA130) exposed to BPA or MP were treated with OHT for 24 h prior to Annexin V staining and compared with untreated controls. All experiments were performed in triplicate. Plots illustrate average values and the standard deviation for each culture group under conditions of no treatment, XE exposure followed by OHT or OHT treatment alone. Values demonstrating the effect of XEs in reducing OHT-induced apoptosis are statistically significant in all cases (P < 0.002). (B) FACS profiles of representative samples. M1 fraction—autofluorescence; M2—Annexin V-positive cells (which increase with OHT treatment). (C) Dose–response measurements (shown as decreasing XE concentrations from left to right) in early passage HRBECs.
lines, HRBECs more closely reflect the diversity of genetics, exogenous hormone use, life events such as pregnancy, etc. of women encountered in clinical practice. Our demonstration that HRBECs from all subjects displayed prosurvival changes after XE exposure does not claim that all exposed persons will develop cancer, only that such exposure can cause changes that may facilitate malignant progression.

all cases, the protection from OHT-induced apoptosis (apoptotic evasion) was calculated as a fraction of the apoptotic response in the absence of XEs (set to 1). Each data point represents an average of eight independent HRBEC samples (shown individually in supplementary Figure S1 is available at Carcinogenesis Online). Error bars display the variation between samples in the protection from OHT-induced apoptosis.

Fig. 4. XE exposure alters oxidative stress levels in breast epithelial cells. (A) Comparative analysis of intracellular ROS levels measured and quantified by FACS analysis of C400-stained cancer cell lines (T47D and SKBR3), HRBEC lines (IMM-PA024, IMM-PA025 and IMM-PA115) and early passage HRBEC cultures (PA094, PA099, PA103, PA106, PA107 and PA130) exposed to BPA or MP. A post-XE 24 h treatment with tamoxifen (OHT) was used to induce ROS. All experiments were performed in triplicate. Averaged data representing the MFI of C400 are plotted, and standard deviations are shown. Values demonstrating the effect of XEs in reducing OHT-induced ROS are statistically significant in all cases (P < 0.0001). (B) FACS profiles of representative samples. The area under each curve reflects C400-positive cells. Note the right shift of the C400 peak in OHT-treated samples (indicating higher MFI) when compared with untreated control populations (top two panels) and the mild reduction of MFI in cells exposed to XEs (bottom two panels). (C) ROS levels measured as C400 MFI in early passage HRBECs exposed to various concentrations of BPA or MP. Results are expressed as percent reduction from baseline MFI of no XE controls. Each data point represents an average of six independent HRBEC samples (shown individually in supplementary Figure S2, available at Carcinogenesis Online). Error bars display the variation between cases.
Human cell samples that maintain phenotypes relevant to clinical targets of interest are essential for carcinogenicity testing. For example, to evaluate the role of XEs in the initiation of breast cancer, HRBECs are particularly well suited because they consistently express low/moderate levels of both ER\(_a\) and ER\(_b\). Earlier studies that did not distinguish ER isoforms (as continues in current clinical practice where primarily ER\(_a\) is measured) typically reported low ER positivity in normal or benign breast tissue (32–34), suggesting that this phenotype is sufficient for agonistic activity of natural and synthetic estrogens, as well as for antagonistic effects of tamoxifen in reducing breast cancer incidence in randomized trials (35,36), even in patients harboring ER-negative atypia (37). Thus, high ER\(_a\) levels are not a prerequisite for XEs to exert their biological effects either clinically or \textit{in vitro}. Similarly, reversal of tamoxifen toxicity by exposure to the common XEs, BPA and MP, implies that the ER profile of HRBECs is also sufficient for the induction of cancer-associated phenotypes by estrogenic chemicals.

Against the backdrop of non-malignant ER levels simulated by HRBECs, BPA-induced transcriptional and protein alterations characteristic of breast cancer were discernible. For example, activation by phosphorylation of AKT is a key regulatory step in subsequent mTOR activation and induction of cell growth (25). During \textit{in vitro} morphogenesis and differentiation of acini from non-malignant cells in Matrigel, pAKT localizes to peripheral cells, away from the central apoptotic region (38). Moreover, constitutive pAKT expression prevents lactogenic differentiation (39). Expectedly therefore, pAKT is increased during RAS-induced carcinogenesis of MCF10 xenografts (40) and pAKT is necessary for carcinogenic progression in this model since blocking pAKT results in apoptotic cell death (40). Clinically, a similar progression is observed whereby pAKT is significantly higher in malignant than benign breast tissue (41) and pAKT levels in cancer correlate directly with poor prognosis (42,43). Conversely, \textit{in vitro} PTEN inactivation by phosphorylation and reduction in protein levels parallel Cowden’s syndrome where PTEN loss of function is associated with an increase in breast and other cancers (44,45). Downstream, mTOR acts by phosphorylating (activating) 4EBP1 and RPS6 (indirectly through p70S6K activation). Both 4EBP1 and its target elf4E increase during experimental carcinogenesis (40); p4EBP1 is higher in cancer than in benign biopsies (41) and associated with higher tumor grade (46); and consequently, p4EBP1 correlates directly with tumor recurrence in women (46). In \textit{vitro}, pRPS6 is downregulated by exposure to rapamycin (a defining mTOR inhibitor) in 12/12 cell lines (46,47). In one series, pRPS6 did not correlate with prognosis, but it was upregulated in the majority (77%) of breast cancers (46). mTOR activation by BPA exposure is further defined by inhibition of rapamycin-induced hallmarks of cancer.
apoptosis (48), which is the converse of the therapeutic benefit of rapamycin analogs achieved through suppression of the mTOR pathway (49,50). In vitro pretreatment of HRBECs with BPA essentially replicates the continuous environmental exposure underlying positive urine tests in the general population. The failure of rapamycin to induce apoptosis after BPA treatment raises concern that activation of mTOR by continuous XE exposure could limit the effectiveness of this drug and its analogs that are being tested in clinical trials (49,50). Thus, a single estrogen mimic undermines multiple cell control mechanisms, as demonstrated here for tamoxifen and rapamycin and by others for the chemotherapeutic agents, doxorubicin and cisplatin (12).

Evasion of apoptosis and increased S-phase, induced by test XEs in HRBECs, are accepted hallmarks of cancer (51) with wide applicability in clinical cancer management. Apoptosis occurs rapidly after initiation of successful chemotherapy (52,53). Tamoxifen induces apoptosis within the first 24 h of treatment in animal models (54,55). Radiation therapy works by triggering apoptosis (56). High S-phase is an established indicator of poor tumor outcome (57), and maintenance of proliferation in the face of therapy indicates inadequate treatment response (58). Moreover, a drop in the ratio of proliferating to apoptotic cells (Cell Turnover Index) is indicative of a response to hormone-based therapy in benign (59) and malignant breast tissue (60). Thus, the ability of HRBECs to evade apoptosis and continue DNA replication after XE exposure portrays the acquisition of two fundamental phenotypes of cancer. Another fact supporting the causative role of BPA and MP is that both evasion of apoptosis and maintenance of S-phase are induced in a dose-dependent manner in HRBECs.

In this study, BPA and MP exposures also led to reduced ROS in live HRBECs. This is consistent with the finding that inhibition of the AKT/mTOR complex correlates with a marked increase in ROS production (61), and conversely, induction of the S6 kinase promotes resistance to oxygen and glucose deprivation and reduction of ROS levels (62). Being certain of the effects of free radicals is complicated because, depending on the level of ROS, cellular oxidative stress promotes either apoptosis or DNA damage (63,64). Low levels of ROS that accumulate depending on the level of ROS, cellular oxidative stress promotes either apoptosis or DNA damage, whereas high levels of ROS are necessary for induction of apoptosis by hormone therapy (65), chemotherapy (66) and ionizing radiation (67).

Expansion of clinical samples with limited cellularly undoubtedly requires additional effort compared with the convenience of infinite cell yields from immortalized lines. However, renewable samples, such as HRBECs obtained by RFPA, are readily available and represent a significantly wider population than rare immortalized human cell lines. Even in just this study, if confined to cell lines alone, the majority of test samples would have been unavailable to reveal the consistent XE effects that occurred despite variable baseline levels of proteins such as PTEN, AKT and 4EBP1 (Figure 2B). HRBECs are uniquely suited to capture and ultimately to investigate the basis of this variability among humans. We conclude: (i) testing ‘renewable’ samples from the at-risk population allows a wider sampling of different genetics, hormonal history, etc. than occur in a limited class of experimental models, (ii) employing HRBECs in experimental studies facilitates hypothesis generation and confirmation in independent sample sets (as we have done) and (iii) the complexity of xenoestrogenic effects requires evaluation of multiple cellular end points at this time for impact assessment instead of relying on a single receptor or functional assay.

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