Acetaminophen Exhibits Weak Antiestrogenic Activity in Human Endometrial Adenocarcinoma (Ishikawa) Cells

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The purpose of this study was to test the hypothesis that acetaminophen would alter an estrogen-regulated process in human cells that express endogenous estrogen receptor α and β (ERα and ERβ). Specifically, the extent to which acetaminophen altered the expression of estrogen-inducible alkaline phosphatase in endometrial adenocarcinoma (Ishikawa) cells and directly interacted with ERβ and ERα was determined. Ishikawa cells were exposed to estradiol and/or to a range of concentrations of acetaminophen for four days, and alkaline phosphatase activity was measured spectrophotometrically. Acetaminophen inhibited both basal and estradiol-induced alkaline phosphatase activity in Ishikawa cells in a concentration-dependent manner. The reduction of Ishikawa cell alkaline phosphatase was not due to direct inhibition of enzyme activity by acetaminophen. Toxic effects of acetaminophen on Ishikawa cells were determined by measuring loss of cellular lactate dehydrogenase to culture medium. High concentrations of acetaminophen (>0.5 mM) induced lactate dehydrogenase release from cells and reduced the amount of cellular protein in culture dishes, indicating some acetaminophen-induced reduction of alkaline phosphatase activity in the absence of detectable toxicity. Acetaminophen also augmented 4-hydroxy-tamoxifen reduction of alkaline phosphatase activity. Competition binding assays with human ERα and ERβ demonstrated 104-fold molar excess acetaminophen did not directly interact significantly with the ligand-binding domain of either receptor. These studies indicate acetaminophen exerts weak antiestrogenic activity in Ishikawa cells without directly binding ERα or ERβ.

Key Words: acetaminophen; Ishikawa cells; estrogen receptors; alkaline phosphatase; endocrine disruption.

Acetaminophen, an analgesic and antipyretic drug, contained in more than 800 over-the-counter and prescription formulations, is taken acutely, as well as chronically, by millions of adults and children. Metabolism of acetaminophen, primarily in the liver by mixed function oxidases, can result in the production of a highly reactive metabolite, N-acetyl-p-benzoquinoneimine (NAPQI). At high doses acetaminophen elicits well-characterized hepatotoxic effects due to the production of NAPQI, which are exacerbated by ethanol consumption (Prescott, 1996). Acetaminophen has also been shown to have a variety of other effects upon cells/tissues both in vitro and in vivo. In vitro acetaminophen inhibits DNA synthesis (Hongso et al., 1991) and cell proliferation (Boulares et al., 1999; Dai and Cederbaum, 1995; Holme et al., 1988; Wiger et al., 1997), downregulates c-myc and bcl-2 mRNA (Boulares et al., 1999; Wiger et al., 1997), and increases chromosomal breaks (Holme et al., 1988). Many of these effects do not appear to be a result of NAPQI formation, because most of the cells in these studies do not metabolize acetaminophen (Boulares et al., 1999; Dai and Cederbaum, 1995; Kappers et al., 2000). In vivo studies in mouse and rat indicate that cytotoxic concentrations of acetaminophen bind DNA, increase single strand breaks, and interfere with DNA repair and DNA synthesis (Hongso et al., 1994). Furthermore, in humans acetaminophen significantly increases chromatid breaks in peripheral lymphocytes (Hongso et al., 1991).

Other studies suggest acetaminophen may alter some hormone-regulated processes. In rats, high doses of acetaminophen reduce testis DNA synthesis resulting in decreased testicular weight, decreased number of spermatocytes, and deterioration of sperm chromatin structures (Wiger et al., 1995). In humans, acetaminophen use is associated with a decreased risk of ovarian cancer (Cramer et al., 1998a), as well as reduced gonadotropin and estradiol (E2) levels (Cramer et al., 1998b). Although acetaminophen does not exert a positive or negative effect in rodent E2-dependent uterotrophic (wet weight) assays (Harnagea-Theophilus et al., 1999a; Isenhower et al., 1986; Patel and Rosengren, 2001), some studies indicate therapeutic levels of this drug can specifically alter E2-regulated processes. In cultured trout liver cells acetaminophen decreases E2-dependent vitellogenin mRNA and protein production in a concentration-dependent manner (Miller et al., 1999). Similarly, in immature mice moderate doses of acet-
aminophen reduce E2-induced uterine peroxidase activity and nuclear progesterone receptor protein levels (Patel and Rosen- gren, 2001). Furthermore, therapeutic concentrations of acet- aminophen stimulate proliferation of estrogen receptor (ER)- positive breast cancer cells (MCF-7, T47D, ZR-75-1), but not ER-negative (MDA-MB-231, HS578T) breast cancer cells, via a mechanism inhibited by antiestrogens (Harnagea-Theophilus and Miller, 1998; Harnagea-Theophilus et al., 1999a,b). It is not yet clear why acetaminophen exerts antiestrogenic activity on some processes in some cells, while it mimics estrogen in stimulating proliferation of breast cancer cells.

To further explore the influence of acetaminophen on E2-regulated processes in human cells, the hypothesis that acetaminophen would alter expression of alkaline phosphatase activity in Ishikawa cells was determined. Ishikawa cells are endometrial adenocarcinoma cells that express functional ERα and ERβ (Bhat and Pezzuto, 2001; Enmark et al., 1997; Kassan et al., 1989). Estradiol has a variety of effects upon Ishikawa cells including upregulation of progesterone receptors (Holinka et al., 1986b) and c-fos mRNA (Gadd et al., 2002); increased c-erbB2/NEU proto-oncogene protein expression (Markogiannakis et al., 1997); increased intracellular en- dorphin release (Makrigiannakis et al., 1992); modest stimula- tion of cell proliferation (Holinka et al., 1986a; Holinka et al., 1989; Nishada et al., 1985); and increased alkaline phos- phatase enzyme activity and mRNA (Albert et al., 1990; Holinka et al., 1986b; Littlefield et al., 1990). The sensitivity of Ishikawa cell alkaline phosphatase activity to induction by E2 provided a well-characterized system in which to assess the E2-agonistic and E2-antagonistic activity of acetaminophen. In addition, this system was used to determine the extent to which acetaminophen impacted the effect of an antiestrogen, 4-hy- droxy-tamoxifen, on expression of an E2-regulated gene. Be- cause many of the cells/tissues in which acetaminophen alters E2-regulated processes express different levels of ERα and ERβ, the ability of acetaminophen to directly bind the ligand-binding site of purified ERα and ERβ is reported for the first time.

**MATERIALS AND METHODS**

**Cell culture.** Ishikawa cells (a generous gift from Jacques Simard, CHUL Research Center, Quebec, Canada) were maintained in MEM medium (Gibco) supplemented with 10% fetal bovine serum and 0.1% gentamycin. Cells were incubated at 37°C, 5% CO₂ and passaged by trypsinization at 80% confluency. Several weeks prior to initiating experiments, as well as during experi- ments, Ishikawa cells were cultured in estrogen-free medium (Holinka et al., 1989) to achieve low, basal levels of estrogen-inducible alkaline phosphatase activity. Estrogen-free medium is phenol red-free Ham’s F12:DMEM (1:1) supplemented with 2% fetal bovine serum that had been treated with charcoal/ dextran (Stoib et al., 1994). Cells were treated with indicated concentrations of acetaminophen and/or E2 (positive control). Acetaminophen was dissolved directly in medium; an E2 stock solution was prepared in ethanol and added to medium with the final concentration of ethanol in cultures ≤ 0.00003%, which had no detectable effect on any parameters measured.

**Alkaline phosphatase activity and protein determination.** Ishikawa cells in estrogen-free medium were trypsinized and plated in CoStar 96-well plates at 7 × 10⁵ cells/well. Twenty-four h later the indicated compounds were added in fresh medium, and cells were exposed to these compounds for four days, with one medium change two days after the first addition of compounds. At the end of the exposure period wells were assayed for cellular alkaline phosphatase activity as previously described (Littlefield et al., 1990) using a Spectramax 340PC plate reader. Alkaline phosphatase activity was determined for n = 5 wells per treatment in each experiment and is presented as mean pmol p- nitrophenol (pNP) formed/min/well ± SE. Total cellular protein in cultures was determined using fluorescamine reagent by measuring fluorescence (360 nm ex/460 nm em) in a Cytofluor 400, as previously described (Udenfriend et al., 1972). Total cellular protein determinations were performed in triplicate and the amount of protein was extrapolated from an albumin standard curve. Results are presented as mean µg protein/well ± SE. The effects of com- pounds on alkaline phosphatase activity and on total cellular protein are reported as independent parameters, to illustrate how the compounds differ- entially altered each of these parameters.

To assess the effect of acetaminophen directly on alkaline phosphatase activity, an extract from E2-treated cells was prepared. Alkaline phosphatase activity in the extract was measured in the absence and presence of various concentrations of acetaminophen.

**Lactate dehydrogenase cytotoxicity assay.** Ishikawa cells were plated into CoStar 6-well plates at 1.3 × 10⁴ cells/well and allowed to adhere for 24 h. Cells were then treated with various concentrations of acetaminophen for indicated periods of time. Medium was carefully removed from cells, and lactate dehydrogenase (LDH) released from cells was measured in culture medium. Cellular LDH activity was determined by disrupting cells with 1 ml 0.1% Triton X-100 and measuring LDH in cell lysates. LDH activity was measured as described in Reeves and Finogni (1966), and protein in cell lysates was determined by Bio-Rad Protein Assay (Bio-Rad, CA). LDH activity in both cell supernatant and cell lysate was normalized to protein concentration in cell lysates.

**ER binding assays.** In vitro competition binding assays utilized purified human recombinant ERα and ERβ (Pan Vera). Briefly, reactions containing 1 pmol ER, 1 pmol [³H]-E2 (Amersham), and indicated concentrations of com- peting, unlabeled E2 or acetaminophen in 10 mM Tris, pH 7.5, 10% glycerol, 2 mM dithiothreitol (DTT), 1 mg/ml bovine serum albumin were incubated in a 37°C water bath for 30 min. ER with bound [³H]-E2 was separated from free [³H]-E2 by the addition of hydroxyapatite and repeated centrifugation and wash- ing. Liquid scintillation counting determined the amount of [³H]-E2 bound to ER.

**Western blots.** Western blots were used to assess the relative abundance of ERα in MCF-7 cells and in Ishikawa cells, using antibodies specific for ERα (Santa Cruz). Ishikawa and MCF-7 (human breast cancer) cells were plated into 100-mm dishes and maintained in E2-free medium for four days with one medium change on day 2. On day 4, cells were rinsed with ice-cold phosphate buffered saline and lysed in 1 ml of SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 50 mM DTT, 0.1% bromphenol blue). Protein concentration was determined by 50% trichloroa-etic acid precipitation of an aliquot of cell lysate, followed by the Coomassie Plus Protein Assay (Pierce). Proteins were separated via electrophoresis in denaturing 7.5% polyacrylamide gels and electroblotted to PVDF membrane. Blots were blocked with 5% powdered milk for 1 h at room temperature and then incubated overnight at 4°C with anti-ERα antibody (Santa Cruz) diluted in 5% albumin. Visualization was achieved through incubation with horserad- ish peroxidase-linked donkey antirabbit IgG (Pierce) in 5% powdered milk for 1 h at room temperature and then washed with Tris buffered saline. Blots were stripped and then reprobed for β-catenin (Santa Cruz). Optimas 6.2 software was used to quantitate the optical density of each band of interest.

**Statistical analysis.** Statistical analysis of alkaline phosphatase activity, protein concentration, and LDH assays were conducted by one-way ANOVA followed by Tukey’s Test; significant differences (p < 0.05) between group means are indicated. For ER-binding assays, one-way ANOVA was per- formed, followed by the Dunnett’s test; significant differences (p < 0.05) are indicated.
RESULTS

Acetaminophen Reduces E2-Inducible Alkaline Phosphatase Activity in Ishikawa Cells

Initial studies determined the extent to which acetaminophen altered basal alkaline phosphatase activity and cellular protein in cultures of Ishikawa cells maintained in E2-depleted medium. The concentrations of acetaminophen investigated included those achieved in serum with therapeutic doses (0.03–0.3 mM), as well as a higher concentration (1 mM) associated with toxicity; all of these acetaminophen concentrations stimulated proliferation of ER-positive breast cancer cells (Harnagea-Theophilus and Miller, 1998; Harnagea-Theophilus et al., 1999a,b) and inhibited E2-dependent vitellogenin production in trout liver cells (Miller et al., 1999). In our studies, the level of basal alkaline phosphatase activity varied ~10-fold, and in all studies acetaminophen reduced the basal alkaline phosphatase activity in a concentration-dependent manner (Fig. 1A). In three other experiments acetaminophen exerted comparable effects on alkaline phosphatase activity. However, the effect of acetaminophen on cellular protein was more variable. In one study (Fig. 1B) 0.3 and 1.0 mM acetaminophen significantly reduced cellular protein and contributed to the reduction of enzyme activity (Fig. 1A), while in two other studies cellular protein levels were not significantly altered by any concentration of acetaminophen tested, and in another study 0.3 and 1.0 mM acetaminophen only reduced total cellular protein ~20% (not shown).

The effect of acetaminophen on E2-induced alkaline phosphatase activity was then investigated; initial studies established the response of alkaline phosphatase activity to increasing concentrations of E2. Significant induction of alkaline phosphatase activity, ~2-fold greater than control activity, was observed with 0.01 nM E2; 0.1 nM E2 increased alkaline phosphatase activity ~4-fold, and 1 nM E2 maximally induced activity (Fig. 2A), in agreement with other studies (Littlefield et al., 1990). The effect of E2 on the number of cells/well, reflected in the amount of cellular protein/well, was variable; in some studies E2 significantly increased cellular protein (Figs. 2B and 5B), and in other studies E2 did not significantly alter cellular protein (Figs. 3B, 4B, and 4D). In subsequent studies, 0.1 nM E2 was chosen because it induced robust stimulation of alkaline phosphatase activity without “saturating” the enzyme induction observed with higher concentrations of E2. Figure 3A shows that acetaminophen reduced E2-induced alkaline phosphatase activity in a concentration-dependent manner, and cellular protein was decreased by ~3 mM acetaminophen (Fig. 3B). The acetaminophen-induced reduction of Ishikawa cell alkaline phosphatase activity (Figs. 1 and 3) was not due to direct inhibition of enzyme activity by this drug; alkaline phosphatase activity was not reduced when acetaminophen (0.25–3 mM) was included in the enzyme assay (not shown).

High Levels of Acetaminophen Cause LDH Release from Cells

Acetaminophen is known to induce toxic effects in various cells and tissues (Prescott, 1996), and the reduction of cellular protein in cultures of Ishikawa cells treated with high concentrations of acetaminophen (Figs. 1 and 3) could reflect toxicity. LDH release assays were undertaken to better determine if acetaminophen was eliciting a toxic response in Ishikawa cells. Ishikawa cells were exposed to various concentrations of acetaminophen for four days, then assayed for alkaline phosphatase activity (A) or cellular protein (B); values are means ± SE. *Significantly different from control (p < 0.05).
that concentrations of acetaminophen ≥0.5 mM increased the amount of LDH activity in culture medium four days after addition, and this was accompanied by a reduction in intracellular LDH activity. After only 24 h exposure of cells to acetaminophen the effects on released and intracellular LDH activity were less pronounced and more variable.

**Acetaminophen Augments Antiestrogen Reduction of Alkaline Phosphatase Activity**

Because nontoxic concentrations of acetaminophen (≤0.3 mM) were shown to downregulate E2-inducible alkaline phosphatase activity in Ishikawa cells (Figs. 1 and 3), we wanted to determine if acetaminophen modified antiestrogen inhibition of alkaline phosphatase. Initial studies established the effects of a range of concentrations of the partial antiestrogen 4-hydroxytamoxifen and the pure antiestrogen ICI 182,780 on E2-induc-
cellular protein ~20%, and higher concentrations of ICI 182,780 further reduced cellular protein in a concentration-dependent manner (Fig. 4D).

Hydroxy-tamoxifen was used in further studies because it reduced alkaline phosphatase activity without significantly altering cellular protein. Figure 5A shows that 0.01 mM hydroxytamoxifen, a nontoxic concentration (Fig. 4), reduced the E2-induced alkaline phosphatase activity ~60%, and acetaminophen further reduced alkaline phosphatase activity in a concentration-dependent manner between 0.1 and 1.0 mM. Although acetaminophen also reduced the amount of protein in cell cultures (Fig. 5B), the reduced cellular protein only partially accounted for the decreased alkaline phosphatase activity mediated by 0.1–1.0 mM acetaminophen.

**Acetaminophen Does NotCompete with E2 Binding ERα or ERβ**

Ishikawa cells express both high- and low-affinity E2-binding sites (Kassan et al., 1989), and recent studies demonstrated that both ERα and ERβ are expressed in these cells (Bhat and Pezzuto, 2001). To determine if the acetaminophen-mediated reduction of E2-regulated alkaline phosphatase in Ishikawa cells may be attributed to nonproductive binding of acetaminophen to ERα or ERβ, competition binding studies were performed using purified human ERα and ERβ. Even the highest concentrations of acetaminophen tested (10^6-fold molar excess) did not compete significantly with [3H]-E2 for binding ERα or ERβ (Fig. 6). In addition, the acetaminophen-mediated inhibition of E2-induced alkaline phosphatase activity (Fig. 3) was not significantly altered by increasing the concentration of E2 100-fold (not shown), further indicating that acetaminophen and E2 are not directly competing for binding ER. The relative level of ERα expression in Ishikawa and MCF-7 cells was also investigated. Figure 7 demonstrates that ERα is expressed at a lower level in Ishikawa cells than in MCF-7 cells. In two independent studies, the optical density of immunoreactive ERα was ~3-fold higher in MCF-7 cells than in Ishikawa cells with equal amounts of total cellular protein.
DISCUSSION

The purpose of this study was to further investigate the extent to which acetaminophen impacts E2-regulated processes in ER-positive human cells. The same concentrations of acetaminophen that mimic E2 in stimulating the proliferation of three ER-positive (MCF-7, T47D, ZR-75-1) breast cancer cell lines (Harnagea-Theophilus and Miller, 1998; Harnagea-Theophilus et al., 1999a,b) did not induce Ishikawa cell alkaline phosphatase activity (Fig. 1). Rather, acetaminophen reduced the activity of basal and E2-stimulated alkaline phosphatase in Ishikawa cells in a concentration-dependent manner (Figs. 1A and 3A). The reduction of alkaline phosphatase activity by acetaminophen was not due to direct inhibition of enzyme activity; however, some of this inhibitory effect of acetaminophen could be attributed to reduction of cellular protein (Figs. 1B and 3B), especially at concentrations ≥0.3 mM. In addition, concentrations of acetaminophen ≥0.5 mM resulted in loss of LDH from cells (Table 1), clearly indicating a toxic effect. Nonetheless, acetaminophen significantly reduced basal and E2-induced alkaline phosphatase activity at concentrations that did not appear to exert detectable toxicity (Figs. 1A and 3A, Table 1). In this regard, the effect of acetaminophen on Ishikawa cell alkaline phosphatase activity resembles the effects of acetaminophen on vitellogenin production in trout liver cells (Miller et al., 1999) and on uterine peroxidase and progesterone receptors in mice (Patel and Rosengren, 2001). In trout liver cells, concentrations of acetaminophen that did not exert detectable toxic effects (≥0.3 mM) significantly inhibited the E2-dependent production of vitellogenin protein and mRNA ~50% (Miller et al., 1999). In immature mice, a recent in vivo study demonstrated acetaminophen was a weak inhibitor of E2-induced uterine peroxidase activity and nuclear progesterone receptor protein (Patel and Rosengren, 2001).

The mechanism by which acetaminophen inhibits E2-induced gene expression without binding ERα or ERβ is not established, but may be mediated by PPAR (peroxisome proliferator-activated receptor) and RXR (retinoid X receptor).

Note. Cells were cultured in medium with no additions (control) or with the indicated concentrations of acetaminophen for one and four days. Following exposure, medium removed from cells was assayed for released lactate dehydrogenase (LDH) activity and cell lysates were prepared and assayed for intracellular LDH activity and total cellular protein. Total LDH is the sum of intracellular and released LDH. Intracellular LDH represents total cellular LDH activity (ΔAbs340nm/min/mg total cellular protein). Released LDH represents total LDH activity released into medium (ΔAbs340nm/min/mg total cellular protein). Percentage released of total LDH is the amount of LDH activity released into the media as a percentage of the total LDH.

TABLE 1
Effect of Acetaminophen on Lactate Dehydrogenase Release from Ishikawa Cells

<table>
<thead>
<tr>
<th>Acetaminophen</th>
<th>0.3 mM</th>
<th>0.5 mM</th>
<th>1 mM</th>
<th>10 mM</th>
<th>20 mM</th>
</tr>
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<tr>
<td>Total LDH</td>
<td>1.49</td>
<td>1.43</td>
<td>1.16</td>
<td>1.09</td>
<td>1.03</td>
</tr>
<tr>
<td>Intracellular</td>
<td>1.46</td>
<td>1.41</td>
<td>1.14</td>
<td>1.06</td>
<td>0.95</td>
</tr>
<tr>
<td>Released</td>
<td>0.03</td>
<td>0.02</td>
<td>0.02</td>
<td>0.03</td>
<td>0.08</td>
</tr>
<tr>
<td>Total LDH released (%)</td>
<td>2.05</td>
<td>2.33</td>
<td>2.19</td>
<td>1.69</td>
<td>1.09</td>
</tr>
<tr>
<td>Intracellular</td>
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<td>2.24</td>
<td>1.76</td>
<td>0.91</td>
<td>0.89</td>
</tr>
<tr>
<td>Released</td>
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<td>0.09</td>
<td>0.14</td>
<td>0.25</td>
<td>0.20</td>
</tr>
<tr>
<td>Total LDH released (%)</td>
<td>3.9</td>
<td>3.9</td>
<td>7.4</td>
<td>21.0</td>
<td>18.4</td>
</tr>
</tbody>
</table>

A. Alkaline Phosphatase Activity (μmol/min/mg well)

B. Cell Protein (μg/well)

FIG. 5. Acetaminophen augments 4-hydroxy-tamoxifen inhibition of alkaline phosphatase activity. Ishikawa cells were plated in 96-well plates and treated with medium containing no additions (control), 0.1 nM E2 alone, or a combination of 0.1 nM E2 and 0.01 μM 4-hydroxy-tamoxifen without or with indicated concentrations of acetaminophen for four days, then assayed for alkaline phosphatase activity (A) or cellular protein (B); values are means ± SE. *Significantly different from control cells. #Significantly different from E2-treated cells. +Acetaminophen-induced changes significantly different from E2 + 4-hydroxy-tamoxifen treated cells (p < 0.05).
PPAR and RXR have been shown to be expressed in ER-positive tissues (Núñez et al., 1997), and PPAR*RXR heterodimers can modulate transcription of genes containing PPREs (PPAR response elements) or EREs (estrogen response elements; Keller et al., 1995; Núñez et al., 1997). Binding of PPAR*RXR heterodimers to natural ERE-containing promoters inhibits transactivation by E2*ER complex through competition for ERE binding (Keller et al., 1995). Therefore, the distribution of ERα and ERβ in different cells/tissues may alter the biological response to an ER-ligand. Thus, the observations that acetaminophen stimulates human breast cancer cell proliferation, mimicking E2 (Harnagea-Theophilus and Miller, 1998; Harnagea-Theophilus et al., 1999a,b), but exerts E2-antagonistic activity in human endometrial adenocarcinoma cells (Figs. 1 and 3), as well as in trout liver (Miller et al., 1999) and mouse uterus (Patel and Rosengren, 2001), could in part be due to differential expression of ERα and ERβ in those cells/tissues. Ishikawa cells express both ERα and ERβ (Bhat and Pezzuto, 2001), whereas MCF-7 cells predominantly express ERα; and ERα is expressed ~3-fold higher in MCF-7 cells than in Ishikawa cells (Fig. 7). The density of ERβ is estimated to be ~10-fold higher than ERα in Ishikawa cells (Kassan et al., 1989); therefore we estimate that total ER (α + β) may be ~3–4-fold higher in Ishikawa cells than in MCF-7 cells. Differences in the proportion of ERα and ERβ or the total level of ER in Ishikawa and MCF-7 cells may impact the response to acetaminophen. However, acetaminophen does not directly compete significantly with E2 binding human ERα or ERβ (Fig. 6), indicating acetaminophen does not differentially interact with the ligand-binding domain of these receptors.

FIG. 6. Acetaminophen does not compete with E2 binding ERα or ERβ. ER-binding studies were conducted as described with 1 pmol human ERα (A) or ERβ (B) incubated with 1 pmol [3H]-E2 and increasing amounts of unlabeled E2 (filled squares) or acetaminophen (open circles). Data points represent the mean value ± SE from two separate experiments in which each condition was assayed in triplicate. *Significantly different from control (no competitor; p < 0.05).

PPAR*RXR have been shown to be expressed in ER-positive tissues (Núñez et al., 1997), and PPAR*RXR heterodimers can modulate transcription of genes containing PPREs (PPAR response elements) or EREs (estrogen response elements; Keller et al., 1995; Núñez et al., 1997). Binding of PPAR*RXR heterodimers to natural ERE-containing promoters inhibits transactivation by E2*ER complex through competition for ERE binding (Keller et al., 1995). The addition of acetaminophen, a known peroxisome proliferator (Lee et al., 1997), to Ishikawa cells could initiate PPAR*RXR heterodimer formation and binding to ERE(s) in the alkaline phosphatase gene promoter, resulting in inhibition of E2-induced alkaline phosphatase. On the other hand, acetaminophen may inhibit E2-induction of alkaline phosphatase by altering an arachidonic acid metabolism pathway. Acetaminophen is a weak inhibitor of cyclooxygenase 1 (COX-1) and COX-2 and a more potent inhibitor of COX-3 (Chandrasekharan et al., 2002), reducing prostaglandin production in most tissues (Prescott, 1996). However, acetaminophen elevates COX-2 and prostaglandin production in liver (Reilly et al., 2001). Alteration of arachidonic acid metabolism via COX can generate potent mediators (prostaglandins, leukotrienes, hydroxyeicosapentaenoic acids) that could alter other signal transduction pathways (Serhan et al., 2000), possibly culminating in inhibition of E2-induced alkaline phosphatase.

In addition, ERα and ERβ are differentially expressed in different tissues (Enmark et al., 1997; Kuiper et al., 1997); and gene expression can be differentially regulated by a ligand binding to ERα or ERβ (Paech et al., 1997). Therefore, the distribution of ERα and ERβ in different cells/tissues may alter the biological response to an ER-ligand. Thus, the observations that acetaminophen stimulates human breast cancer cell proliferation, mimicking E2 (Harnagea-Theophilus and Miller, 1998; Harnagea-Theophilus et al., 1999a,b), but exerts E2-antagonistic activity in human endometrial adenocarcinoma cells (Figs. 1 and 3), as well as in trout liver (Miller et al., 1999) and mouse uterus (Patel and Rosengren, 2001), could in part be due to differential expression of ERα and ERβ in those cells/tissues. Ishikawa cells express both ERα and ERβ (Bhat and Pezzuto, 2001), whereas MCF-7 cells predominantly express ERα; and ERα is expressed ~3-fold higher in MCF-7 cells than in Ishikawa cells (Fig. 7). The density of ERβ is estimated to be ~10-fold higher than ERα in Ishikawa cells (Kassan et al., 1989); therefore we estimate that total ER (α + β) may be ~3–4-fold higher in Ishikawa cells than in MCF-7 cells. Differences in the proportion of ERα and ERβ or the total level of ER in Ishikawa and MCF-7 cells may impact the response to acetaminophen. However, acetaminophen does not directly compete significantly with E2 binding human ERα or ERβ (Fig. 6), indicating acetaminophen does not differentially interact with the ligand-binding domain of these receptors.

FIG. 7. Western blot analysis of ERα in Ishikawa and MCF-7 cell extracts. Ishikawa and MCF-7 cells maintained in estrogen-free media for four days were subjected to Western blotting with anti-ERα and anti-β-catenin primary antibodies, as described in Materials and Methods. Equal amounts of MCF-7 and Ishikawa cell lysate protein (20 µg) from one experiment were analyzed in each lane: Lanes 1 and 2 are Ishikawa cell lysates; lanes 3 and 4 are MCF-7 cell lysates.
This result does not eliminate the possibility that acetaminophen (or a metabolite) may differentially interact directly or indirectly with an ER-accessory protein/factor in the different cells. Alternatively, because the membrane-associated forms of EROs or ERβ may exhibit ligand specificity that differs from that of the soluble receptor forms (Wade et al., 2001), acetaminophen may differentially affect signaling via the membrane-associated forms of EROs or ERβ in different cells.

The ability of acetaminophen to exert mitogenic activity on select cells may depend on both the internal cell environment and the presence of ER. Acetaminophen and E2 both stimulate ER-positive breast cancer cells (MCF-7, T47D, ZR-75-1) to proliferate via an ER-mediated mechanism (Harnagea-Theophilus and Miller, 1998; Harnagea-Theophilus et al., 1999a,b) and induce c-myc expression (Gadd et al., 2002). In contrast, in Ishikawa cells E2 marginally stimulates c-myc expression (Gadd et al., 2002) and cell proliferation (Holinka et al., 1986a), while acetaminophen slightly reduces c-myc expression (Gadd et al., 2002) and does not stimulate proliferation, reflected in cellular protein (Fig. 1), at any concentration tested. In addition, ER-positive breast cancer cells are relatively resistant to acetaminophen toxicity (Harnagea-Theophilus and Miller, 1998; Harnagea-Theophilus et al., 1999a,b), while Ishikawa cells are more sensitive to acetaminophen toxicity (Table 1), likely reflecting differences in metabolism of acetaminophen to the toxic NAPQI. Furthermore, a recent report indicates acetaminophen-stimulated cell proliferation is not restricted to ER-positive breast cancer cells. Acetaminophen was reported to stimulate the proliferation of human endometrioid ovarian cancer (MDA2774) cells (Bilir et al., 2002), which are reported to lack ER (Thompson et al., 1991). Therefore the presence of ER may not be sufficient or necessary for acetaminophen to induce cell proliferation. MDAH cells carry mutations in p-53 and MSH-2 genes (Orth et al., 1994; Santoso et al., 1995), prompting the idea that alteration of the oxidative state of these cells by acetaminophen may promote a mitogenic signal (Bilir et al., 2002). In breast cancer cells, acetaminophen may activate a proliferation signal transduction pathway that requires ER to culminate in a mitotic signal.

Tamoxifen is a chemotherapeutic agent commonly used in the treatment of E2-responsive breast cancer; tamoxifen and metabolites, including 4-hydroxy-tamoxifen exert their beneficial effects as ER-antagonists. Because women receiving tamoxifen often take acetaminophen to manage pain, it was of interest to determine the effect of the combination of these agents on an ER-regulated process. Therapeutic and toxic concentrations of acetaminophen (0.1–0.3 and 1 mM, respectively) augmented the hydroxy-tamoxifen inhibition of Ishikawa cell alkaline phosphatase activity (Fig. 5). Additional studies are required to determine if the combination of tamoxifen (or other antiestrogens) chemotherapy and the use of acetaminophen can affect the drug efficacy or patient health.

References


