PLAB induction in fenretinide-induced apoptosis of ovarian cancer cells occurs via a ROS-dependent mechanism involving ER stress and JNK activation

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Introduction

Retinoids, natural and synthetic analogs of vitamin A, modulate various cell functions including growth, differentiation and apoptosis (1). N-(4-hydroxyphenyl)-retinamide (4HPR), a synthetic analog of all-trans retinoic acid, has emerged as a promising antitumor agent in cancer therapy (2). 4HPR may exert these effects through a mechanism dependent on nuclear retinoid receptors or via other mechanisms (3). 4HPR may induce apoptosis in ovarian cancer cells (4). In preclinical studies, 4HPR-induced reactive oxygen species (ROS) are involved in PLAB upregulation and apoptosis, both events abrogated by the antioxidants vitamin C and butylated hydroxyanisole. We analyzed the expression and activation of endoplasmic reticulum (ER) stress-associated molecules and show that 4HPR-induced ER stress is a consequence of ROS generation. Salubrinal, an ER stress inhibitor, abrogated 4HPR-induced PLAB upregulation and protected the cells from apoptosis. Downstream of ROS generation and ER stress, 4HPR activated c-Jun N-terminal kinase (JNK), which was inhibited by vitamin C and salubrinal. The JNK inhibitor SP600125 reduced 4HPR-induced PLAB upregulation, by decreasing PLAB mRNA half-life, and protected the cells from apoptosis. These data indicate that 4HPR-induced PLAB upregulation occurs downstream of a signaling cascade involving ROS generation, ER stress induction and JNK activation and that these steps are mediators of 4HPR-induced apoptosis.

Materials and methods

Cell lines and reagents

The origins and culture conditions of human ovarian carcinoma cell lines A2780, A2780/HPRO, IGROV-1 and OVCAR-3 are described elsewhere (15). The human cervical carcinoma cell line C33A was purchased from American Type Culture Collection (Rockville, MD) and maintained in a 1:1 (vol:vol) mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12 medium supplemented with 5% fetal bovine serum. 4HPR, kindly provided by Dr J. Crowell (National Cancer Institute, Rockville, MD), was dissolved at 10 mM in dimethyl sulfoxide prior to dilution in culture medium and stored at −80°C in the dark. Vitamin C (Sigma, St Louis, MO), JNK inhibitor SP600125 (Calbiochem, San Diego, CA) and p38 pathway inhibitor SB203580 (Sigma) were added to cells 15 min, 60 min or 90 min before 4HPR. Butylated hydroxyanisole (BHA) (Sigma) and the ER stress response inhibitor salubrinal (Calbiochem) were added together with 4HPR. Actinomycin D was from Sigma.

Reactive oxygen species

Intracellular ROS production was determined using the oxidation-sensitive dye 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (Molecular Probes, Eugene, OR) as described elsewhere (15). Briefly, cells (8 × 10^5 per well) were plated into 6-well cell culture plates and incubated for 4 h in the presence of 4HPR. Medium was discarded, under low light conditions, and replaced with 50 μM 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate in whole medium for 20 min at 37°C. Cells were harvested, transferred to foil-wrapped tubes and immediately analyzed by flow cytometry. Unstained A2780 cells were used as negative control and 4HPR-treated C33A cells, known to generate ROS after 4HPR exposure (16), were used as positive control.

Immunoblot

Proteins were extracted by lysing cells in sodium dodecyl sulfate sample buffer [62.5 mM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate] containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, 12.5 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM sodium orthovanadate and 1 mM sodium molybdate. Cell extracts were processed for western blotting as described elsewhere (17). The following antibodies were used: PLAB, p38 and glucose-regulated protein 78KDa (GRP78)/immunoglobulin-binding protein (BiP) from Santa Cruz Biotechnology (Santa Cruz, CA); phospho-JNK (Th183/Y185), JNK, phospho-heat shock protein (HSP)27 (Ser82), phospho-p38 (Thr180/Tyr182), phospho-alpha-subunit of eukaryotic initiation factor 2 (eIF2 α) (Ser51), eIF2 α from Cell Signaling Biotechnology (Beverly, MA); poly(adenosine diphosphate-ribose)polymerase (PARP) from Calbiochem and actin from Sigma.

**Abbreviations**

ARE, AU-rich element; BHA, butylated hydroxyanisole; BiP, immunoglobulin-binding protein; eIF2 α, alpha-subunit of eukaryotic initiation factor 2; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; GRP78, glucose-regulated protein 78KDa; HSP, heat shock protein; 4HPR, N-(4-hydroxyphenyl)-retinamide; IC50, inhibiting concentration 50%; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PARP, poly(adenosine diphosphate-ribose)polymerase; PCR, polymerase chain reaction; PLAB, placental bone morphogenetic protein; ROS, reactive oxygen species; XBP-1, X-box binding protein-1.

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Apoptosis assays

DNA fragmentation was determined by photometric enzyme immunoassay using the Cell Death Detection ELISAplus kit (Roche, Penzberg, Germany) following the manufacturer’s instructions. Briefly, cells (1 × 10^6 per well) were seeded in 96-well cell culture plates and treated the day after. Adherent and floating cells were then lysed and centrifuged, and cytoplasmic fractions containing fragmented DNA were transferred to streptavidin-coated microtiter plates and incubated for 2 h at room temperature with a mixture of anti-histone-biotin and anti-DNA-peroxidase antibodies. The quantity of colored product (immobilized antibody–histone complex) was determined photometrically with 2,2′-azino-di-(3-ethylbenz-thiazoline-sulfonate-6-diammonium salt) as peroxidase substrate. DNA fragmentation in control and treated cells was expressed as absorbance at 405 nm. Activation of caspase-3 was determined using the caspase-3 colorimetric assay kit (MBL International, Woburn, MA). Briefly, cells (8 × 10^5 per well) were plated in 6-well cell culture plates and treated the day after. Whole-cell lysate from adherent and floating cells was centrifuged and the supernatant was assayed for protein concentration. An aliquot of 200 µg protein was incubated for 1 h at 37°C with the p-nitroanilide-labeled caspase-3-specific substrate [DEVD (Asp-Glu-Val-Asp)/p-nitroanilide]. Cleavage of the caspase substrate by active caspase and the release of p-nitroanilide were quantified in control and treated cells by measuring absorbance at 405 nm.

Semiquantitative reverse transcription–polymerase chain reaction

Semiquantitative reverse transcription polymerase chain reaction (PCR) analysis was performed as described elsewhere (17). PLAB and control (β-actin) gene sequences were co-amplified in the same reaction; CIEBP homologous protein (CHOP) was amplified separately. The amplification was performed using the following gene-specific oligonucleotide primers: PLAB forward primer, 5′-ACGCTGATGGCCTCAGAT-3′; CHOP forward primer, 5′-GGCAATAATCAGACCTGGAACCT-3′; CHOP reverse primer, 5′-ACAGTGCCTCGAGGAGAAAGG-3′; PLAB reverse primer, 5′-TGTTCAACTCTTCCAGCTG-3′; β-actin forward primer, 5′-GGAATCGTGCGTGACATTAAG-3′ and β-actin reverse primer, 5′-CTAGAACATTTGCGGTGGACGATGGAGGGGCC-3′.

Fig. 1. Effect of antioxidants vitamin C and BHA on 4HPR-induced ROS generation, apoptosis and PLAB in A2780 cells. (A) ROS production in cells treated 4 h with 5 or 10 µM 4HPR, with or without 100 µM vitamin C or 100 µM BHA. Analysis was performed by flow cytometry after addition of the redox-sensitive dye 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate. The graphs show representative flow cytometry fluorescence profiles of untreated and treated cells. A shift to the right from control indicates increased ROS levels. As positive control of ROS generation, 4HPR-treated C33A cells, known to generate ROS after 4HPR exposure (16), were used. (B) Apoptosis in cells treated 24 h with 5 or 10 µM 4HPR, with or without 100 µM vitamin C or 100 µM BHA. Apoptosis was evaluated by enzyme-linked immunosorbent assay (left) and caspase-3 activation (right). Data are means of three independent experiments; vertical bars are standard deviations. Asterisks indicate significant differences (P ≤ 0.05). (C) Reverse transcription–PCR (above) and western blot (below) to evaluate PLAB mRNA and protein expression, respectively, in cells treated as in (B). For reverse transcription–PCR, β-actin was also amplified as internal control. For western blot, actin, probed with actin antibody, served as indicator of how much protein was loaded.
Thirty cycles were used for PLAB amplification and 20 for β-actin. The PCR products were electrophoresed in agarose gel and visualized by UV after ethidium bromide staining.

X-box-binding protein-1 reverse transcription–PCR splicing assay

X-box-binding protein-1 (XBP-1) cleavage assay was performed as described elsewhere (18). Briefly, XBP-1 complementary DNA was amplified using the gene-specific oligonucleotide primers: XBP-sense, 5’-AAACAGAGTAGCAGCTCAGACTGC-3’ and XBP-antisense, 5’-TCCTTCTGGTAGACTCTGGGAG-3’. The PCR was performed by 2 min of denaturation at 94°C followed by 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s. To distinguish the unspliced (473 bp) from the spliced (450 bp) band, the PCR products were separated on a 3% agarose gel and visualized by UV after ethidium bromide staining.

Statistical analysis

All experiments were repeated at least three times, and each experiment was carried out at least in triplicate. The significance of differences between means was assessed by Student’s t-test with P < 0.05 considered significant.

Results

Antioxidants inhibit 4HPR-induced ROS generation, PLAB upregulation and apoptosis in A2780 cells

We showed previously that 4HPR treatment of A2780 ovarian cancer cells induces upregulation of the proapoptotic gene PLAB and that PLAB is a mediator of 4HPR-induced apoptosis (9). Since ROS are induced by 4HPR in various cells, including ovarian cancer cells (19), we here investigated ROS involvement in this signaling cascade by determining the effect of the antioxidants vitamin C and BHA on 4HPR-induced PLAB expression and apoptosis in A2780 cells, which are highly sensitive to the retinoid (20). In cells exposed for 4 h to 5 or 10 μM 4HPR, ROS increased 2.5- and 2.9-fold, respectively, over controls (Figure 1A); addition of vitamin C or BHA to 4HPR strongly reduced ROS generation (Figure 1A). Furthermore, both antioxidants protected 4HPR-treated cells from apoptosis, as observed after 24 h.
(Figure 1B), and abrogated the 4HPR-induced upregulation of PLAB mRNA and protein (Figure 1C). These findings suggest that ROS production, increased by 4HPR, mediates cell death and plays a role in PLAB upregulation.

4HPR activates the ER stress response downstream of ROS generation

ROS is known to induce the ER stress response (21). We hypothesized that 4HPR-induced ROS generation induces ER stress in A2780 cells. We therefore measured the effects of 4HPR, with and without vitamin C, on the following ER stress-specific signals: post-transcriptional splicing of XBP-1 transcription factor; transcription of the CHOP transcription factor; expression of the chaperon protein GRP78/BiP and phosphorylation of the initiation factor eIF2α. Treatment with 4HPR for 24 h induced splicing of a 25 bp intron from XBP-1 precursor mRNA, upregulation of CHOP mRNA and GRP78/BiP protein and phosphorylation of eIF2α, all prevented by vitamin C (Figure 2A). 4HPR-induced XBP-1 splicing and CHOP upregulation were not dose dependent: the XBP-1 spliced band and CHOP mRNA were more abundant after treatment with 5 μM than with 10 μM 4HPR. Thus, 4HPR induces ER stress that is prevented by vitamin C, indicating that ROS-dependent mechanisms are involved in 4HPR-induced ER stress in A2780 cells. To investigate whether ER stress is only a consequence of ROS generation or also contributes to ROS production, we analyzed the effects of the ER stress inhibitor salubrinal (22) on 4HPR-induced ROS generation. In A2780 cells exposed for 4 h to 10 μM 4HPR, the addition of 10 μM salubrinal did not alter the level of ROS generation (Figure 2B), implying that 4HPR-induced ER stress was not involved in ROS generation.

Pharmacological inhibition of ER stress prevents 4HPR-induced PLAB upregulation and apoptosis

ER stress has been shown to be involved in apoptosis (23). We determined whether ER stress is involved in 4HPR-induced PLAB upregulation and apoptosis in A2780 cells. We tested the effects of the ER stress inhibitor salubrinal on PLAB expression and apoptosis induced by 10 μM 4HPR. Addition of 10 μM salubrinal protected cells from 4HPR-induced apoptosis, assayed as DNA fragmentation (Figure 2C) and PARP cleavage (Figure 2D), and abrogated 4HPR-induced PLAB upregulation (Figure 2D). These results indicate that in A2780 cells, 4HPR-induced PLAB upregulation and apoptosis are dependent on prior activation of ER stress.

4HPR activates JNK downstream of ROS generation and ER stress induction

ROS generation and ER stress have been shown to activate JNK which itself has been shown to participate in the apoptosis of damaged cells

Fig. 3. 4HPR-induced JNK activation and effect of inhibition of JNK activation on 4HPR-induced PLAB upregulation and apoptosis in A2780 cells. (A) Western blot of whole-cell extracts of A2780 cells analyzed for total and phosphorylated JNK after treatment with 5 or 10 μM 4HPR for 24 h (left panel) and with 10 μM 4HPR, with or without 100 μM vitamin C or 10 μM salubrinal for 24 h (right panel). (B) Western blot of whole-cell extracts of A2780 cells to analyze for total and phosphorylated JNK, PLAB and PARP cleavage (left panel) and phosphorylated HSP27, PLAB and PARP cleavage (right panel). Actin, probed with actin antibody, served as indicator of how much protein was loaded. The cells were pretreated with 10 μM SP600125 for 15 min (left panel) or with 10 μM SB203580 (right panel) for 90 min, 10 μM 4HPR was then added to the culture medium and the cells were incubated for an additional 24 h before obtaining the cell extracts. (C) Detection of apoptosis by enzyme-linked immunosorbent assay (DNA fragmentation assay). Data are means of three independent experiments; vertical bars are standard deviations. The asterisk indicates a significant difference (P < 0.05). The cells were pretreated with 10 μM SP600125 for 15 min or with 10 μM SB203580 for 90 min, 10 μM 4HPR was then added to the culture medium and the cells were incubated for an additional 24 h before performing enzyme-linked immunosorbent assay.
We investigated whether 4HPR treatment leads to JNK activation in A2780 cells, determining its phosphorylation status. Western blot using a phospo-specific antibody that detects the phosphorylated (active) form of JNK showed that 24 h treatment with 5 μM or 10 μM 4HPR caused kinase phosphorylation and that phosphorylation is to some extent dose dependent (Figure 3A, left panel). To determine whether 4HPR-induced ROS increase and ER stress are involved in JNK phosphorylation, we tested the effects of vitamin C and salubrinal on JNK activation induced by 10 μM 4HPR. Treatment of cells with either vitamin C or salubrinal reduced 4HPR-induced JNK phosphorylation (Figure 3A, right panel), implying that JNK activation is dependent on 4HPR-induced ROS generation and ER stress.

Pharmacological inhibition of JNK suppresses PLAB induction and protects against 4HPR-induced apoptosis

To analyze whether JNK activation plays a role in 4HPR-induced PLAB upregulation and apoptosis, the effects of SP600125, a pharmacological inhibitor of JNK, were tested. Addition of 10 μM SP600125 suppressed JNK phosphorylation induced by 10 μM 4HPR, as observed after 24 h (Figure 3B, left panel). SP600125 also reduced PLAB upregulation in response to 4HPR (Figure 3B, left panel) and resulted in reduction of 4HPR-induced apoptosis assayed as PARP cleavage (Figure 3B, left panel) and DNA fragmentation (Figure 3C). Although SP600125 has been described as a specific inhibitor of JNK activation, other kinases may also be affected by this drug. To ascertain whether inhibition of JNK activity alone contributes to the reduced 4HPR-induced PLAB upmodulation and apoptosis, we examined the other two MAPK members, p38 and extracellular signal-regulated kinase (ERK), for possible involvement. To analyze the role of p38 in 4HPR apoptotic activity, the effect of SB203580, a specific pharmacological inhibitor of the p38 pathway, was assayed by testing the phosphorylation of HSP27, a specific downstream target of p38 (26). Addition of 10 μM SB203580 to A2780 cells suppressed 4HPR-induced phosphorylation of HSP27 (Figure 3B, right panel), but had no effect on 4HPR-induced PLAB upregulation and apoptosis measured as PARP cleavage (Figure 3B, right panel) and DNA fragmentation (Figure 3C). Involvement of ERK in the 4HPR apoptotic cascade was also excluded because, in time course experiments, following 4HPR treatment PLAB upmodulation and apoptosis onset were present at 15 h, whereas ERK phosphorylation only occurred at 24 h (data not shown). These findings suggest that PLAB upregulation and apoptosis induced by 4HPR are in part dependent on JNK activation.

Regulation of PLAB expression by JNK in A2780 cells

We found previously that 4HPR increases the half-life of the PLAB transcript (9). Since JNK is known to regulate mRNA stability (27), we wondered whether JNK might be involved in the stabilization of PLAB mRNA by 4HPR. We investigated the effect of JNK inhibition by SP600125 on PLAB mRNA stability. A2780 cells were treated with 4HPR or dimethyl sulfoxide dilution in culture medium, with or without SP600125. Transcription was then inhibited by 5 μg/ml actinomycin D. As expected, 4HPR had a major effect on PLAB mRNA levels (Figure 4A). Addition of SP600125 did not alter the half-life of PLAB mRNA (~1.5 h) in cells without 4HPR, but decreased its half-life in 4HPR-treated cells from >6 h to ~4.5 h (Figure 4A and B). These findings indicate that JNK contributes to the increase in PLAB mRNA stability induced by 4HPR.

Activation of the 4HPR-signaling cascade from ROS to PLAB occurs in 4HPR-sensitive cells but not in resistant cells

To determine whether the events leading to 4HPR-induced PLAB upregulation and apoptosis were restricted to A2780 cells, we assessed ROS generation, ER stress induction and JNK activation in the human ovarian cancer cell lines IGROV-1, OVCAR-3 and A2780/HPR, shown previously to have different 4HPR sensitivities, and to vary in extent of 4HPR-induced PLAB expression (9). As summarized in Figure 5A, IGROV-1 cells were inhibited by 4HPR [inhibiting concentration 50% (IC50) = 5 μM], while less than A2780 cells (IC50 = 1 μM), and 4HPR increased PLAB expression in IGROV-1 cells, though less than in A2780 cells (data not shown). OVCAR-3 cells are resistant to 4HPR (IC50 = 10 μM) and 4HPR did not increase PLAB expression in these cells. In A2780/HPR, a 4HPR-resistant cell line (IC50 > 10 μM), obtained by continuous exposure of A2780 cells to the retinoid (19), 4HPR caused only a slight upregulation of PLAB expression, whose final levels were much lower than observed in 4HPR-sensitive cells. Analysis of ROS generation in these cell lines exposed to 10 μM 4HPR for 4 h showed a slight ROS increase in IGROV-1 and A2780/HPR cells (1.5 and 1.3 times, respectively, compared with controls) and no change in ROS production in OVCAR-3 cells (Figure 5B). 4HPR induced ER stress (evidenced by XBP-1 splicing, upregulation of CHOP mRNA, GRP78/Bip protein, and elf2β phosphorylation) in IGROV-1 and in A2780/HPR cells, though less than in A2780 cells (data not shown), but not in OVCAR-3 cells (Figure 5C) and caused JNK phosphorylation only in IGROV-1 cells. Thus, activation of all the events of the signaling cascade from ROS generation to PLAB appears to occur only in IGROV-1 cells, which are 4HPR sensitive. To ensure that the slight increase in 4HPR-induced ROS generation in IGROV-1 cells was relevant to 4HPR signaling, we evaluated the effect of the antioxidant vitamin C on PLAB expression. Vitamin C reduced PLAB upmodulation and abrogated PARP cleavage (Figure 5D) induced by 4HPR, indicating that ROS generation, although slight, also plays a role in PLAB upregulation in IGROV-1 cells.

Discussion

The proapoptotic gene PLAB is highly upregulated by 4HPR in human ovarian cancer cell lines sensitive to 4HPR, where it mediates 4HPR-induced apoptosis (9). PLAB expression has also been linked to anti-inflammatory activity and the growth arrest and apoptosis of other cancer cells (11); several anticancer and proapoptotic compounds target PLAB expression (11–14). Nevertheless, the signaling pathways of this protein remain largely unknown. In the present study we have delineated the signaling pathway that contributes to 4HPR-induced upregulation of PLAB in ovarian cancer cell lines sensitive to 4HPR.
The most commonly described characteristic of 4HPR-induced apoptosis is that it is inhibited by antioxidants implicating oxidative stress and ROS generation in the apoptotic mechanism (3). Our previous study demonstrated that 4HPR increases ROS production in ovarian cancer cells (19). In the present study, we have shown that ROS are involved in 4HPR-induced upregulation and apoptosis in the human ovarian carcinoma cell line A2780 (20) since inhibition of 4HPR-induced ROS with antioxidants vitamin C or BHA also abrogated PLAB upregulation and protected the cells from apoptosis. In human A549 lung carcinoma cells, hydrogen peroxide exposure induced PLAB mRNA (28) providing further evidence of a relationship between oxidative stress and PLAB regulation.

4HPR-induced ROS production activates the ER stress response in cancer cells of various types (29–31) and there is evidence that ER stress contributes to 4HPR-induced apoptosis (30–32). Furthermore, 4HPR upregulates HSP70 in A2780 cells (9) and this chaperone is known to increase during ER stress and to promote 4HPR-mediated apoptosis (30). We therefore hypothesized that 4HPR-induced ROS generation might induce ER stress in A2780 cells and that ER stress was also involved in PLAB upregulation and apoptosis. We found that 4HPR did indeed induce ER stress as revealed by XBP-1 splicing, GRP78/BiP, pJNK, and pERF2 expression—and all markers of ER stress (23). Furthermore, the ER stress response occurred downstream of ROS induction, since, when A2780 cells were cotreated with vitamin C, XBP-1 remained unspliced, and GRP78/BiP and pJNK phosphorylation were strongly reduced. In fact, ER stress was a consequence of ROS generation and did not contribute to ROS production since the ER stress inhibitor salubrinal (22) has no effect on 4HPR-induced ROS generation in A2780 cells. The relationship between oxidative stress and ER stress is poorly understood. Contrary to the present findings and those of others (29–31) that ER stress is a consequence of ROS generation, it has been reported in Saccharomyces cerevisiae that prolonged ER stress causes ROS accumulation, indicating that ROS generation is a downstream event of ER stress (33).

![Fig. 5. Effect of 4HPR on ROS generation, induction of ER stress markers and JNK activation in IGROV-1, OVCAR-3 and A2780/HPR cell lines. (A) IC₅₀ values of 4HPR and 4HPR-induced PLAB expression in IGROV-1, OVCAR-3 and A2780/HPR cells. (B) Analysis of ROS production in IGROV-1, OVCAR-3 and A2780/HPR cells treated for 4 h with 10 µM 4HPR. The analysis was performed by flow cytometry after addition of the redox-sensitive dye 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate. The figure shows representative flow cytometry fluorescence profiles of untreated and treated cells. A shift to the right from control indicates increased ROS levels. (C) Reverse transcription–PCR gels (top) showing splicing of the 25 bp intron from XBP-1 transcript and expression of the CHOP transcript, and (bottom) western blot detection of GRP78/BiP, pERF2 and pJNK proteins in IGROV-1, OVCAR-3 and A2780/HPR cells treated 24 h with 10 µM 4HPR. For reverse transcription–PCR, β-actin was also amplified as internal control. For western blot, actin, probed with actin antibody, served as indicator of how much protein was loaded. (D) Western blot to evaluate PLAB protein expression and PARP cleavage in IGROV-1 cells treated 24 h with 10 µM 4HPR, with or without 100 µM vitamin C. Actin, probed with actin antibody, served as indicator of how much protein was loaded.](image-url)
The present study also provides evidence that activation of ER stress is involved in the 4HPR-signaling cascade leading to PLAB upregulation and apoptosis. Thus, inhibition of ER stress by salubrinal, a selective inhibitor of eIF2α dephosphorylation (22), abrogated 4HPR-induced PLAB upregulation and protected the A2780 cells from apoptosis. To our knowledge, this is the first evidence of a causal relationship between ER stress and PLAB upregulation.

The protein JNK has been shown to be induced by ER stress (34) and to participate in apoptosis induction in cells with irreparable ER stress (25). JNK activation also seems to mediate apoptotic signaling induced by 4HPR in various cancer cell lines (35–38) and to be a downstream event of 4HPR-induced ROS production in head and neck squamous cell carcinoma (35). We found that 4HPR treatment activated (phosphorylated) JNK in A2780 cells, and the phosphorylation was dependent on ROS generation and ER stress since it was abrogated by addition of both vitamin C and salubrinal. Furthermore, JNK activation was partly responsible for 4HPR-induced PLAB upregulation and consequent apoptosis, as shown by the reduction in these events when the JNK inhibitor SP600125 was present. Although SP600125 abrogated 4HPR-induced JNK phosphorylation, PLAB expression and apoptosis were reduced but not abolished (as occurred when vitamin C and salubrinal were present). SP600125 is a fairly non-specific inhibitor; thus, other members of the MAPK family might also be affected by this compound. To determine whether inhibition of JNK activity alone contributes to the reduced 4HPR-induced PLAB upmodulation and apoptosis, we assessed whether the other two MAPK members, p38 and ERK, were involved. Like JNK, p38 activation was also induced by 4HPR treatment; however, we were able to exclude its involvement in 4HPR apoptotic activity since specific pharmacological inhibition of p38 pathway did not alter cell sensitivity to 4HPR and 4HPR-induced PLAB upregulation. 4HPR treatment also activated ERK but its phosphorylation is subsequent of PLAB upregulation and apoptosis. We therefore conclude that JNK, but not p38 and ERK, participates in PLAB upregulation and 4HPR-induced apoptosis, even though it is not the sole mediator of these events. A limitation of our study is that JNK involvement in PLAB-mediated apoptotic pathway induced by 4HPR has been determined only by kinase chemical inhibitors. Two different genetic approaches were also tried to inhibit 4HPR-induced JNK activation in A2780 cells: transfection with siRNAs for JNK1 and JNK2 and transfection with JNK1- and JNK2-dominant negatives. However, we were not able to reduce JNK expression (and therefore not to inhibit 4HPR-induced JNK phosphorylation) by transfecting siRNA and we did not obtain expression of the exogenous proteins by transfecting with dominant negative-JNK constructs. We do not have explanations for the failure of these approaches, but speculate that in A2780 cells the expression of JNK might be essential for cell vitality. In addition, from our experience, A2780 are very difficult to transfect.

4HPR stabilizes PLAB expression by increasing PLAB mRNA half-life (9). In the present study, we have shown that phosphorylated JNK is involved in this process since inhibition of phosphorylation reduced 4HPR’s ability to delay PLAB mRNA decay. JNK is known to play a role in the stabilization of RNA that contains AU-rich elements (AREs) in its 3′-untranslated region (39). The 3′-untranslated region of PLAB mRNA contains highly conserved ARE sequences (40) and the 4HPR-induced increase in PLAB mRNA stability may therefore be due to JNK-dependent stabilization of its ARE sequences. Interestingly, ARE sequences in PLAB RNA have recently been reported to be stabilized by MAPKs in colorectal and prostate cancer cells treated with the novel peroxisome proliferator-activated γ ligand MCC-555 (40) and with vitamin E succinate (41), respectively.

We present the proposed signaling cascade from 4HPR to PLAB-mediated apoptosis in A2780 cells in Figure 6. This cascade could plausibly be a characteristic of ovarian cancer cells responsive to 4HPR since it (ROS generation, ER stress, JNK activation and PLAB upregulation) occurred in another 4HPR-responsive human ovarian carcinoma cell line (IGROV-1) but not the 4HPR-resistant OVCAR-3 or A2780/HPR cell lines. The increase in ROS generation by 4HPR in IGROV-1 cells was modest (1.5-fold compared with untreated control cells); however, it was sufficient to activate 4HPR signaling, as indicated by the fact that vitamin C reduced both PLAB upmodulation and PARP cleavage induced by 4HPR in these cells. Thus, this small increase in ROS production by IGROV-1 cells seems attributable to their sensitivity to 4HPR, although they were markedly less sensitive than A2780 cells, where 4HPR caused a 2.9-fold increase in ROS generation. In A2780/HPR cells, 4HPR provoked a small ROS increase (1.3 times compared with controls), a slight modulation of ER stress markers (less than in A2780 cells), but no detectable JNK phosphorylation. Thus, acquisition of the 4HPR-resistant phenotype was associated with inability to phosphorylate JNK upon 4HPR treatment.

It is important to note that, contrary to the present findings, in our previous study (19), A2780 and A2780/HPR cells exhibited similar levels of ROS generation after 4HPR treatment. The explanation is probably that A2780/HPR cells, which are continuously maintained in 4HPR-supplemented medium, have newly acquired the ability to reduce ROS generation in response to 4HPR.

To conclude, the novelty of our study is that it expands knowledge of signaling pathways involving the PLAB proapoptotic protein, showing for the first time that PLAB induction and apoptosis following 4HPR treatment occurs via ROS-dependent mechanisms involving ER stress induction and JNK phosphorylation in ovarian cancer cells.

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