Grp78 is essential for 11-deoxy-16,16-dimethyl PGE$_2$-mediated cytoprotection in renal epithelial cells

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$^1$Department of Pharmacology and Toxicology, College of Pharmacy, University of Arizona Health Sciences Center, Tucson, Arizona 85721-0207; $^2$Division of Pharmacology and Toxicology, College of Pharmacy, University of Texas at Austin, Austin 78712; $^3$University of Texas M. D. Anderson Cancer Center, Science Park-Research Division, Smithville, Texas 78957; and $^4$Eli Lilly and Company, Greenfield, Indiana 46140

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Jia, Z., M. D. Person, J. Dong, J. Shen, S. C. Hensley, J. L. Stevens, T. J. Monks, and S. S. Lau. Grp78 is essential for 11-deoxy-16,16-dimethyl PGE$_2$-mediated cytoprotection in renal epithelial cells. Am J Physiol Renal Physiol 287: F1113–F1122, 2004. First published June 29, 2004; 10.1152/ajprenal.00138.2004.—11-Deoxy-16,16-dimethyl PGE$_2$ (DDM-PGE$_2$) protects renal proximal tubule epithelial cells (LLC-PK$_1$) against the toxicity induced by 2,3,5-trithiolatathion-S-ythydroquinone (TGHQ), a potent nephrotoxic and nephrocarcinogenic metabolite of hydroquinone. We have now determined the ability of DDM-PGE$_2$ to protect against other renal toxicants and report that DDM-PGE$_2$ only protects against oncocytic cell death, induced by H$_2$O$_2$, iodoacetamide, and TGHQ, but not against apoptotic cell death induced by cisplatin, mercuric chloride, or tumor necrosis factor-α. DDM-PGE$_2$-mediated cytoprotection is associated with the upregulation of at least five proteins, including the major endoplasmic reticulum (ER) chaperone glucose-regulated protein 78 (Grp78). To elucidate the role of Grp78 in oncocytic cell death, we used LLC-PK$_1$ cells in which induction of grp78 expression was disrupted by stable expression of an antisense grp78 RNA (pkASgrp78). As anticipated, DDM-PGE$_2$ failed to induce Grp78 in pkASgrp78 cells, with a concomitant inability to provide cytoprotection. In contrast, DDM-PGE$_2$ induced Grp78 and afforded cytoprotection against H$_2$O$_2$, iodoacetamide, and TGHQ in empty vector transfected cells (pkNEO). These data suggest that Grp78 plays an essential role in DDM-PGE$_2$-mediated cytoprotection. Moreover, TGHQ-induced p38 MAPK activation is disrupted under conditions of a compromised ER stress response in pkASgrp78 cells, which likely contributes to the loss of cytoprotection. Finally, using two-dimensional gel electrophoresis coupled to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, we found that DDM-PGE$_2$ induced several proteins in pkNEO cells, but not in pkASgrp78 cells, including retinol-binding protein, myosin light chain, and heat shock protein 27. The findings suggest that additional proteins may act in concert with Grp78 during DDM-PGE$_2$-mediated cytoprotection against oncocytic cell death.

Prostaglandins (PGs) play important yet diverse roles in mammalian cell signaling, participating in cellular homeostasis, differentiation, proliferation, stress response signaling pathways, inflammation, apoptosis, and carcinogenesis (47). The diverse cellular effects of PGs are tissue dependent. Much work has been conducted on PG-mediated cytoprotection in the gastrointestinal tract and liver (39, 44). A number of PGs, and their analogs, protect target cells against various toxic, ischemic, and infectious injuries. Indeed, several synthetic PG analogs are used in the clinic to treat nonsteroidal anti-inflammatory drug-induced gastropathy, acute liver failure, liver transplantation, and chronic liver disease (39, 43, 54). However, the molecular mechanisms underlying the cytoprotective effect of PGs remain largely unknown. In particular, little is known about PG-mediated protective effects in the kidney (37).

PGE$_2$, a major metabolite of arachidonic acid in mammalian kidneys, regulates renal metabolism, sympathetic nerve activity, hemodynamics, and water and ion transport (47). PGE$_2$ is cytoprotective in the kidney in vivo and in cultured renal cells, suggesting the cytoprotective effects occur, at least in part, at the cellular level, independent of effects on the inflammatory response, blood flow, or neural innervation factors (46). Several cytoprotective mechanisms have been proposed, including changes in renal blood flow, increases in membrane stability, alterations in toxicant metabolism, regulation of tight junctions, inhibition of spontaneous apoptosis, and enhancement of tissue regenerative capacity (46, 52).

Pretreatment of renal proximal tubule epithelial cells (LLC-PK$_1$) with either PGE$_2$ or 11-deoxy-16,16-dimethyl PGE$_2$ (DDM-PGE$_2$), a stable synthetic analog of PGE$_2$, protects them against 2,3,5-trithiolatathion-S-ythydroquinone (TGHQ)-mediated cytotoxicity. TGHQ is a selective and potent nephrotoxic (42) and nephrocarcinogenic metabolite of hydroquinone (20). The DDM-PGE$_2$-mediated cytoprotective effect against TGHQ does not involve any of the four known PGE$_2$ receptors (EP), since EP agonists do not induce cytoprotection (55). In contrast, the protection appears to be mediated via a thromboxane receptor (TP) acting through a protein kinase C (PKC) signal transduction pathway coupled to NF-κB (56). Thus U-46619 (a TP agonist) and TPA (a potent PKC activator) also induce cytotoxicity against TGHQ in LLC-PK$_1$ cells, and TP antagonists abolish the cytoprotective effect of DDM-PGE$_2$. These findings indicate that DDM-PGE$_2$-mediated cytoprotection is mediated by events, including transcriptional control, mediated by the TP receptor (55, 56). $^{35}$S)methionine labeling, coupled to mass spectrometric protein sequencing, and confirmatory Western blot analyses revealed that at least five proteins are induced in LLC-PK$_1$ cells by DDM-PGE$_2$ treatment.
These proteins included elongation factor 2, heat shock protein 90B, elongation factor 1α, glucose-regulated protein 78 (Grp78), and actin. The known functions of these proteins suggest that cytoprotection may involve changes in the cytoskeleton, maintenance of cell polarity, activation of the unfolded protein response, or possibly increases in proximal tubule cell regenerative capacity (41, 50).

Among the DDM-PGE₂ proteins identified, Grp78 was of particular interest since induction of Grp78 and other endoplasmic reticulum (ER) molecular chaperones confers protection to epithelial cells against various insults (25, 50). In addition, overexpression of Grp78 prevents ER stress-mediated transcriptional activation of the ER stress response and protects cells against death caused by calcium depletion from the ER (8). Conversely, prevention of Grp78 induction sensitizes cells to oxidative stress, hypoxia, and cell-mediated toxicity (22). Therefore, we hypothesized that Grp78 may mediate the cytoprotective effects to DDM-PGE₂.

The present study was designed to determine the role of Grp78 in DDM-PGE₂-mediated cytoprotection (24, 25). Utilizing LLC-PK₁ cells in which induction of grp78 expression was disrupted by stable expression of an antisense grp78 RNA (pKASgrp78) as a model, we also determined the relative ability of DDM-PGE₂ to protect against known renal toxicants through either oncotic [TGHQ, H₂O₂, and iodoacetamide (IDAM)] or apoptotic [cisplatin, mercuric chloride, and tumor necrosis factor-α (TNF-α)] cell death (9, 18, 59). The results indicate that Grp78 is involved in DDM-PGE₂-mediated cytoprotection and provide new insights into the selectivity of DDM-PGE₂ cytoprotection against apoptotic and oncotic cell death.

**MATERIALS AND METHODS**

**Cell Line**

Renal proximal tubule epithelial cells (LLC-PK₁) were purchased from the American Type Culture Collection (Rockville, MD). LLC-PK₁ cells in which induction of grp78 expression was disrupted by stable expression of an antisense grp78 RNA (pKASgrp78) have been described (24, 25). Two independent clones of pKASgrp78 and a single clone of pKNEO cells were used in our study to avoid experimental errors that might arise from random selection of the clones. Cells were maintained in DMEM with 4.5 g/l glucose (GIBCO-BRL, Grand Island, NY) supplemented with 10% FBS (Atlanta Biologicals, Atlanta, GA) in a 37°C/5% CO₂ humidified incubator. The pKASgrp78 and pKNEO cells were maintained in complete medium with 400 μg/ml G418 (GIBCO). Cells were subcultured every 4–6 days at 90% confluence. All experiments were conducted using postconfluent cells (3–5 days postconfluence).

**Materials**

TGHQ was synthesized and purified according to established protocols (19). DDM-PGE₂, all other PG analogs, and U-46619 were purchased from Cayman Chemical (Ann Arbor, MI). HPLC-grade solvents were purchased from EM Science (Cincinnati, OH); acetic acid was from Aldrich (Milwaukee, WI); sequencing-grade trypsin was from Promega (Madison, WI); and α-cyano-4-hydroxycinnamic acid and calibration mixture 1 were from the Sequazyme Peptide Mass Standards Kit (Perseptive Biosystems, Framingham, MA). Primary antibodies and the manufacturers are listed as follows: caspase-3, retinol-binding protein (RBP; BD Transduction Laboratories, San Jose, CA); total p38 MAPK, phospho-p38 MAPK, MAPKAPK-2, phospho-MAPKAPK-2 (Cell Signaling Technology, Beverly, MA); GAPDH (a generous gift from Dr. K. Kline, University of Texas at Austin); heat shock protein 27 (Hsp27; Stressgen Biotechnologies, Victoria, BC, Canada); and phospho-Hsp27: Ser15 (Santa Cruz Biotechnology). Ser78 (Stressgen Biotechnologies), and Ser82 (Cell Signaling Technology). Antibody recognizing porcine Grp78 was raised against a 17-amino-acid COOH-terminal peptide from the porcine sequence (CAGFPPTGDEESADKDEL) with an NH₂-terminal cysteine added for coupling to keyhole-limpet hemocyanin; detailed characterization of the antibody will appear elsewhere (S. Assmelash, J. L. Stevens, and T. Ichimura, unpublished observations). With the exception of the secondary anti-rabbit antibody for Grp78 (Stressgen Biotechnologies), all of the secondary antibodies were purchased from Santa Cruz Biotechnologies.

**Compound Treatment**

TNF-α was dissolved in 1.0% BSA in PBS. Stock DDM-PGE₂ and U-46619 solutions were made in 100% ethanol. All other toxicants were dissolved in distilled and deionized water. Treatment with TNF-α for 2 h after pretreatment with 100 ng/ml gliotoxin for 30 min was used as a positive control for apoptosis in LLC-PK₁ cells (59). A dose that induced 50–60% cell death in cell culture was selected for each toxicant.

**Ethidium Bromide/Acridine Orange Staining**

The fluorescent DNA-binding dyes, acridine orange and ethidium bromide (Sigma-Aldrich), were used for the morphological assessment of apoptosis and oncrosis. Concentrations of both dyes in the mixture were 100 μg/ml in PBS. Cells were grown on sterilized coverslips in six-well plates. At the end of each treatment, cells were washed in ice-cold PBS three times and the dyes (6 μl) were then added to each cover slip, which were then inverted and viewed under a Nikon E800 fluorescent microscope. The images were captured using a Nikon Cool Snap photo system and processed using Photoshop 6.0 software from Adobe (San Jose, CA).

**Caspase-3 Assay**

Caspase-3 activity was measured according to an established protocol (6). Briefly, Ac-DEVD-pNA (A2559; Sigma) was used as the substrate for activated caspase-3, and 1–2 × 10⁶ cells were typically used. Reactions were conducted in 96-well plates at 37°C for 2 h in the dark. Absorbance was determined with a microplate reader (model FL600FA; Bio-Tex Instruments) at 405 nm. p-Nitroaniline (pNA) concentrations were calculated by comparison with a standard curve. The linear standard curve was constructed using different concentrations of freshly prepared pNA.

**Western Blot Analysis**

At the end of each experiment, cells were washed three times with ice-cold PBS, scraped, and lysed in homogenization buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM sodium fluoride, 5 mM EDTA, 1% Triton X-100, 40 mM β-glycerophosphate, 0.5 mM sodium orthovanadate, 0.25 mM phenylmethylsulfonyl fluoride, and 1 Complete Mini Protease Inhibitor Cocktail tablet/10 ml lysis buffer) for 15–30 min on ice. Cell lysates were clarified by centrifugation at 14,000 g for 20 min at 4°C. Supernatant containing total protein were collected and stored at −80°C. For caspase-3 immunoblotting, cells were lysed in Chaps Cell Extraction Buffer (catalog number 9852; Cell Signaling Technology) and subjected to a freeze-thaw cycle three times. Supernatants obtained after centrifugation were designated as cytosolic extracts. Protein concentrations were measured with the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Proteins were separated on 10% denaturing polyacrylamide gels (SDS-PAGE) and transferred to a nitrocellulose membrane by electroblotting (wet transfer). The appropriate blocking and incubation
procedures were modified from the manufacturer’s instructions for all the antibodies.

Neutral Red Uptake Assay

Cell viability was measured with the neutral red lysosomal uptake assay (28). Cells were grown in 24-well plates 3–5 days postconfluence before treatment. At the end of each experiment, cells were washed one time with Hanks’ balanced salt solution, 1.0 g/l d-glucose, 0.4 g/l KCl, 0.06 g/l KH₂PO₄, 8.0 g/l NaCl, 0.05 g/l Na₂HPO₄, 0.01 g/l phenol red, and 0.35 g/l NaHCO₃. Cells were then incubated with 0.05 mg/ml neutral red solution for 1 h at 37°C/5% CO₂. The neutral red solution was aspirated, and 1 ml 1% formaldehyde/1% CaCl₂ solution (wash-fixation solution) was added to each well for 45 s. One percent glacial acetic acid/50% ethanol solution (1 ml each well) was added and incubated with cells for 15 min at room temperature in the dark to remove excess neutral red dye. The amount of neutral red dye accumulation in lysosomes was assessed by determining the absorbance at 540 nm.

Two-Dimensional PAGE Analysis

Two-dimensional (2D) PAGE was performed according to established protocols (26). Briefly, cells were lysed in a modified RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.5% Triton X-100, and 10 mM EDTA). Protein samples were loaded on an 11-cm focusing tray. 2D SDS-PAGE was performed using a Bio-Rad PROTEAN IEF Cell. Precast IPG dry strips were rehydrated with protein sample for 12 h at 50 volts in the PROTEAN IEF Cell. Isoelectric focusing was carried out automatically using Immobilized pH gradient strips (pH 3–10) under conditions suggested by the manufacturer. SDS-PAGE was carried out using a precast Criterion 8–16% gradient gel in a Criterion Cell, at 20 volts for 10 min and then at 200 volts for 45–55 min. The gels were stained with SYPRO Ruby protein stains (Bio-Rad) overnight. The 2D images were captured and quantitatively assessed on a Kodak Image Station 440 CF (Eastman Kodak, Rochester, NY). The differentially expressed protein spots were excised manually from the SDS-PAGE gels and subject to in-gel trypic digestion, based on a modification of standard protocols (45).

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Analysis

Sample preparation. Tryptic digests were analyzed on a Voyager-DE PRO matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Perseptive Biosystems). The samples were dried to <5 μl in a SpeedVac (ThermoSavant, Holbrook, NY) and mixed with matrix at a 1:1 ratio on a hydrophobic-coated steel MALDI target (Perseptive Biosystems) in 1 μl total volume. The matrix was a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid in distilled, deionized water. Samples were desalted with C₁₈ 0.6-μl ziptips (Millipore) according to the manufacturer’s protocol, using the matrix solution as the elution buffer, with direct target spotting. The calibration mixture was prepared in matrix solution, according to the manufacturer’s recommendations. Automated MALDI-TOF spectra acquisition was performed for both calibrants and samples in the range of 1,000–100,000, 100 ppm peptide mass tolerance for trypptic digest, and a maximum of two missed cleavages, carbamidomethylation of the cysteines, and a minimum of four peptides matched required for a hit. After Intelect recalibration, a second search was performed with 20 ppm peptide mass tolerance. For proteins not identified in the automated analysis, manual acquisition, and/or manual calibrations were performed using the trypsin autolysis peaks at mass-to-charge 842/51, 1045/5642, and 2211/1046. When a manual database search was performed, the number of peptides submitted varied depending on the spectrum. The protein represented by the highest number of peptide masses matched, or the highest scoring match is reported.

Post source decay. For samples that could not be definitively identified by peptide mass mapping, one ion from the MS spectrum was subjected to post source decay (PSD) analysis for protein identification by peptide sequencing. The PSD spectra of each ion are a composite of several segments of spectrum, each acquired at a different PSD mirror ratio. Each segment of the spectrum was averaged over ~400 laser shots. For MALDI-PSD spectra, the raw data were smoothed, and the mass list was exported to the MS-Tag peptide fragmentation database search engine in the Protein Prospector suite. Similar search parameters were used vide infra, except that the parent ion mass tolerance was 20 ppm and the fragment ion tolerance was 1,500 ppm. Y (COOH-terminal peptide fragments), b (NH₂-terminal peptide fragments), a (b − c = 0), neutral loss, immionium, and internal fragment ions were matched. The protein identified in each case was the highest-scoring match.

Statistical Analysis

For individual comparisons, one-way ANOVA followed by Student-Newman-Keul’s post hoc analysis was used. P < 0.05 were considered to be significant.

RESULTS

DDM-PGE₂-mediated Cytoprotection is Dependent on the Mode of Cell Death

Both morphological and biochemical determinations for oncosis and apoptosis in LLC-PK₁ cells after toxicant treatments were assessed at early (2 h) and later (24 h) time points. In TGHQ₂-, IDAM⁻, or H₂O₂-treated LLC-PK₁ cells, the predominant form of cell death was oncosic (Fig. 1A and B), as evidenced by the uptake of ethidium bromide resulting in loss of membrane integrity and the pyknotic appearance of the nuclei at both time points. DDM-PGE₂ pretreatment protected the cells against the morphological changes at both the 2- and 24-h time points, since the cells still maintained plasma membrane integrity, as indicated by resistance to ethidium bromide staining (Fig. 1A and B). In contrast, LLC-PK₁ cells treated with either cisplatin or mercuric chloride underwent apoptotic cell death evidenced by condensed and fragmented chromatin stained with acridine orange (Fig. 1C). TNF-α was used as a positive control for apoptotic cell death in LLC-PK₁ cells (Fig. 1C). DDM-PGE₂ pretreatment failed to prevent the morphological changes induced by these three apoptosis-inducing toxicants. Consistent with these data, Western blot analysis revealed that cleavage of pro-caspase-3 to active fragments was observed only in those treatments where morphological correlates of apoptosis were present (Fig. 2). Caspase-3 activ-
Gir78 IS ESSENTIAL FOR DDM-PGE2-MEDIATED CYTOPROTECTION

Fig. 1. Morphological evaluation of oncotic/necrotic and apoptotic cell death and 11-deoxy-16,16-dimethyl PGE2 (DDM-PGE2) cytoprotection. Initial studies indicated that DDM-PGE2 protects only against oncotic cell death using the neutral red assay to quantitate cell killing. Consistent with morphological evaluations and the caspase-3 data, pretreatment of LLC-PK1 cells with either DDM-PGE2 (2 μM, 24 h) or the TP agonist U-46619 (10 μM, 24 h) protected against TGHQ-, IDAM-, and H2O2-, but not against cisplatin-, mercuric chloride-, or TNF-α-mediated cell death (Fig. 4).

Effects of DDM-PGE2 in pkASgrp78 Cells

The induction of grp78 mRNA and protein is disrupted in pkASgrp78 cells by stable expression of a 500-bp antisense grp78 RNA (pkASgrp78). Therefore, we tested the ability of DDM-PGE2 to induce Grp78 protein and to protect pkASgrp78 or control cells transfected with only the empty vector (pkNEO) against toxicants. As expected, DDM-PGE2 induced Grp78 in both naive (LLC-PK1 cells without transfection) and pkASgrp78 cells (Fig. 3). We also confirmed that DDM-PGE2 protects only against oncotic cell death using the neutral red assay to quantitate cell killing. Consistent with morphological evaluations and the caspase-3 data, pretreatment of LLC-PK1 cells with either DDM-PGE2 (2 μM, 24 h) or the TP agonist U-46619 (10 μM, 24 h) protected against TGHQ-, IDAM-, and H2O2-, but not against cisplatin-, mercuric chloride-, or TNF-α-mediated cell death (Fig. 4).

Fig. 2. Caspase-3 cleavage in LLC-PK1 cells undergoing oncotic/necrotic or apoptotic cell death. LLC-PK1 cells were lysed in CHAPS cell extract buffer and pkASgrp78 RNA (pkASgrp78). Therefore, we tested the ability of pkASgrp78 cells by stable expression of a 500-bp antisense DDM-PGE2 to induce Grp78 protein and to protect pkASgrp78 cells were more sensitive to toxicants (data not shown), as might be expected if Grp78 affords protection. Therefore, toxicant concentrations were adjusted to achieve a similar degree of cell killing in all cell lines tested. Pretreatment of pkNEO cells with DDM-PGE2 or U-46619 afforded full cytoprotection against TGHQ, IDAM, or H2O2. In contrast, cytoprotection against TGHQ, IDAM, or H2O2 was totally abolished in pkASgrp78 cells (Fig. 6). These findings clearly demonstrate that Grp78 plays a critical role in DDM-PGE2-mediated cytoprotection.

2D-PAGE Analysis of Protein Expression in pkASgrp78 and pkNEO Cells

To gain insights into cytoprotective events downstream of Grp78 induction, we compared the pattern of DDM-PGE2-induced proteins in pkASgrp78 and pkNEO cells. 2D-PAGE analysis of protein expression patterns in pkNEO and pkASgrp78 cells was similar (2 representative gel images are shown in Fig. 7). However, a number of proteins (spots a-d) were changed after DDM-PGE2 pretreatment of pkNEO cells, but not of pkASgrp78 cells (Fig. 8, A, B, E, and F). Mass spectral analysis identified these proteins as retinol binding protein (RBP) (d, 11-fold), myosin light chain (a, 2.2-fold), phosphoheat shock protein 27 (Hsp27; b, 2.8-fold), and Hsp27 (c, 4.0-fold), phosphoheat shock protein 27 (Hsp27; b, 2.8-fold), and Hsp27 (c,
1.75-fold). These proteins were identified by either PSD or best hit from the database search. The inductions of RBP and Hsp27 were confirmed by Western blot (Fig. 9).

**TGHQ Induces Phosphorylation of Hsp27 in pkNEO but not in pkASgrp78 Cells**

Two spots (b and c) exhibiting the same molecular weight but with differing PI values were identified as Hsp27. The intensity of spot b increased after TGHQ treatment in pkNEO but not in pkASgrp78 cells. The shift in PI from high to low (spot c to b) suggested a posttranslational modification induced upon TGHQ treatment (Fig. 8, A–D). We further confirmed the modification was a result of the phosphorylation of Hsp27 on all three known sites (Ser15, Ser78, and Ser82) by Western blot analysis (Fig. 9). Interestingly, TGHQ-induced Hsp27 phosphorylation occurred in pkNEO but not in pkASgrp78 cells (Fig. 8, E–H, and Fig. 9), suggesting that Grp78 induction is required for both the induction and phosphorylation of Hsp27.
**p38 MAPK Signaling Pathway is Abrogated in pkASgrp78 Cells**

p38 MAPK and a direct downstream substrate, the kinase MAPKAPK-2, were phosphorylated upon TGHQ treatment in pkASgrp78 cells. Eight 2D gel-cropped image panels (A-H) focusing on the local areas containing inducible proteins are shown. Both pkASgrp78 and pkNEO cells were exposed to either vehicle control [ethanol (EtOH)] or DDM-PGE2 pretreatment (2 μM, 24 h) followed by TGHQ treatment (150 or 200 μM for 2 h). Protein spots identified are myosin light chain (a), phospho-heat shock protein (Hsp) 27 (b), Hsp27 (c), and retinol-binding protein (d).

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**Fig. 8.** Differences in protein expression in pkNEO and pkASgrp78 cells. Eight 2D gel-cropped image panels (A-H) focusing on the local areas containing inducible proteins are shown. Both pkASgrp78 and pkNEO cells were exposed to either vehicle control [ethanol (EtOH)] or DDM-PGE2 pretreatment (2 μM, 24 h) followed by TGHQ treatment (150 or 200 μM for 2 h). Protein spots identified are myosin light chain (a), phospho-heat shock protein (Hsp) 27 (b), Hsp27 (c), and retinol-binding protein (d).

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**Fig. 9.** Western blot analysis of retinol-binding protein, phospho-Hsp27 (Ser15, Ser82, Ser78), and total Hsp27 expression in pkNEO and pkASgrp78 cells. Both pkNEO and pkASgrp78 cells were exposed to either ethanol (E) or 2 μM DDM-PGE2 (D) for 24 h, followed by TGHQ challenge (ET, ethanol pretreatment followed by TGHQ; DT, DDM-PGE2 pretreatment followed by TGHQ). TGHQ treatments are 200 μM for 2 h for pkNEO cells and 150 μM for 2 h for pkASgrp78 cells to achieve similar toxicity/biological effect.

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**Fig. 10.** Western blot analysis of phospho-p38, total p38, phospho-MAPKAPK-2, and total MAPKAPK-2 expression in pkNEO and pkASgrp78 cells. Cells were treated as described in the legend to Fig. 9. Briefly, E and ET refer to ethanol (vehicle control for DDM-PGE2) pretreatment followed by either water as a vehicle control or TGHQ, respectively. D and DT refer to DDM-PGE2 pretreatment followed by either water as vehicle control of TGHQ, respectively.
pkNEO cells. DDM-PGE2 pretreatment enhanced the phosphorylation. In contrast, TGHQ did not induce phosphorylation of either p38 MAPK or MAPKAPK-2 in pkASgrp78 cells, despite the fact that the total levels of these two kinases are similar in both cell types (Fig. 10). These data are consistent with the hypothesis that the loss of Hsp27 phosphorylation in the pkASgrp78 cells results from an inability to activate p38 MAPK and its downstream target MAPKAPK-2. Thus the p38 MAPK signaling response is disrupted in pkASgrp78 cells after TGHQ treatment. We conclude that a disrupted grp78-dependent ER stress response interferes with p38 MAPK signaling upon ROS generation.

DISCUSSION

DDM-PGE2 affords cytoprotection against ROS-induced toxicity in LLC-PK1 cells via a TP or TP-like receptor (55, 56). We have now determined the specificity of DDM-PGE2-mediated protection with respect to a variety of toxicants that induce cell death via different mechanisms: TGHQ [an oxidant and electrophile (51)]; H2O2 (a pure oxidant); IDAM [an alkylating agent and oxidative stressor (4)]; cisplatin [a DNA-damaging (23) and mitochondrial toxicant (3)]; mercuric chloride [a potent thiol-binding agent (35)]; and TNF-α [a prototypical apoptosis-inducing agent (2)]. We also evaluated the role of Grp78 in cytoprotection by examining the effects of the selective disruption of DDM-PGE2-mediated Grp78 induction. Finally, we linked the ER stress pathway to upstream signaling events mediated by a TP-like receptor and to downstream signaling events. A number of key observations are worthy of additional discussion.

DDM-PGE2 only offered cytoprotection against agents that induced oncotic/necrotic cell death (TGHQ, IDAM, or H2O2) and failed to protect against apoptotic cell death (cisplatin, mercuric chloride, or TNF-α). Moreover, the TP agonist U-46619 also only provided cytoprotection against the chemicals that caused oncotic cell death, confirming that cytoprotection is dependent on the mode of cell death. van De Water et al. (53) reported that prior ER stress is able to prevent IDAM-induced apoptosis. However, the experimental protocol used in those studies required the inclusion of antioxidant treatment as a prerequisite for the engagement of the apoptotic cell death pathway. Regardless, the studies reported herein support the conclusion that DDM-PGE2 pretreatment selectively prevents oncotic cell death.

A proteomic analysis revealed that Grp78 levels increased in response to DDM-PGE2 treatment (50). Grp78 is a major ER chaperone present in all cells and also plays an important role in calcium storage in addition to its function in the translocation, folding, and assembly of nascent proteins (22). Grp levels are regulated at the transcriptional level by ER stress, but also in response to growth factor stimulation or withdrawal. PG-mediated Grp78 induction is dependent on chemical structure. Thus Δ12-PGJ2 and other synthetic PGs possessing a cyclopentenone structure induce Grp78 gene expression in HeLa cells and in normal rat kidney cells (34, 49). In contrast, a synthetic cyclopentanone PG does not induce Grp78 expression (49). DDM-PGE2 is a structurally distinct synthetic cyclopentanone PG, and the mechanism by which it induces Grp78 expression likely differs from that of the cyclopentenone PGs.

Prior ER stress induces Grp78 and protects renal epithelial cells against a subsequent challenge with a variety of toxicants (24, 25). Indeed, treatment of LLC-PK1 cells with H2O2, IDAM, or TGHQ significantly increased Grp78 expression, suggesting that these agents all activate the ER stress response (data not shown). Thus pretreatment of LLC-PK1 cells with DDM-PGE2 primes the ER stress response before the TGHQ/IDAM/H2O2 exposure, thereby providing cytoprotection. Prior ER stress also protects against IDAM-induced oncotic and apoptotic cell death in LLC-PK1 cells (53). This protection is reduced in pkASgrp78 cells, a LLC-PK1 cell line in which induction of grp78 expression is disrupted by stable expression of an antisense grp78 RNA (pkASgrp78; see Refs. 24 and 25). pkASgrp78 cells were more sensitive to TGHQ-, IDAM-, or H2O2-induced toxicity than the corresponding pkNEO cells, which retain an appropriate Grp78 response (Fig. 5). Thus Grp78 is critical to DDM-PGE2-mediated cytoprotection (Fig. 6).

2D PAGE analysis coupled to MALDI-TOF MS revealed that proteins other than Grp78 were induced in DDM-PGE2-treated pkNEO but not in pkASgrp78 cells. Thus disruption of appropriate Grp78 induction also interferes with the induction of complementary cytoprotective proteins. Comparison of the protein profiles of DDM-PGE2-treated pkNEO and pkASgrp78 cells revealed that several proteins were induced differentially by DDM-PGE2 in pkNEO cells, including Hsp27, myosin light chain, and RBP.

Other PGs, such as PGF2α and TPA, have been reported to induce Hsp27 synthesis (14, 16). Hsp27 belongs to the small heat shock/heat shock/crystallin protein family. It is also a molecular chaperone that binds denatured proteins and inhibits the aggregation and precipitation of nonnative proteins. The enhanced expression of Hsp27 promotes cell survival against a variety of stresses, including heat shock, oxidative stress, and cytotoxic agents (11, 17). Although studies on the function and regulation of Hsp27 have been extensive, the exact mechanisms underlying the cytoprotective effects of this protein remain elusive. Three properties of Hsp27 may contribute to its cytoprotective effects as follows: 1) Hsp27 can regulate intracellular ROS and GSH content when present as large, nonphosphorylated aggregates (27); 2) Hsp27 acts as a molecular chaperone (15); and 3) Hsp27 may regulate actin filament dynamics and stabilize microfilaments upon dissociation from large to small aggregates, and when phosphorylated by p38 MAPK-activated MAPKAP-2/3 (13). Hsp27 appears to interact with cytoskeletal proteins and may confer cytoprotection by modulation of cytoskeletal organization, which is critical for the maintenance of cell morphology and normal function (5). Oxidant-induced damage to the actin network is considered to be a critical and sensitive target of oxidant stress (5), and Hsp27 promotes the stabilization of the microfilament system (21). Thus, in combination with our previous findings that DDM-PGE2 induces cytoskeletal protein expression, including filamin, myosin, and actin, the induction of Hsp27 may coordinate cytoskeletal reorganization, thereby contributing to cytoprotection.

TGHQ-induced Hsp27 phosphorylation (Fig. 9) is consistent with previous reports of ROS-mediated Hsp27 phosphorylation. Phospho-Hsp27 also regulates microfilament dynamics during the protective response to oxidative stress, since substantial evidence suggests that the modulation of microfilament...
Because the phosphorylation of Hsp27 and of upstream kinases, including MAPKAPK-2 and p38 MAPK, only occurred in pkNEO but not in pkASgrp78 cells, the inability to induce Grp78 expression clearly interferes with the p38 MAPK signaling pathway (including Hsp27 phosphorylation), thereby contributing to the loss of cytoprotection in pkASgrp78 cells. Although Hsp27 is also phosphorylated by PKC-mediated ERK activation, TGHQ-mediated Hsp27 and MAPKAPK-2 phosphorylation are blocked by a p38 MAPK inhibitor, SB-202190, but not by PD-98059, an ERK inhibitor (data not shown). Furthermore, dominant-negative transfected p38 MAPK totally abolished MAPKAPK-2 and Hsp27 phosphorylation (7). These data strongly suggest that the upstream MAPK responsible for TGHQ-induced Hsp27 phosphorylation in LLC-PK1 cells is p38 MAPK not ERK. pkASgrp78 cells express less ERK and more JNK activation in response to H₂O₂, suggesting that the efficiency of the ER stress response contributes to the balance in the response of the ERK and JNK signaling pathways to oxidative stress (12). Although p38 MAPK is activated upon H₂O₂ exposure in LLC-PK₁ cells, the authors did not examine p38 MAPK expression in pkASgrp78 cells (12). Thus we report for the first time that p38 MAPK signaling is disrupted in Grp78-dependent ER stress response compromised cells. However, Hsp27 phosphorylation alone is insufficient to afford cytoprotection, since phospho-Hsp27 is induced by TGHQ in both vehicle control and DDM-PGE₂-pretreated pkNEO cells (Fig. 9). DDM-PGE₂ pretreatment increased phosphorylation of p38 MAPK and MAPKAPK-2 upon TGHQ treatment (Fig. 10). The inability to couple Hsp27 phosphorylation to increases in Hsp27 expression suggests that the chaperone function of unphosphorylated Hsp27 is also important for cytoprotection.

RBP was robustly induced (11-fold) by DDM-PGE₂ treatment in pkNEO but not in pkASgrp78 cells. The major organ for RBP synthesis is the liver, but the kidney is also a significant site of synthesis (48). RBP is responsible for transporting retinol/vitamin A from the storage pool in liver to extrahepatic epithelial tissue. Retinol subsequently binds to cellular RBP inside the cell where it is metabolized to retinoic acids. Retinoids are indispensable for a variety of fundamental biological functions, including growth, vision, reproduction, embryo development, epithelial differentiation, immune system function, and bone remodeling (32). Two principal retinoic acid metabolites that contribute to its biological activity are all-trans-retinoic acid [a ligand for the retinoic acid receptor (RAR)] and 9-cis-retinoic acid [a ligand for RAR and the retinoid X receptor (RXR); see Ref. 33]. Retinoids are critical for epithelial differentiation. The retinoid acid family activates nuclear receptors RAR and RXR, which in turn act as transcription factors and regulate many genes containing retinoic acid response element (RARE) in their regulatory region (1). The RBP gene promoter region contains the RARE (38). RBP has been implicated in repair and regeneration after partial hepatectomy in rats (29), during skin wound healing (57), and after sciatic nerve injury (58). Thus increased RBP synthesis suggests that the retinoid signaling pathway is likely involved in DDM-PGE₂-mediated cytoprotection. In addition, retinoids also induce reorganization of the actin cytoskeleton (40). Because the α-actin gene contains a T3 response element (TRE) that interacts with RXR-α in a sequence-specific manner, and the α-actin and myosin TREs interact with identical nuclear factors in muscle cells (31), it seems likely that DDM-PGE₂ is interacting with retinoid signaling.

In summary, DDM-PGE₂ selectively induces a group of proteins, including RBP, cytoskeletal proteins, and cellular chaperones such as Grp78 and Hsp27 in pkNEO but not pkASgrp78 cells. These proteins may act in concert in DDM-PGE₂-mediated cytoprotection against oncotic/necrotic but not apoptotic cell death. Because tissue damage arising as a result of necrotic cell death is implicated in many types of renal injury, such as ischemic reperfusion, hypoxia, and acute renal failure (10, 36), our studies have the potential to provide insights into novel therapeutic strategies for kidney disease.

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