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Select cyclopentenone prostaglandins trigger glutathione efflux and the role of ABCG2 transport

Heather M. Brechbuhl^c, Elysia Min^e, Chirag Kariya^d, Barbara Frederick^d, David Raben^d, and Brian J. Day^{a,b,c,e}

^aDepartment of Medicine, University of Colorado Denver Health Sciences Center, Denver, Colorado, USA

^bDepartment of Immunology, University of Colorado Denver Health Sciences Center, Denver, Colorado, USA

^cDepartment of Pharmaceutical Sciences, University of Colorado Denver Health Sciences Center, Denver, Colorado, USA

^dDepartment of Radiation Oncology, University of Colorado Denver Health Sciences Center, Denver, Colorado, USA

^eDepartment of Medicine, National Jewish Health, Denver, Colorado, USA

Abstract

Electrophilic cyclopentenone prostaglandins (cyPGs), such as 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15dPGJ₂), initiate redox-based cell signaling responses including increased intracellular glutathione (GSH) synthesis. We investigated whether cyPGs facilitated GSH efflux and if members of the ATP-cassette binding (ABC) protein family mediated the efflux. Four human cell lines were treated with 1–6 μ M of cyPGs for 48 hours. Media and cells were harvested for GSH measurements using HPLC-EC. CyPG treatment increased extracellular GSH levels 2- to 3-fold over controls in HN4 and C38 cells and 5- to 6-fold in SAEC and MDA 1586 cells and were dependent on increased GSH synthesis. Our studies show that prostaglandin D₂ (PGD₂) and its metabolites, prostaglandin J₂ (PGJ₂) and 15dPGJ₂ specifically induce GSH efflux as compared to other eicosanoids. These higher extracellular GSH levels were associated with protection from tert-butyl hydroperoxide. Superarray analysis of ABC transporters suggested only ABCG2 expression had a positive relationship in the 4 cell types when compared with extracellular GSH increases after cyPG treatment. The ABCG2 substrate Hoechst 33342 inhibited extracellular GSH increase after 15dPGJ₂ treatment. We report for the first time that ABCG2 may play a role in GSH efflux in response to cyPG treatment, and may link inflammatory signaling with antioxidant adaptive responses.

Keywords

15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂; ABC proteins; ATP-cassette transporter proteins; BCRP; breast cancer resistance protein; glutathione; prostaglandins

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Corresponding author: Brian J. Day, Ph.D., Professor of Medicine, National Jewish Health, 1400 Jackson St., Denver, CO 80206. Phone: 303-398-1121; Fax: 303-270-2263; E-mail: dayb@njc.org..

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Introduction

Eicosanoid family members include the prostaglandins (PGs), prostacyclins, thromboxanes, and leukotrienes, which are derived from arachidonic acid. Owing to their prominent role in various immune responses, eicosanoids are often referred to as mediators of inflammation that function in the development, progression, and/or resolution of multiple diseases [1-5]. Inflammation is comprised of a complex series of events that can involve a redox response in both the intracellular and often extracellular compartments [6,7]. Redox changes in the extracellular matrix are very important, as fluctuations can alter cell proliferation, cell-to-cell signaling, and even promote cell death [8]. Many eicosanoids, including PGE₂, 8-iso PGE₂, PGA₂, and the PGD₂ metabolites PGJ₂, and 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15dPGJ₂), are capable of inducing signaling pathways that alter the redox environment within and surrounding cells [9-15]. We examined possible changes in glutathione (GSH) associated with eicosanoid exposure because PG signaling frequently leads to the build up of reactive oxygen, nitrogen, and electrophilic compounds in the extracellular matrix.

GSH is the major endogenous thiol antioxidant having an extensive role in the preservation of cellular redox balance as well as the detoxification of exogenous and endogenous compounds [16]. The cell maintains millimolar concentrations of GSH in the intracellular compartment by tightly regulating its synthesis and turnover. The cell has several enzymes required for GSH production and the synthesis of these enzymes is often induced through activation of various regulatory response elements, including the electrophile-responsive element (EpRE) and the xenobiotic response element (XRE) [17,18]. A wide variety of transcription factors can activate EpRE and XRE including, AP-1, Nrf2, NF-kappaB, p53, HIF-1, and others. The response elements regulate transcription of a wide variety of stress response proteins, including glutamate-cysteine ligase (GCL), glutathione S-transferase (GST), heme oxygenase 1 (HO-1), glutaredoxins, thioredoxins, and peroxiredoxins, among others [19]. All of these mechanisms help protect the cell from intracellular and extracellular oxidative stress.

In order to adapt to extracellular oxidative stress, the cell must have mechanisms by which it can signal antioxidant responses within the extracellular compartment. One such response includes GSH efflux, as is evident in the lung's response to cigarette smoke and bacterial infection [20,21]. In order for GSH efflux to occur and be protective, the cell must have at least two functional components: a molecule(s) capable of signaling the cell to synthesize GSH, and a transporter(s) through which GSH can move.

ABC proteins play a major role in the efflux of endogenous and exogenous compounds. The ABCC subfamily contains transporters commonly referred to as the multi-drug resistant proteins (MRPs). Within this subfamily, six members have been linked to GSH and/or GSH conjugate transport: ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, and ABCC7 [22-27]. Previous studies have shown that ABCC1, ABCC3, and ABCC4 modulate cellular PG activity, largely through efflux mechanisms [28-30]. Furthermore, evidence indicates that low concentrations (<10 μ M) of electrophilic cyclopentenones such as 15dPGJ₂ invoke redox-based cell signaling responses, including increased synthesis of intracellular GSH [12,31-33]. The ability of 15dPGJ₂ to increase intracellular GSH and the active transport capacity of many ABC proteins to efflux GSH and GSH conjugates motivated us to examine whether this system may link inflammatory responses to antioxidant adaptive responses.

We sought to determine whether alterations in intracellular GSH content upon PG exposure have translational effects in the extracellular compartment. Therefore, the primary objectives of this investigation were: (1) to determine if eicosanoids induce increases in extracellular GSH; (2) to identify if this induction is specific to a group of eicosanoids or if it is a general characteristic of eicosanoid exposure; (3) determine whether efflux of GSH in the extracellular

space is cytoprotective against oxidant injury; and (4) to identify candidate transporters that mediate the ability of a cell to efflux GSH in response to eicosanoid exposure. To this extent our data shows that PGD₂ and its metabolites, in particular 15dPGJ₂, significantly increase extracellular GSH concentrations, and that this increase significantly protected cells from tert-butyl hydroperoxide exposure. The GSH efflux is dependent on increased intracellular GSH concentrations. Finally, we have identified the ABC transporter, ABCG2, as one of the possible mediators in GSH efflux induced by 15dPGJ₂ exposure.

Experimental Procedures

Cell Culture and Treatment

All cell lines were cultured at 37°C with 5% CO₂, and grown in media and supplements purchased from Mediatech (Manassas, VA) unless otherwise stated. FBS was purchased from Gemini Bio-Products (West Sacramento, CA). Human primary small airway epithelial cells (SAEC) were purchased and grown in SAGM BulletKit from Lonza (Rockland, MA). Human head and neck cancer MDA 1586, cells were grown in RPMI media containing L-glutamine and supplemented with 5% (v/v) FBS. Human head and neck HN4 cancer cells were grown in DMEM containing L-glutamine and supplemented with 5% (v/v) FBS. Human lung epithelial C38 cells were grown in LHC-8 media containing L-glutamine and supplemented with 10% (v/v) FBS and 5% (v/v) Penstrep.

All eicosanoid compounds were purchased from Cayman Chemical (Ann Arbor, MI) and all other reagents were purchased from Sigma-Aldrich Corporation (St. Louis, MO) unless otherwise stated. Eicosanoids were either received as a solution or we reconstituted them in ethanol or methyl acetate and stored each in aliquots at -80°C to minimize evaporation and concentration changes between experiments. The concentrations of individual PGs were determined based on the molecular weight of each compound. The concentrations of PG and leukotriene mixtures were determined based on the average molecular weights of each individual component in their respective mixture. At the time of treatment, compounds along with vehicle supplement (if needed) were added to fresh media, 1mL of this mixture was added to each well of a 24-well plate.

Cells were grown in T-75 flasks then plated into 24-well plates and treated at 70-85% confluence with fresh media containing the treatment compound and 100μM acivicin (gamma-glutamyl transpeptidase inhibitor to prevent the breakdown of extracellular GSH per well [34]. For Bisbenzimidazole H 33342 (Hoechst dye) and tert-butyl hydroperoxide (t-BOOH) studies, cells were plated into 96-well plates and treated at 90-100% confluence. Diluted stock solutions of both were prepared in sterile water and prepared fresh at the time of treatment. After indicated treatment periods, media was removed and centrifuged at 2,000 g for 5 minutes at 4°C. The cells were rinsed once with PBS and lysed with a sonicating probe (Cole-Parmer Instruments, Vernon Hill, IL). Lactate dehydrogenase (LDH) activity was determined in both media and lysate samples, and percent release was calculated as a measure of cell injury as previously described [35]. Protein values using cell lysates were required for the normalization of our GSH values and were determined spectrophotometrically with the Coomassie Protein Assay Kit according to the manufacturer's protocol (Pierce, Rockford, IL). The proteins were then precipitated using a 10% (w/v) meta-phosphoric acid added to both media and lysates. The acidified samples were vortexed and centrifuged at 23,000 g for 5 minutes at 4°C, and the supernatant was transferred into autosampler vials and analyzed for GSH using an HPLC-EC system. All samples awaiting analysis in the sample tray were kept at 4°C, otherwise they were stored at -20°C until ready for processing.

HPLC

Reverse phase HPLC was performed using an ESA system (CoulArray model 5600; ESA, Chelmsford, MA) coupled with an electrochemical detector as previously described [20]. A Synergi 4u Hydro-RP 80A, 150 × 4.6mm column in addition to a guard column (Phenomenex, Torrance, CA), and a 4 channel electrochemical cell (ESA Laboratories Inc., Chelmsford, MA) was used to detect GSH. The mobile phase, consisted of 0.125M sodium phosphate solution with 0.7% HPLC-grade methanol (Fisher Scientific, Pittsburg, PA) brought to pH 3 with concentrated phosphoric acid (Fisher Scientific, Pittsburg, PA) and was sterile filtered. An isocratic method with a flow rate of 0.5mL/min and electrode voltages set to 250mV, 525mV, 575mV, and 800mV for channels 1 through 4, respectively, was used for GSH separation and detection. After a minimum electrode equilibration time of 24hrs, 10μL of sample and GSH standard solutions prepared in either media or PBS were injected. Under these conditions, GSH had an approximate retention time of 7.5 minutes, which was quantitated using the dominant signal on channel 3.

Western Blotting

Cells were grown and treated as described previously in preparation for western blotting of ABCG2 using anti-BRCP clone BXP-21 (Kamiya Biomedical Company, Seattle, WA), γ -glutamylcysteine synthetase (GCS), commonly called γ -glutamylcysteine ligase (GCL) (Abcam Inc., Cambridge, MA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Abcam Inc., Cambridge, MA). After treatment periods, media was removed and GSH was analyzed by HPLC as described above. Cells were washed once with 1mL of PBS. PBS (200μL) containing protease inhibitors (Roche Applied Science, Indianapolis, IN) was added to the cultured cells and cells were lysed by sonication. Proteins were determined using the Coomassie Protein Analysis Kit (Fisher Scientific, Pittsburg, PA). All proteins were run on 15% tris-acrylamide gels and transferred to PVDF membranes which were blocked with 8% milk in tris-buffered saline containing 1% Tween 20 (TBS-T) solution at 4°C overnight with gentle rocking. Room temperature TBS-T was added to wash the membranes for 7 minutes, and all washes were repeated 3 times. Primary antibody was added for 2.5 hours at room temperature with gentle rocking (ABCG2 and GAPDH 1:5000, GCL 2.5μg/mL). Membranes were washed again at room temperature with fresh TBS-T. Appropriate secondary antibodies, (ABCG2 and GAPDH use peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG from Jackson ImmunoResearch Laboratories Inc.; West Grove, PA; and GCL uses peroxidase-conjugated rabbit polyclonal to Rat IgG from Abcam Inc.; Cambridge, MA), were applied at a 1:45,000 dilution for 30 minutes at room temperature with gentle rocking. Proteins were detected using ECL Plus Western Blotting Detection Reagents Kit (GE Healthcare, Piscataway, NJ) according to the manufacturer's protocol. Films were scanned and relative protein density was determined using Image J software provided by the NIH.

Real time reverse transcriptase (SYBR Green) SuperArray assay

Cells were grown to confluence in T-75 flasks as previously described. At the time of harvest, cells were trypsinized from the flask with 0.125% Puck's EDTA trypsin, washed once with media containing FBS to deactivate the trypsin, and then washed twice with PBS to completely remove residual media. Cells were pelleted at 2,000 rpm for 7 minutes after each wash. Prior to total RNA isolation, all residual PBS was removed from the cell pellets by careful aspiration. Isolation of mRNA was accomplished using the Qiagen RNeasy Mini Spin Column - QIAshredder Kit (Qiagen, Valencia, CA), according to the manufacturer protocol. Purity and quantity of total RNA was determined using the Nanodrop (ND-100) UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE). First strand DNA was created using RT² First Strand Kit (SABiosciences Corporation, Frederick, MD), protocol and cycling times were followed according to the manufacturer's protocol. The reverse transcriptase step

utilized a 9800 FAST 96-well block PCR (Applied Biosystems, Foster City, CA). We ordered a custom designed 96-well format RT² SuperArray (SABiosciences Corporation, Frederick, MD) to run a two-stage real time RT-PCR on ABC family members and several GSH and SLC family members (refer to Supplemental Tables 1-3 for list of super array targets). The super arrays were designed for the Bio-Rad (MJ) Opticon 2 thermal cycler (Bio-Rad, Hercules, CA) and sample preparation and cycle times were used according to the RT² SuperArray protocol. All solutions, including the SYBR green RT PCR mixture were purchased from SABiosciences Corporation, as recommended by the SuperArray protocol. Relative mRNA abundance for each target was determined by comparing the cycle time of the target against a 6-point (1:1 to 1:100,000) GAPDH standard curve created by serial dilutions of loaded cDNA template within each plate.

Statistical Analysis

Data are expressed as the mean $\pm$ standard error of the mean (S.E.M.), and significance was set at a p value < 0.05 . All experiments included at least triplicate treatment groups and each experiment was repeated at least three times. One way analysis of variance (ANOVA), Tukey post comparison test, and Linear regressions and Pearson correlation were performed using Prism version 5 (GraphPad, San Diego, CA).

Results

CyPGs increase intracellular and extracellular GSH levels

To examine the ability of cyPGs to induce GSH synthesis and GSH efflux, we screened 3 cell lines (HN4, MDA 1586, C38), and human primary lung epithelial cells (SAEC) to three concentrations (250nM, 500nM, and 1000nM) of a cyPG mixture. There was no significant cytotoxicity detected at these concentrations, as determined using the LDH assay (data not shown). The cyPG mixture contained a 1:1 ratio of PGA₂, PGB₂, PGD₂, PGE₂, PGJ₂, and 15dPGJ₂. Although PGs and other lipid mediators are not biologically present in a 1:1 ratio, the use of commercially available HPLC standard mixtures allowed us to maximize the number of compounds we could screen in our initial experiments. Individual components of mixtures resulting in significant intracellular and/or extracellular GSH changes were further examined if positive in our initial screen.

In the positive cyPG mixture, only PGA₂, PGJ₂, and 15dPGJ₂ contain electrophilic carbons. The cyPG mixture induced GSH changes in both the intracellular and extracellular compartments of all 4 epithelial cell systems over a 48hr treatment period (Fig. 1). No significant changes in the levels of oxidized glutathione (GSSG) were noted (data not shown). At the highest cyPG concentration, intracellular GSH in the cells increased approximately 2- to 4-fold (C38<HN4~MDA 1586<SAEC) and extracellular GSH increased approximately 2- to 6-fold (HN4<C38<SAEC<MDA 1586) compared to respective control levels. To evaluate specificity, we chose to test 1-6 μ M of several other PGs in the MDA 1586 and HN4 cells because these two cell types come from similar cell origins (head and neck cancer) and because they showed a strong differential GSH efflux response to the cyPG HPLC mixture.

We compared the response of MDA 1586 and HN4 cells to a variety of eicosanoid mixtures containing 8-iso PGE₂, 5-oxoETE, 8-iso PGF_{2 α} , and two cysteinyl leukotriene mixtures (mixture 1 contained LTC₄, LTD₄, N-acetyl LTE₄, LTE₄, and LTF₄; mixture 2 contained PGB₂, LTC₄, LTD₄, N-acetyl LTE₄, LTE₄, and LTF₄). There were no significant intracellular or extracellular GSH changes in either the MDA 1586 or HN4 cell types in response to any concentration of the tested eicosanoids (data not shown), indicating that the intracellular and extracellular GSH response was highly specific to the cyPG mixture.

The cyPG mixture was proficient in its ability to alter intracellular and extracellular GSH concentrations in part because of the electrophilic nature of some of the cyPGs contained in the mixture (PGA₂, PGJ₂, and 15dPGJ₂). Unlike the other eicosanoids we screened, only this mixture contained members with α,β -unsaturated carbonyl electrophilic carbons. Electrophilic cyPGs have been shown to exhibit a wide range of activity that is not shared by similar PGs that do not have electrophilic carbons [36]. Some cyPGs, such as 15dPGJ₂, do not act via receptors, but effect changes in cellular behavior through direct interaction with proteins at thiol sensitive sites [37]. 15dPGJ₂ has been shown to form covalent adducts with Keap1 which initiates nrf2 release to the nucleus where it can act on the ERE and induce transcription of GCL [11,12]. These factors led us to believe that the PGD₂ series of cyPGs in the mixture were responsible for the observed GSH alterations. To test this hypothesis we treated MDA 1586 and HN4 cells with each individual mixture component.

GSH export is specific to PGD₂ and its metabolites

To determine if the GSH response to the cyPG mixture was attributed to a specific cyPG within the mixture, we examined each component individually. PGA₂, PGB₂, and PGE₂ exposures did not alter intracellular or extracellular GSH in either the MDA 1586 or HN4 cell types (data not shown). In contrast, PGD₂, PGJ₂, and 15dPGJ₂ treatments increased intracellular and extracellular GSH in both cell types in a dose dependent manner (Fig. 2). 15dPGJ₂ is derived from PGJ₂ and contains 2 electrophilic carbons, PGJ₂ contains 1 electrophilic carbon and is derived from PGD₂ which contains no electrophilic carbons. The order of potency with these compounds is not surprising given the number of electrophilic carbons present in each (PGD₂~PGJ₂<15dPGJ₂).

Intracellular GSH increased approximately 5.5-fold over control in the MDA 1586 cells and 5-fold over control in the HN4 cells in response to a 6 μ M 15dPGJ₂ treatment. No changes were observed in the levels of GSSG in either the intracellular or extracellular compartments with cyPG treatments (data not shown). Of interest, the intracellular GSH fold-change response in both MDA 1586 and HN4 cells was approximately equivalent between the cell types (Fig. 2 A&B). In contrast, treatment with PGD₂, PGJ₂, and 15dPGJ₂ over a concentration range of 1-6 μ M resulted in an approximate 2- to 3-times greater GSH fold-change in extracellular GSH efflux in MDA 1586 cells, as compared to HN4 cells (Fig. 2 C&D). The difference was best illustrated at the 6 μ M treatment with 15dPGJ₂ which induced a 25.6-fold increase in extracellular GSH in the MDA 1586 cells, as compared to a 7.7-fold change in extracellular GSH efflux in the HN4 treated cells. From this data, we conclude that 15dPGJ₂ is the major component of the cyPG mixture responsible for intracellular and extracellular GSH changes. In order to better understand the relationship between the intracellular and extracellular GSH changes, we examined the role of GCL as a contributing factor in the ability of 15dPGJ₂ to induce both intracellular GSH synthesis and in maintenance of the extracellular GSH efflux.

15dPGJ₂ induced intracellular and extracellular GSH changes require elevated GCL activity

In order to determine if GSH synthesis was a prerequisite to GSH export, we measured both intracellular and extracellular GSH changes induced by 15dPGJ₂ over a 48 hour time course. Both MDA 1586 and HN4 cell types responded to 15dPGJ₂ in a time dependent manner, with initial intracellular GSH elevations beginning at 8 hours and peaking at 48 hours (Fig. 3A). Changes in extracellular GSH, in both cell types, did not occur until the 24 hour time point (Fig. 3B). This data supports the hypothesis that GSH synthesis must precede extracellular GSH export when the cells respond to 15dPGJ₂. Indeed, when comparing 15dPGJ₂ treated and untreated samples in both HN4 and MDA 1586 cell types, GCL protein expression significantly increased in a dose dependent manner, 2-fold in HN4 and 3-fold in the MDA 1586 cells at the maximum concentration of 15dPGJ₂ (Fig. 3C-F). Taken together, the data shows that GCL expression and subsequent GSH synthesis is a major component of the cellular response to

15dPGJ₂ treatment. We next asked whether the GSH adaptive response initiated by 15dPGJ₂ was protective against an extracellular added oxidant.

15dPGJ₂ induced extracellular GSH efflux significantly protected MDA 1586 but not HN4 cells from t-BOOH exposure

To determine if the increases in extracellular GSH levels had a functional effect, we exposed MDA 1586 and HN4 cells to the strong pro-oxidant t-BOOH and measured cell viability using the LDH assay. Pilot studies determined a steep toxicity response curve to t-BOOH that occurred at 24 hours with 500μM. Cells were treated for 24 hours with 15dPGJ₂ (1μM and 3μM) followed by the addition of 500μM t-BOOH by directly to the media for another 24 hour time period. Treatment of cells with 500μM t-BOOH increased total LDH release from 20-25% in controls to 90-95% in MDA 1586 and HN4 cells, indicating significant t-BOOH toxicity (Fig. 4). MDA 1586 cells treated with 15dPGJ₂ showed a dose-dependent decrease in t-BOOH-mediated toxicity where 1μM 15dPGJ₂ pre-treatment reduced total LDH release from 90% to 38% and 3μM 15dPGJ₂ resulted in complete protection from the toxic effect of t-BOOH (Fig. 4A). Unlike the MDA 1586 cells, 15dPGJ₂ pre-treatment did not protect HN4 cells from t-BOOH toxicity (Fig. 4B). These results demonstrate a functional consequence for increasing extracellular GSH levels following 15dPGJ₂ exposure especially since both cell lines showed significant and similar increases in intracellular GSH levels with 15dPGJ₂ treatments. The final question we addressed was to identify potential transporter(s) involved in the GSH efflux response.

ABCG2 expression is correlated with 15dPGJ₂ induced GSH efflux

As noted previously, when we treated MDA 1586, SAEC, HN4 and C38 cell types with the cyPG mixture, all cell types responded to some degree over their control values by increasing both intracellular and extracellular GSH. However, when we compared individual cell type basal levels of extracellular GSH using concentrations normalized to cell protein, (μmols/g protein), the MDA 1586 cells had 1.9-fold more extracellular GSH than SAEC cells, 2.0-fold more than HN4 cells, and 4.9-fold more than the C38 cells. At the 1000nM cyPG treatment, the MDA 1586 cells increased extracellular GSH concentrations 1.9-fold more than SAEC cells, 5.1-fold more than HN4, and 6.7-fold more than the C38 cells (Fig. 5A). The varying basal concentrations in extracellular GSH and the differences in response to the cyPG treatment, prompted us to question whether GSH transport could be correlated to the ABC transporter expression profile in the cells. To address this question, we designed a custom 96-well-plate SYBR green real-time reverse transcriptase array (SuperArray) that included primers for all of the currently identified ABC proteins (Supplemental Table 1), many GSH related proteins (Supplemental Table 2), some solute carrier transporter (SLC) family members, the GTPase ral binding protein RALBP1 and the major vault protein (MVP) (Supplemental Table 3). All of the proteins on our array were chosen based on their potential role in GSH metabolism, catabolism and/or transport.

Because many, though not all of the proteins in the ABCC (CFTR/MRP) subfamily of ATP-cassette binding proteins have been shown to transport reduced and/or oxidized GSH as well as GSH conjugates, we hypothesized that positive correlations between transporter and GSH export would be most likely identified in the ABC superfamily. These proteins include ABCB1 (MDR1/Pgp), ABCG2 (BCRP/MXR) and the ABCC (MRP/CFTR) subfamily proteins which include ABCC1 (MRP1), ABCC2 (MRP2, cMOAT), ABCC3 (MRP3), ABCC4 (MRP4, MOATB), ABCC5 (MRP5, MOATC), ABCC6 (MRP6), ABCC7 (CFTR), ABCC8 (SUR1), ABCC9 (SUR2), ABCC10 (MRP7), ABCC11 (MRP8), and ABCC12 (MRP9). When comparing all four cell lines, our results indicate that the only ABC protein with a significant correlation to GSH export and basal protein expression in all treatment groups including controls was ABCG2 (Fig. 5B). When we analyzed ABCG2 protein expression in the MDA

1586 and HN4 cell types, we found that MDA 1586 expressed approximately 2-fold more protein than HN4 cells, which is approximately equivalent to the 2- to 3-times difference in GSH efflux between the cell types when treated with 15dPGJ₂ as previously discussed (Fig. 6). Based on GSH export correlation data in the MDA 1586, SAEC, HN4 and C38 cell types in addition to the differential ABCG2 protein expression between MDA 1586 and HN4 cell types, we decided to further test the possible role of ABCG2 in the extracellular GSH response to 15dPGJ₂ exposures.

The ABCG2 substrate Hoechst dye inhibits extracellular GSH increases after 15dPGJ₂ exposure

Hoechst dye is a well-documented ABCG2 substrate [38,39]. As an indirect method for determining if ABCG2 was important for the extracellular GSH increases after 15dPGJ₂ exposure, we tested the ability of MDA 1586 cells to increase extracellular GSH in the presence of both Hoechst dye and 15dPGJ₂. Cells were co-treated with 15dPGJ₂ (0μM, 1μM, or 3μM) and Hoechst dye (0μM, 1.25μM, or 2.5μM) for 48 hours and then harvested for intracellular and extracellular GSH measurements. Hoechst dye did not affect the ability of the cells to increase intracellular GSH levels in response to 15dPGJ₂ treatment (Fig. 7A). Although intracellular GSH levels in cells co-treated with 1μM 15dPGJ₂ and 2.5μM Hoechst dye reached similar concentrations, extracellular GSH in the cells with Hoechst dye did not increase above control levels (Fig. 7B). When cells were co-treated with 3μM 15dPGJ₂ and 2.5μM Hoechst dye, again intracellular GSH levels increased to similar levels, but extracellular GSH increase in cells with Hoechst dye was blunted by approximately 77% (Fig. 7B). These results suggest that the Hoechst dye is competitively inhibiting GSH transport through ABCG2.

The ABCG2 mRNA superarray expression profiles in the four epithelial cell types SAEC, C38, HN4, and MDA 1586 all corresponded with extracellular GSH responses to the cyPG mixture. More specifically, high ABCG2 mRNA expression corresponded to high extracellular GSH increases in response to cyPG treatment. A differential extracellular GSH response to 15dPGJ₂ treatment was also observed in the low ABCG2 protein expressing HN4 cells compared to the high ABCG2 protein expressing MDA 1586 cells. Furthermore, when the MDA 1586 cells were co-treated with 15dPGJ₂ and the ABCG2 substrate Hoechst dye, the extracellular GSH response was significantly inhibited. Based on these results, we report for the first time that GSH may be a novel substrate for ABCG2.

DISCUSSION

The ability of 15dPGJ₂ to increase intracellular GSH concentrations has been well documented and is one reason why 15dPGJ₂ is widely recognized as a PG involved in the resolution of inflammation [32,33,40,41]. One possible mechanism by which 15dPGJ₂ can alter cellular GSH synthesis is through interactions with the transcription factor complex Nrf2/Keap1. Previous investigators have shown that the electrophilic nature of 15dPGJ₂ allows it to oxidize cysteine thiols in Keap1 [11,12]. The oxidation of Keap1 results in the subsequent release of Nrf2, which allows translocation of Nrf2 to the nucleus where it binds to antioxidant response elements (AREs) to induce transcription of numerous antioxidant proteins including gamma-glutamylcysteine-ligase (GCL) [18,42-46]. Indeed, our results corroborate the idea that 15dPGJ₂ increases expression of antioxidant proteins, as illustrated by a significant increase of GCL protein expression in both the MDA 1586 and HN4 cells after 15dPGJ₂ treatment. In addition to 15dPGJ₂, the eicosanoid subfamily of cyPGs contains several electrophilic PGs, so it is reasonable to hypothesize that those PGs with similar electrophilic properties may also be capable of oxidizing Keap1, and ultimately resulting in similar intracellular GSH increases. Interestingly, after testing a broad range of PGs with electrophilic properties, our results indicate that only PGD₂ and its metabolites PGJ₂ and 15dPGJ₂ are capable of altering

intracellular GSH. PGD₂ is readily converted to PGJ₂ via non-enzymatic dehydration of its cyclopentenone ring, and further ring dehydration results in the formation of 15dPGJ₂. Not surprisingly, the order of potency in our HN4 and MDA 1586 cell types in regard to intracellular and extracellular GSH increase was PGD₂~PGJ₂<15dPGJ₂.

One of the important findings of this work is that the large induction of GSH synthesis, specifically in response to 15dPGJ₂ treatment, can induce dramatic changes in extracellular GSH concentrations. Our data also suggest that cells that have higher expression of GSH transporters have higher levels of extracellular GSH and these levels may be related to the transporters' K_m for GSH. These results suggest a possible function for 15dPGJ₂ in extracellular GSH adaptive responses during periods of inflammation, especially in organ systems exposed to high levels of oxidants, such as the lung.

The lung represents a major organ system in which GSH export into the extracellular space is vital. The extracellular space of the airways is comprised of epithelial lining fluid (ELF), a major component of the lung's host defense system. GSH is an important antioxidant in the ELF and can reach concentrations 1000 times greater than serum levels [21]. Many acute and chronic lung diseases (such as idiopathic pulmonary fibrosis, cystic fibrosis, acute respiratory distress syndrome, lung transplantation, and HIV infected subjects) are known to have decreased GSH concentrations in the ELF and/or a decreased ability to stimulate GSH production in the lung [20,47-50]. 15dPGJ₂ has been shown to effectively mitigate acute lung injury via increased synthesis of intracellular GSH [51]. Our results indicate that intracellular GSH increases may not be the sole purpose of the 15dPGJ₂ response. Because redox balance in the lung is very important and because GSH is a major thiol that helps maintain this balance, it is possible that 15dPGJ₂ assists in maintaining a high extracellular GSH concentration in the ELF via increased GSH synthesis resulting in increased GSH efflux.

The ABC protein family contains several members in the subfamilies ABCB, ABCC, and ABCG that are highly proficient efflux pumps. In fact, many of the proteins in these three subfamilies were identified based on their ability to confer multi-drug resistance in a wide number of cancers, and were originally given the nomenclature MRPs to indicate multi-drug resistance proteins [52]. The first multidrug resistance protein identified was ABCB1 (MDR1/P-glycoprotein) by Juliano and Ling in 1976 [53]. As outlined in the introduction, ABCC1, ABCC2, ABCC3, ABCC4, and ABCC5 have been identified as capable of transporting GSH, GSSG, or GSH conjugates. Interestingly, cancer cells prefer a slightly oxidative environment and regulation of GSH levels may be critical for the overall survival and propagation of cancer cells [54-56]. Indeed the importance of GSH depletion in cancer cells as a means to sensitize them to chemotherapy has been recognized for many years and has prompted the development of GSH synthesis inhibitors such as BSO [52]. It is interesting to note that a cancer cell's attempt to evade chemotherapy treatments via increased ABC transporter expression may come at the potential cost of GSH loss.

In the lung, export of GSH into the ELF is partly dependent on an active transport process involving ABCC7, commonly named the cystic fibrosis transmembrane regulator protein (CFTR) [20,27]. However, CFTR only accounts for a portion of GSH efflux into the ELF. ABCG2 has been shown to be localized to the lung, predominantly in the stem cell side population, and moderately in epithelial cells, seromucinous glands, and in endothelial capillaries [57-59]. ABCG2 is also localized to the apical membrane of kidney proximal tubule cells [60], hematopoietic cells [61], amnion and fetal membranes [62,63], in intestinal cells [64], at the blood-brain barrier [65], and it is a popular marker for multiple stem cell types [66]. In all of these examples, GSH plays an active part in maintaining redox balance in both the intracellular and extracellular compartments. Our results indicate that ABCG2 mRNA expression in the 4 cell types we examined positively correlates with extracellular basal GSH

concentration, and with increased extracellular GSH concentrations induced by treatment with the cyPG mixture. This is the first time GSH has been implicated as an ABCG2 substrate.

There is limited knowledge on GSH transporters, particularly in the lung. Because of the wide array of ABCG2 tissue expression, its role in xenobiotic transport, and its position on many side population stem cells and cancer cells, we believe that the correlation between ABCG2 and GSH efflux warrants further investigation. Our data suggests that 15dPGJ₂ may play a critical role in linking inflammatory signaling events with adaptive responses that may have important functions in resolving inflammation, injury repair and tissue remodeling. Our findings suggest a possible inflammatory resolution pathway by which 15dPGJ₂ and ABC transporter function may be linked. Given the importance of dysregulated inflammatory responses in many human diseases, this pathway may provide new understanding and drug targets to resolve inflammation associated pathogenesis of disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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The abbreviations used are

ABC, ATP-binding cassette; 15dPGJ₂, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂; PG, prostaglandin; γ -GCL, γ -glutamylcysteine ligase; GSH, glutathione; MRP, multi-drug resistant protein; BCRP, breast cancer resistance protein; SAEC, small airway epithelial cells; SLC, solute carrier protein.

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Fig. 1. cyPG treatment increases intracellular and extracellular GSH levels in epithelial cells
Cells were treated for 48 hours with 0nM, 250nM, 500nM, or 1000nM of the cyPG mixture containing a 1:1 ratio of PGA₂, PGB₂, PGD₂, PGE₂, PGJ₂, and 15dPGJ₂. Intracellular GSH (white bars) and extracellular GSH (black bars) levels were measured using HPLC-EC detection. The MDA 1586 cells (A) had the greatest change in intracellular and extracellular GSH levels in response to cyPG treatment at all concentrations as compared to SAEC (B), HN4 (C), and C38 (D) cells. The final concentration of the vehicle (Veh), methyl acetate, in each treatment group was 0.2% (v/v). Data are presented as the mean $\pm$ S.E.M. and bars with different letter superscripts are significantly different ($p < 0.05$) from each other, as determined using ANOVA with the Tukey post comparison test.

Fig. 2. Comparison of changes in intracellular and extracellular GSH levels in MDA 1586 and HN4 cells exposed to individual CyPGs

Treatment of MDA 1586 and HN4 cells with the individual cyPG mixture components, PGD₂, PGJ₂, and 15dPGJ₂ revealed that they were capable of significantly altering intracellular (A and B) and extracellular (C and D) GSH concentrations. The order of reactivity in both cell systems was PGD₂ ≈ PGJ₂ < 15dPGJ₂. Each eicosanoid was delivered using the same vehicle (Veh), methyl acetate, and the final concentration in each treatment was 0.2% (v/v). Data are presented as the mean ± S.E.M. and bars with different letter superscripts are significantly different ($p < 0.05$) from each other, as determined using ANOVA with the Tukey post comparison test.

Fig. 3. Time dependent changes in intracellular and extracellular GSH levels in response to 15dPGJ₂ treatment

HN4 (open squares) and MDA 1586 (open circles) cells were treated with 6 μM of 15dPGJ₂ and (A) intracellular and (B) extracellular GSH levels were measured by HPLC-EC over a 48 hour time course. Intracellular GSH (A) increased at 8 hours and peaked at 48 hours in both HN4 and MDA 1586 cells. Intracellular GSH peaked at approximately equal concentration in the two cell lines. Extracellular GSH (B) began to rise at 24 hours and peaked at 48 hours in both HN4 and MDA 1586 cells. Extracellular GSH increased 7.7-fold more at 48 hours in MDA 1586 cells as compared to HN4 cells. Intracellular GSH synthesis is accompanied by increased GCL protein expression in both MDA 1586 (C and D) and HN4 (E and F) cells. Densitometry quantification of GCL protein expression as normalized to GAPDH. The final concentration of the vehicle (Veh), methyl acetate, in each treatment group was 0.2% (v/v). Data are presented as the mean ± S.E.M. and bars with different letter superscripts are significantly different ($p < 0.05$) from each other, as determined using ANOVA with the Tukey post comparison test.

Fig. 4. 15dPGJ₂ treatment selectively protects MDA 1586 cells with high extracellular GSH levels from t-BOOH exposure

(A) MDA 1586 cells were pre-treated for 24 hours with 0μM (white bars), 1μM (light grey bars), or 3μM (black bars) 15dPGJ₂, and then exposed to 500μM t-BOOH added directly to the media for 24 hours. 15dPGJ₂ protected the MDA 1586 cells from t-BOOH exposure. (B) HN4 cells were pre-treated for 24 hours with 0μM (white bars), 1μM (light grey bars), or 3μM (black bars) 15dPGJ₂, and then exposed to 500μM t-BOOH added directly to the media for 24 hours. 15dPGJ₂ did not protect the HN4 cells from t-BOOH exposure. The vehicle (Veh) group was treated with 0.2% (v/v) methyl acetate, the PGJ₂ vehicle, and with 0.2% (v/v) water, the t-BOOH vehicle. Data are presented as the mean ± S.E.M. and bars with different letter superscripts are significantly different ($p < 0.05$) from each other, as determined using ANOVA with the Tukey post comparison test.

Fig. 5. Positive correlation between ABCG2 and GSH export in C38, HN4, SAEC, and MDA 1586 cells after cyPG treatment

(A) Increasing extracellular GSH efflux in 4 epithelial cell systems treated with 0nM, 250nM, 500nM, and 1000nM cyPG reveals a positive linear trend with GSH efflux and CyPG mixture. Linear regression indicates that the slopes of each line are all significantly different from one another. (B) Basal levels of ABCG2 mRNA expression in each cell system also indicates a positive correlation with basal extracellular GSH, indicating a potential role for ABCG2 in the maintenance of basal GSH levels as well as the CyPG induced GSH adaptive response. There were no significant deviations from linearity with any line for all linear trend lines and dashed lines represent the 95% confidence interval. Correlations were determined by Pearson correlation analysis with significance set at $p < 0.05$.

Fig. 6. ABCG2 protein expression in 15dPGJ₂ treated MDA 1586 and HN4 cells
 (A) ABCG2 apoprotein expression in MDA 1586 and HN4 cells treated with 0 μ M and 6 μ M 15dPGJ₂ for 48 hours. (B) Quantification by densitometry of ABCG2 protein expression, normalized to GAPDH, in MDA 1586 and HN4 cells. MDA 1586 have approximately 2-fold more ABCG2 basal expression than HN4 cells. Data are presented as the mean $\pm$ S.E.M. and bars with different letter superscripts are significantly different ($p < 0.05$) from each other, as determined using ANOVA with the Tukey post comparison test.

Fig. 7. Hoechst dye is an ABCG2 substrate that inhibits the ability of MDA 1586 cells to increase extracellular GSH after 15dPGJ₂ treatment

Cells were co-treated with 15dPGJ₂ and the ABCG2 substrate Hoechst dye for 48 hours. (A) Consistent with previous results, intracellular GSH synthesis was significantly increased by 15dPGJ₂ treatment. (B) Hoechst dye significantly blunted 15dPGJ₂-mediated increases in extracellular GSH. Data are presented as the mean $\pm$ S.E.M. and bars with different letter superscripts are significantly different ($p < 0.05$) from each other, as determined using ANOVA with the Tukey post comparison test.