Identification of Aldo-Keto Reductase AKR1B10 as a Selective Target for Modification and Inhibition by Prostaglandin A1: Implications for Antitumoral Activity

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Abstract

Cyclopentenone prostaglandins (cyPG) are reactive eicosanoids that may display anti-inflammatory and anti-proliferative actions, possibly offering therapeutic potential. Here we report the identification of members of the aldo-keto reductase (AKR) family as selective targets of the cyPG prostaglandin A1 (PGA1). AKR enzymes metabolize aldehydes and drugs containing carbonyl groups and are involved in inflammation and tumorigenesis. Thus, these enzymes represent a class of targets to develop small molecule inhibitors with therapeutic activity. Molecular modeling studies pointed to the covalent binding of PGA1 to Cys299, close to the active site of AKR, with His111 and Tyr49, which are highly conserved in the AKR family, playing a role in PGA1 orientation. Among AKR enzymes, AKR1B10 is considered as a tumor marker and contributes to tumor development and chemoresistance. We validated the direct modification of AKR1B10 by biotinylated PGA1 (PGA1-B) in cells, and confirmed that mutation of Cys299 abolishes PGA1-B incorporation, whereas substitution of His111 or Tyr49 reduced the interaction. Modification of AKR1B10 by PGA1 correlated with loss of enzymatic activity and both effects were increased by depletion of cellular glutathione. Moreover, in lung cancer cells PGA1 reduced tumorigenic potential and increased accumulation of the AKR substrate doxorubicin, potentiating cell-cycle arrest induced by this chemotherapeutic agent. Our findings define PGA1 as a new AKR inhibitor and they offer a framework to develop compounds that could counteract cancer chemoresistance. Cancer Res; 71(12): 4161–71. ©2011 AACR.

Introduction

Cyclopentenone prostaglandins (cyPG) are endogenous reactive eicosanoids which display varied biological actions including inhibition of proinflammatory gene expression and modulation of cell proliferation and redox status (1–3). cyPG arise from the spontaneous dehydration of various PG or from nonenzymatic peroxidation of arachidonic acid. These eicosanoids possess an α,β-unsaturated carbonyl group in the cyclopentene ring which confers them a high reactivity toward nucleophiles, such as thiol groups, and can lead to the formation of covalent adducts by Michael addition. This property is essential for cyPG biological actions (4), including PPAR activation (5) and inhibition of NF-kB and AP-1 transcription factors (1, 6). A- and J-series cyPG were initially observed to share several protein targets and biological actions (7–9). However, recent studies have shown that cyPG with different structures may target selective subsets of cellular proteins (10), and even different residues within the same protein. Ras proteins and histone deacetylases constitute examples of this intra- and intermolecular selectivity (9, 11). Identification of the factors involved in the selectivity of protein modification by different cyPG, as well as of the selectively modified targets, could unveil potential opportunities for drug discovery.

Using proteomic approaches we have identified proteins of the aldo-keto reductase (AKR) family as targets for selective modification by PGA1 compared with 15-deoxy-A12,14-prostaglandin J2 (15d-PGJ2). AKR enzymes are involved in the metabolism of several ketones and aldehydes and play important roles in pathophysiology (12). Among them, AKR1B10 exerts key roles in tumor biology. AKR1B10, first identified in human hepatocarcinoma (13), is overexpressed in colorectal, breast and lung cancer, for which it is considered a diagnostic marker (14, 15), and may play a pathogenic role in hepatocellular carcinoma (16) and in tobacco-related carcinogenesis (14, 17). Proposed AKR1B10-mediated tumorigenic mechanisms include retinoic acid depletion and cancer cell dedifferentiation (18, 19) as well as chemoresistance due to metabolism of carbonyl group–bearing anticancer drugs...
(20). Therefore, AKR1B10 constitutes a primary target for the development of inhibitors with anticancer potential.

Here we have confirmed the direct modification of AKR1B10 by PGA1-B and show that this cyPG inhibits AKR activity. Moreover, PGA1 treatment potentiated the effects of the carbonyl group–containing drug doxorubicin. These results provide a novel approach for the inhibition of AKR1B10 which could be exploited in the design of strategies to overcome cancer chemoresistance.

Materials and Methods

Reagents

Prostanoids and their biotinylated analogues [15d-PGJ2-biotin(15d-PGJ2-B) and prostaglandin A1-biotin(PGJ1-B)] were from Cayman Chemical. Horseradish peroxidase (HRP)–streptavidin and enhanced chemiluminescence (ECL) reagents were from GE Biosciences. Neutravidin-agarose was from Pierce. Anti-aldose reductase (sc-33219) and anti-GAPDH (sc-32233) were from Santa Cruz Biotechnology and anti-AKR1B10 from Origene.

Cell culture and treatments

NIH-3T3, H1299, and COS-7 cells were from ATCC. A549 and Calu-3 cells were gifts of Dr. F. Rodríguez-Pascual (21, 22) and Dr. A. Silva, respectively. All cells were used within 6 months after resuscitation. NIH-3T3 and COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% FBS, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. A549 cells were grown in RPMI1640 with the above supplements plus 50 μg/mL gentamycin. For treatments, cells at a confluence of 80% to 90% were incubated in serum-free RPMI1640 with the above supplements plus 50 μg/mL gentamycin. For treatments, cells at a confluence of 80% to 90% were incubated in serum-free RPMI1640 with the above supplements plus 50 μg/mL gentamycin.

Plasmids and transfections

The plasmid pCMV6- XL5 containing human AKR1B10 ORF was from Origene. The insert was cloned into the pCEFL-KZ-AUS plasmid to yield AUS-AKR1B10 wild type (wt). The C299S, H111A, and Y49F mutants were generated by site-directed mutagenesis by using oligonucleotides, 5'-CTGAGCTCATCTTCTCAATTTTG-3', 5'-GGAAGGCGATTATGCGCT-3', and 5'-CTGAGCCTATGTCTTTCAGAATGAACATGAAGTGGG-3', respectively. All constructions were sequenced. COS-7 cells in 6-well dishes were transfected with Lipofectamine 2000, using 2 μg of DNA per well.

AKR activity assay

Cells were lysed in 50 mmol/L sodium phosphate buffer (pH 6.8), and S100 fractions were used for AKR activity measurements. Assay mixtures contained 50 μg of protein, 10 mmol/L N,N1,1-triethylglyceraldehyde, and 200 μmol/L NADPH in 100 mmol/L sodium phosphate buffer (pH 6.8). After 20-minute incubation at room temperature NADPH consumption was monitored by measuring the absorbance at 340 nm in an Ultraspec 4300 pro...
spectrophotometer (GE Biosciences). Enzymatic activity was expressed in nmol/min/mg of protein.

**Doxorubicin detection**

Intracellular doxorubicin was detected by confocal fluorescence microscopy on a Leica SP5 confocal microscope, using excitation and emission wavelengths of 470 and 590 nm (29). Doxorubicin fluorescence was quantitated by flow cytometry on an EPICS Coulter analyzer, using the above excitation and emission settings.

**Cell migration and clonogenic assay**

For wound-healing assays cells were grown to confluence and the monolayer was scratched with a 200 μL pipette tip. The width of the scratch was measured at 3 points in each well, and at various time points after treatment with the indicated agents. Relative migration was calculated as the percentage of distance migrated compared with the control well. Anchorage-independent growth was assessed as described above. Cells were cultured for 3 weeks with 3 weekly medium replacements. Colonies were visualized by ethidium bromide staining on an UV transilluminator.

**Cell-cycle analysis and detection of apoptosis**

Cells treated with the various agents were detached with trypsin/EDTA and fixed with 70% ethanol in PBS. Immediately before flow cytometry analysis, cells were centrifuged and resuspended in 0.5% NP-40, 25 μg/mL propidium iodide and 25 μg/mL RNase A, and incubated in this solution for 15 minutes at room temperature. Apoptosis was detected by binding of Annexin V and propidium iodide staining, as described (30). Cells were treated with the various agents for 3 weeks with 3 weekly medium replacements. Colonies were visualized by ethidium bromide staining on an UV transilluminator.

**Statistical analysis**

Experiments were carried out at least 3 times. Results are presented as average values ± SEM. Average values were compared by Student’s t test for unpaired observations.

**Results**

**PGA1-B selectively modifies AKR enzymes in NIH-3T3 fibroblasts**

We have previously reported that biotinylated analogues of the cyPG 15d-PGJ2 and PGA1, selectively modify specific subsets of cellular proteins in NIH-3T3 fibroblasts (10). To identify selective targets of each cyPG we incubated cells with concentrations of 15d-PGJ2-B or PGA1-B resulting in a prominent labeled band at 37 kDa, appearing selectively in S100 fractions from PGA1-B–treated fibroblasts (Fig. 1A). To identify this selective PGA1-B target we employed a proteomic strategy following the enrichment of biotin-containing proteins by avidin chromatography, as schematized in Figure 1B and detailed in Supplementary Figure S1. Consistent with our previous work, initial proteomic analysis of this band identified glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ref. 23). However, immunoprecipitation of GAPDH did not recover the PGA1-B–selective target, which remained in the nonretained fraction (Supplementary Fig. S1), indicating that the presence of the abundant protein GAPDH was hampering identification of the comigrating PGA1-B target. Consequently, combining GAPDH immunodepletion, avidin chromatography, and LC/MS-MS analysis (Fig. 1B; and Supplementary Fig. S1) allowed the identification of aldose reductase (AKR1B1) as a putative selective target for PGA1-B (Supplementary Fig. S1). To directly evidence covalent addition of PGA1-B to AKR enzymes we conducted 2D-electrophoresis. Samples from control, 15d-PGJ2-B, and PGA1-B–treated cells were resolved in duplicate gels used for transfer and detection of AKR proteins or biotin-positive spots, or for Coomassie blue staining and protein identification (Fig. 1C). Two spots were detected by the anti-AKR antibody: spot 1 showed pl and apparent molecular weight consistent with AKR1B1; MALDI-TOF MS analysis of spot 2 identified aldose reductase-related protein-2 (AKR1B8), an AKR family member expressed in murine fibroblasts. Both spots matched biotin positive spots only in the sample from PGA1-B–treated cells (Fig. 1C, right), confirming the selectivity of their modification by PGA1-B. Moreover, the presence of AKR enzymes in the avidin-bound fraction from PGA1-B–treated cells, but not from control cells was confirmed by Western blot (Fig. 1D).

**Molecular modeling studies of the interaction of PGA1 with AKR enzymes**

In our proposed binding mode for PGA1 in the AKR1B1 active site, the keto-group is accepting hydrogen bonds from both the $N^\delta$-protonated His111 and the hydroxyl of Tyr49 (Fig. 2A). These interactions, which remained stable during the unrestrained MD simulations, facilitate the orientation of the cyclopentenone ring in such a way that the electrophilic carbon is at a suitable distance from the sulfur atom of Cys299 (i.e., ≤4.0 Å) thus making it possible for the Michael reaction to proceed and yield a covalent adduct. Furthermore, in the case of the biotinylated analogue, PGA1-B, the amide bond between PGA1 and the spacer (Fig. 4) can establish an additional hydrogen bonding interaction with the side-chain carboxamide of Gln50 whereas the biotin moiety would be found facing the solvent. Consistent with the observed selectivity of the modification, the interactions predicted by this model could not be established in the case of the J series cyPG, 15d-PGJ2 data not shown).

The AKR family consists of more than 100 members, and significant homology exists among many of them (Fig. 2B). Moreover, the residues predicted by the molecular model to be involved in the interaction of PGA1 with the enzyme are highly conserved among several members of this family. Given the high degree of sequence identity between AKR1B1 and AKR1B10, especially within the active site region (Fig. 2B), the mode of binding of PGA1 and PGA1-B to these 2 enzymes is expected to be identical. Given its significance in tumor biology, we used AKR1B10 for subsequent studies addressing the interactions of PGA1 with AKR enzymes and their functional implications.
Cysteine 299 is essential for PGA₁-B binding to AKR1B10

PGA₁-B clearly formed a stable adduct with AKR1B10 in cells transiently transfected with an AU5-AKR1B10 construct, as assessed by SDS-PAGE and biotin detection (Fig. 3A). Remarkably, mutation of Cys299 to Ser virtually abolished the incorporation of PGA₁-B into AKR1B10, indicating that this residue is critical for PGA₁-B binding. Interestingly, mutation of either Tyr49 or His111 reduced the incorporation of PGA₁-B, suggesting that the presence of these residues favors the interaction of the cyPG with the active site of the enzyme as predicted by the model.

We next addressed the functional consequences of AKR1B10 modification by PGA₁. Endogenous AKR activity was negligible in COS-7 cells (see Fig. 3C). Transfection of AU5-AKR1B10 produced measurable AKR activity, which was inhibited by treatment with either PGA₁ or PGA₁-B by more than 60% (Fig. 3B), without altering AU5-AKR1B10 protein levels. In contrast, 15d-PGJ₂-B at a concentration eliciting a degree of total protein modification similar to that achieved with PGA₁-B, induced only marginal AKR1B10 labeling, and a weak inhibition of AKR activity (15%) was observed in 15d-PGJ₂-treated cells (data not shown). Remarkably, PGA₁-elicted inhibition was abolished in cells transfected with AKR1B10
Cys299Ser, confirming the importance of Cys299 for both PGA1 binding and inhibitory effect (Fig. 3C). Consistent with previous observations, the AKR1B10 Cys299Ser mutant displayed lower activity than the wt variant, reportedly due to reduced affinity for the D,L-glyceraldehyde substrate (32). Mutation of Tyr 49 or His111 abolished activity (data not shown), confirming their importance in catalysis (33).

Inhibition of AKR activity by various cyclopentenones

Given the potential biomedical interest in AKR inhibitors, we characterized PGA1 effect in more detail. PGA1 inhibition of AKR1B10 activity in COS-7 cells was time- and concentration-dependent (IC50, 4 3 μmol/L; Supplementary Fig. S2).

Several PGA1-related compounds including PGA3, 8-isopGA1, and 8-iso-PGA2 inhibited AKR1B10 activity (Fig. 4). Interestingly, the isoprostanes 8-iso-PGA1 and 8-iso-PGA2, isomers of the respective PG, were significantly less effective than their PG counterparts, suggesting that the trans orientation of the PG side chain with respect to the prostane ring is more favorable for interaction with the enzyme. Remarkably, 2-cyclopentenone did not inhibit AKR1B10, suggesting that the chains of the PG are important for docking at the active site. Of the 2 reported inhibitors of AKR enzymes, fenofibrate (34) and AD-5467 (35), only the latter showed a potent inhibitory effect. AKR and actin levels were not affected under the conditions of the assay (Fig. 4).

Figure 2. Proposed binding mode for PGA1 in the aldose reductase active site. A, ribbon representation (green) of AKR1B1 with protein residues enveloped by a semitransparent solvent-accessible surface (PyMOL; DeLano Scientific, LLC). Carbon atoms of NADP+ and PGA1 are colored in magenta and cyan, respectively, and the sphere stands for the biotin moiety. The framed area on the left is enlarged in the view on the right. The protein residues most relevant to the discussion have been labeled, the hydrogen bonds involving PGA1 are displayed as dotted lines, and the arrow points to the site of attack on the cyclopentenone ring for the sulfur atom of Cys299. B, sequences of various AKR family members. AKR1B1, aldose reductase; AR Rel-2, aldose reductase-related protein 2. The extent of identity between the various sequences is shown. Amino acids important for PGA1 or PGA1-B interaction with AR (AKR1B1) are shown in colored boxes. Note that amino acid numbering includes the initial methionine, which is removed in mature AKR1B1.
PGA1 inhibits AKR activity in human lung adenocarcinoma A549 cells

In search for a biologically relevant model to assess PGA1 effects, we explored the levels of AKR1B10 in A549, H1299, and Calu-3 lung cancer cells, of which only A549 cells showed detectable levels (Supplementary Fig. S3). PGA1 reduced cell viability and induced cell-cycle alterations in all cells studied to variable extents (Supplementary Fig. S3). Consistent with a role of AKR1B10 in detoxification and cell survival, A549 cells showed reduced susceptibility to PGA1 effects. Treatment of A549 cells with PGA1-B elicited the selective retention of endogenous AKR proteins, and specifically of AKR1B10, on avidin–agarose beads (Fig. 5A), indicating that PGA1-B modifies AKR enzymes in this cell type. Moreover, PGA1 and PGA1-B effectively inhibited AKR activity without altering AKR protein levels (Fig. 5B). According to previous evidence, reduced glutathione (GSH) forms stable adducts with PGA1-type PG potentially reducing interactions with proteins (36). We previously showed that GSH depletion enhances PGA1-B binding to cellular proteins (10). Consistent with this, treatment of A549 cells with buthionine sulfoximine (BSO), a GSH synthesis inhibitor, improved PGA1-B incorporation into proteins and increased retention of biotin-tagged proteins onto neutravidin beads (Supplementary Fig. S4). Moreover, GSH depletion specifically increased AKR retention on avidin after PGA1-B treatment (Fig. 5C). Furthermore, BSO showed a tendency to reduce AKR activity per se and significantly potentiated PGA1-elicited AKR inhibition (Fig. 5D). Taken together, these results indicate that maintenance of GSH levels or of redox status is important for AKR function. In addition, GSH depletion facilitates AKR modification and inhibition by PGA1.

Effects of PGA1 on A549 cells tumorigenicity and drug resistance

PGA1 inhibited A549 cell migration in a wound healing assay (Fig. 6A) and reduced anchorage-independent clonogenic growth (Fig. 6B), thus suggesting an inhibition of tumorigenic potential. AKR1B10 is involved in the metabolism of a variety of anticancer compounds, including doxorubicin and daunorubicin, potentially leading to chemoresistance (32, 37). Therefore, we explored whether PGA1 could improve doxorubicin efficiency. The main mechanism for doxorubicin action at clinically relevant concentrations is the stabilization of a cleaved ternary topoisomerase II–doxorubicin–DNA complex, resulting in a blockade in the G2 phase of the cell cycle (38–40). Doxorubicin treatment induced a concentration-dependent increase in the proportion of A549 cells present in the G2–M phases of the cell cycle (Fig. 6C, left), an effect that was clearly potentiated by PGA1 (Fig. 6C, right). This effect was associated with an improvement of doxorubicin availability in cells. PGA1 clearly potentiated intracellular accumulation of doxorubicin at several concentrations of the drug, as assessed by flow cytometry (Fig. 6D). Confocal fluorescent microscopy confirmed PGA1-elicited potentiation of doxorubicin retention, mainly at the nuclear compartment (Supplementary Fig. S5). The AKR inhibitor AD-5467 mimicked the effects of PGA1 both on cell-cycle distribution and doxorubicin accumulation (data not shown). Taken together, these results suggest that PGA1 treatment through inhibition of AKR activity in A549 cells, may increase the accumulation of the AKR substrate doxorubicin and potentiate its biological effects, helping to counteract multidrug chemoresistance.

Discussion

Proteomic studies are shedding light into the signaling and targets of endogenous electrophiles, unveiling novel mechanisms of action and providing opportunities for drug discovery (23). Our results identify AKR1B10, an enzyme involved in
tumor development and cancer chemoresistance, as a selective target for modification and inhibition by A-class cyPG. Moreover, we showed that PGA1 inhibits AKR activity in lung cancer cells, reduces their migration and anchorage-independent growth, reflecting reduced invasiveness, and potentiates the effect of the antitumoral agent doxorubicin, a known AKR1B10 substrate. Therefore, the PGA1–AKR1B10 interaction could be explored for the development of novel strategies against tumor chemoresistance.

Insight into the structural basis for the preferential interaction of PGA1 with AKR enzymes was obtained by molecular docking and confirmed by biochemical characterization of site-specific mutants of AKR1B10. AKR1B10, like other AKR enzymes, is a monomeric protein with an (α/β)8 barrel structure containing the active site. Residues His111, Tyr49, K79, and Asp44 line the active site, play a key role in catalysis, and are highly conserved in the AKR family (12). Cys299 is located close to the active site and modulates the interaction...
of substrates and inhibitors with the enzyme. Molecular modeling studies postulated the formation of a Michael adduct between PGA1 and AKR involving Cys299, with Tyr49 and His111 playing a facilitating role. Interestingly, mutagenesis studies confirmed the requirement for Cys299 for covalent addition of PGA1-B to AKR1B10, whereas Tyr49 and His111 were required for optimal binding. Cys299 was also required for PGA1-mediated inhibition of AKR1B10, confirming the functional importance of the interaction. Importantly, both PGA1 and PGA1-B displayed similar inhibitory effects, indicating that, in agreement with the modeling results, the biotin moiety does not interfere with the interaction. Indeed, we have confirmed modification and inhibition of recombinant AKR1B10 by both compounds (B.D-D., unpublished data).

Given the important implications of AKR1B10 in tumor development and cancer chemoresistance, the search for inhibitors of this enzyme is a thriving area of research. Several compounds have been found to display AKR1B10 inhibitory properties in vitro, including some nonsteroidal anti-inflammatory drugs (41), butein (42), and curcumin derivatives (43). Our results suggest that PGA-type cyPG may constitute a novel class of AKR inhibitors. PGA1 binding to AKR1B10 shows unique characteristics among several recently identified inhibitors for this enzyme (41, 43), for which Gln114, Val301, and Gln303 or Ser304 were found to be the critical interacting residues. It would be interesting to explore whether modifications of the PGA1 structure could be designed to increase the specificity or potency of the inhibition. Concentrations of PGA1 used in this work should be considered pharmacologic because they are at least 3 orders of magnitude higher than those measured in biological systems (44).

A549 cells constitute a widely used cellular model of lung carcinoma and express several AKR isoforms, of which AKR1B10 is the most abundant (45). As AKR1B10 is involved in detoxification of various carbonyls, it could also confer increased resistance to PGA1 or to reactive species generated during PGA1 treatment. The finding that blocking GSH
synthesis improves PGA1-elicited modification and inhibition of AKR enzymes suggests that GSH depletion could constitute a strategy to improve the antitumoral action of PGA1-type cyPG. We have also observed that AKR inhibition in A549 cells treated with vehicle or the indicated concentrations of PGA1 in complete medium. Clonogenic efficiency was calculated as the percentage of colonies present with respect to plates treated with vehicle. C, cells were treated with the indicated concentrations of doxorubicin (DOX) for 16 hours (left) or with 10 nmol/L DOX in the absence or presence of 30 μmol/L PGA1 for 16 hours (right). Cell-cycle distribution was analyzed by flow cytometry. Proportions of cells in the G2–M phases shown at the bottom are average values ± SEM of 3 experiments. *, P < 0.05 vs. vehicle by Student’s t test. D, determination of DOX fluorescence by flow cytometry. Left, results from a representative experiment after treatment with 50 nmol/L DOX for 16 hours in the absence or presence of 30 μmol/L PGA1. Right, mean fluorescent intensities of cells treated with various DOX concentrations in the absence or presence of 30 μmol/L PGA1. Results are average values ± SEM of 3 experiments (*, P < 0.05 vs. the same condition in the absence of PGA1 by Student’s t test).
to the improvement of doxorubicin effectiveness, including induction of reactive oxygen species, modulation of GST activity or GSH content, and interference with drug efflux. These possibilities will be the subject of further studies. In summary, our study identifies AKR enzymes as prominent PGA1-selective targets. Moreover, PGA1 binds covalently and inhibits AKR1B10 establishing specific interactions with the enzyme and potentiates the effects of the antimutural drug doxorubicin. These results unveil novel possibilities to overcome cancer chemoresistance.

Disclosure of Potential Conflicts of Interest

D. Pérez-Sala and B. Díez-Dacal are coinventors of a related patent applied for by CSIC. The other authors disclosed no potential conflicts of interest.

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