Nrf2-mediated Induction of Cytoprotective Enzymes by \(15\text{-Deoxy-}\Delta^{12,14}\text{-Prostaglandin J}_2\) Is Attenuated by Alkenal/one Oxidoreductase*

Received for publication, May 15, 2006, and in revised form, June 30, 2006. Published, JBC Papers in Press, July 20, 2006, DOI 10.1074/jbc.M604620200

Xiang Yu1, Patricia A. Egner1, Junko Wakabayashi1, Nobunao Wakabayashi1, Masayuki Yamamoto1, and Thomas W. Kensler1,2,3

From the 1Department of Pharmacology and Molecular Sciences, Johns Hopkins School of Medicine, the 2Division of Toxicology, Department of Environmental Health Sciences, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland 21205 and the 3Center for Tsukuba Advanced Research Alliance and Institute for Basic Medical Sciences, University of Tsukuba, Tsukuba, 305-8577 Japan

NADPH-dependent alkenal/one oxidoreductase (Aor) was discovered to be highly inducible in rat liver following treatment with the cancer chemopreventive agent 3\(H\)-1, 2-dithiole-3-thione. Aor was further characterized as an Nrf2-regulated antioxidative enzyme that reduces carbon–carbon double bonds in a variety of \(\alpha, \beta\)-unsaturated aldehydes and ketones. 15-Deoxy-\(\Delta^{12,14}\text{-prostag-}

landin \(J_2\) (15d-PGJ2) is a reactive membrane lipid metabolite that activates multiple pathways, including Nrf2-mediated induction of cytoprotective enzymes. Physiologically, it is postulated that 15d-PGJ2 alkylates key regulatory proteins via the electrophilic carbon centers found in two \(\alpha, \beta\)-unsaturated ketone moieties. This current study addresses the metabolism of 15d-PGJ2 by rat Aor (rAor) and subsequent deactivation of the Nrf2 signaling pathway by both rat and human AOR. We demonstrate that induction of NADPH-dependent quinone oxidoreductase activity by 15d-PGJ2 is markedly attenuated in mouse embryonic fibroblasts that overexpress rAor. Luciferase reporter assay and quantitative real-time PCR confirmed these findings. Concentrations required for doubling the NADPH-dependent quinone oxidoreductase response are increased from 1.8 \(\mu\)M in wild-type to >10 \(\mu\)M in rat Aor transgenic fibroblasts. 15d-PGJ2 is metabolized by recombinant rAor with a \(K_m\) of 9.6 \(\mu\)M and \(k_{cat}\) of 18.5 min\(^{-1}\). The major product is 12,13-
dihydro-15-deoxy-\(\Delta^{12,14}\text{-prostaglandin J}_2\) (dihydro-15d-PGJ2). The reduction of C=C by Aor yielding dihydro-15d-PGJ2 abolishes the inducibility in an antioxidant response element-driven luciferase assay. Collectively, these results demonstrate that 15d-PGJ2 can be catabolized by Aor, thereby attenuating subsequent Nrf2 signaling and possibly inflammatory and apoptotic processes also influenced by 15d-PGJ2.

Prostaglandins (PG)2 are a subfamily of eicosanoids shown to regulate a variety of physiological processes, including growth,

differentiation, vascular constriction, inflammation, and homeostasis. Prostaglandins are primarily derived from arachidonic acid following phospholipase-catalyzed release from membrane lipids. Arachidonic acid is converted by cyclooxygenases into PGH2, which is further metabolized into PGD2, PGE2, prostacyclin, or thromboxane via various prostaglandin synthases. PGD2 can undergo dehydration reactions to yield the J2 series of prostaglandins, including PGJ2, \(\Delta^{12,14}\text{-PGJ}_2\), and 15d-PGJ2 (1). The J2 series of prostaglandins influences multiple signaling pathways by covalently binding with key signaling molecules (2–6). Among them, 15d-PGJ2 has displayed highest potency as an inducer of gene expression. Discovered as an agonist for peroxisome-proliferator-activated receptor \(\gamma\) (PPAR\(\gamma\)), 15d-PGJ2 was shown to modulate the expression of genes containing a peroxisome-proliferator response element in the promoter region, resulting in adipocyte differentiation (7). In addition, 15d-PGJ2 contains two \(\alpha, \beta\)-unsaturated ketone moieties in tandem within its structure. These moieties covalently modify critical proteins in multiple pathways, such as \(I\kappaB\) kinase, Thioredoxin, and Keap1 (3, 6, 8), resulting in altered protein complex conformations and, subsequently, the expression of effector genes.

That 15d-PGJ2 may serve as an endogenous regulator of Nrf2 signaling is of particular interest. This pathway, centrally composed of two proteins, Keap1 and Nrf2, is responsible for the induction of many cytoprotective and antioxidative enzymes that form a natural defense system against assaults brought by electrophiles and oxidants. Nrf2 belongs to the cap’ n’ collar family of basic leucine zipper transcription factors. Keap1 is an actin-binding protein that sequesters Nrf2 in the cytoplasm under basal conditions. Upon exposure to inducers, the Keap1-Nrf2 binding equilibrium is disrupted, followed by an increased nuclear accumulation of Nrf2, which in turn, heterodimerizes with small Maf proteins to enhance transcription of target genes via a cis-acting antioxidant response element (ARE) in the promoter regions. Animals with impaired Nrf2 expression display a greatly increased susceptibility toward chemically

2-dithiole-3-thione; E3, ubiquitin-protein isopeptide ligase; 15d-PGJ2, 15-deoxy-\(\Delta^{12,14}\text{-prostaglandin J}_2\); Nqo1, NADPH-dependent quinone oxidoreductase; rAor, rat Aor; SH3, Src homology 3; MEF, mouse embryonic fibroblast; PBS, phosphate-buffered saline; PPAR, peroxisome proliferator-activated receptor.
induced carcinogenesis (9). Chemicals that promote Nrf2 nuclear accumulation, such as sulforaphane, oltipraz, and triterpenoids, are under intensive study as experimental and clinical chemopreventive agents against chemically induced carcinogenesis (10–12).

Alkenal/one oxidoreductase (Aor), originally discovered as a leukotriene B$_{4}$-12-hydroxydehydrogenase in porcine kidney (13), is highly inducible in rat liver following treatment with the chemopreventive agent D3T (14). It was later found that, compared with dehydrogenase activity, Aor has a 100-fold higher reductase activity, thereby efficiently catalyzing the hydrogenation of the C=C double bond in a variety of α,β-unsaturated aldehydes and ketones. Such action reduces the key reactive centers, preventing electrophilic attack on macromolecules. Consequently, Aor has been shown to ameliorate acute cytotoxicity of 4-hydroxynonenal, a lipid peroxidation product and a α,β-unsaturated aldehyde, in cultured human embryonic kidney cells (15).

The reactivity of 15d-PGJ$_{2}$ lies in two α,β-unsaturated ketone moieties, one of which is located on the cyclopentenone ring. Both of these electrophilic carbons may attack nucleophilic centers found on protein side chains, such as the sulfhydryl group of cysteine residues (16). Because at least one of these reactive centers can potentially serve as a substrate for Aor, in the present study we examined the possible deactivation by Aor of 15d-PGJ$_{2}$ signaling through the Nrf2 pathway.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—Prostaglandins were purchased from Cayman Chemicals Co. (Ann Arbor, MI). Chemicals were purchased from Sigma unless otherwise specified. All solvents and chemicals were of analytical reagent grade or higher.

**Plasmid Construction**—pCEP4-rAor and 3×NF-E2 luciferase plasmid pRBGP2 were previously constructed as described (17). Plasmid pRL-TK containing a constitutive Renilla luciferase was purchased from Promega (Madison, WI). Human AOR was PCR cloned from a human liver cDNA library (catalogue number 7113–1, Lot 1040535; Clontech, Mountain View, CA) according to NCBI GenBankTM sequence (accession code NM_012212) using the following primer set: Forward, 5'-GTC-GCGGAATTCAGCTTCAGGATGGTGTCTAAGACA TGG, and Reverse, 5'-GTCGCGCTGAGTTACTATCATG CTTTACTATTTGCTTCCC.

PCR product was cleaned and ligated into pBlueScript between EcoRI and Xhol sites. The DNA insert was confirmed by sequencing and subcloned into episomal vector pCEP4.

**Expression of Aor in Escherichia coli and Purification**—Recombinant rAor was purified as previously described with a few modifications (18). Briefly, pTrcHisA containing rAor cDNA with a His$_{6}$ tag at the N terminus was transformed into chemically competent E. coli strain BL21. Aor was expressed in Luria-Bertani medium containing 100 µg/ml carbenicillin. Recombinant Aor was purified using nickel-nitrilotriacetic acid Superflow resin (Qiagen Inc., Valencia, CA) according to the manufacturer’s recommendations. Elutions were analyzed by SDS-polyacrylamide gel electrophoresis using Coomassie Blue staining. Fractions with >95% purity were pooled and dialyzed at 4 °C overnight against 1 liter of 10 mM potassium phosphate buffer, pH 7.3, containing 1 mM dithiothreitol, 5 mg/ml Complete protease inhibitor mixture (Roche Applied Science), 0.1% Tween 20. Protein concentrations were determined by Bio-Rad protein assay. Aliquots were stored at −80 °C for up to 12 months without significant loss of enzymatic activity.

**Cell Culture and Transfection**—Mouse embryonic fibroblasts (MEF) were derived from embryos of Nrf2 null or wild-type littermates (ICR strain), as well as rAor transgenic and corresponding wild-type (C57BL/6+DBA/2) MEFs. Fibroblasts were cultured in Iscove’s modified Eagle’s medium (Invitrogen). Human embryonic kidney cells (293 cells) were obtained from ATCC (American Type Culture Collection, Manassas, VA) and grown in Dulbecco’s modified Eagle’s medium (high glucose; Invitrogen). Both cell types were maintained in 10% fetal bovine serum (Invitrogen) and incubated at 37 °C in a humidified atmosphere containing 5% CO$_{2}$. Plasmids were transfected into 293 cells with Lipofectamine 2000 reagents (Invitrogen) according to the manufacturer’s recommendations.

**Measurement of Nqo1 Activities**—MEF cells were seeded at 5000/well in 96-well microtiter plates 18 h before treatment with 15d-PGJ$_{2}$, in complete growth medium. Nqo1 activity was measured using the “Prochaska” microtiter plate assay 48 h later (19).

**Determination of Aor Enzymatic Activities in Cultured Cells**—Cultured cells were collected and lysed in reaction buffer (0.5 × PBS, pH 7.2, 0.01% Triton X-100) by freezing and thawing three times. Cell lysates were subsequently centrifuged at 12,000 × g for 10 min to collect cytosolic fractions. Aor activities were determined spectrophotometrically by monitoring the rate of NADPH oxidation at 340 nm as previously described (15) using trans-2-nonenal as a substrate on a Beckman DU800 spectrophotometer.

**Dual Luciferase Assay**—3 × NF-E2 firefly luciferase plasmid and constitutive Renilla luciferase plasmids were co-transfected into 293 cells that had been selected after pCEP4 or pCEP4-Aor transient transfection. Cells were seeded into 6-well culture plates at 500,000 cells/well 18 h before treatment. 15d-PGJ$_{2}$ dissolved in ethanol was added to fresh growth medium at a final concentration of 1–10 µM. Final ethanol concentration was kept <0.1%. 24 h after treatment, cells were washed with PBS prior to passive lysis. Harvested lysates were centrifuged at 15,000 × g for 5 min to remove cellular debris. Supernatants were assayed for luciferase activity using the Promega Dual Luciferase assay kit. The ratio of firefly and Renilla luciferase activities was used as the indicator for transcriptional activation.

**Enzymatic Assay and Mass Spectrometric Analysis**—Enzymatic reactions were conducted in 0.5 × PBS supplemented with 0.01% Triton X-100, 0.05% bovine serum albumin, and a saturating NADPH concentration of 150 µM. To measure the $K_{m}$ and $k_{cat}$ for 15d-PGJ$_{2}$, 10 µg of recombinant rAor was incubated with 4–120 µM 15d-PGJ$_{2}$ at 37 °C with shaking. Reactions were terminated at 4, 8, and 12 min by the addition of ice-cold ethyl acetate containing 2 nmol piperine (internal standard). The mixture was vortexed and centrifuged at 13,000 × g for 3 min. The ethyl acetate fraction was removed, taken to dryness under vacuum, and redissolved in 60:40 ace-
tonitrite/H$_2$O. The piperine and remaining 15d-PGJ$_2$ were separated using a Waters 510 HPLC system equipped with a 996 photodiode array detector monitoring 306 and 340 nm. Separation of piperine and 15d-PGJ$_2$ was achieved using a 4.6 $\mu$m × 250 mm Luna C$_{18}$ column (Phenomenex Inc., Torrance, CA) at a 1 ml/min hyperbolic gradient of 60 to 100% acetonitrile in water. Initial velocities were calculated from the consumption of 15d-PGJ$_2$ over time, and the GraphPad Prism 4 program was used to compute the $K_m$ and $k_{cat}$ values via hyperbolic regression analysis. Liquid chromatography electrospray ionization tandem mass spectrometry was conducted using a ThermoFinnigan Deca electrospray mass spectrometer (ThermoFinnigan, San Jose, CA) equipped with a 1 × 15 mm Luna column eluted at 20 $\mu$l/min using a gradient as described above. Tandem mass spectrometry spectra were generated from the appropriately positively charged parent ion ($m/z$ = 301.1, 299.1) using helium as a collision gas.

**Gene Expression Analysis**—Wild-type 293 cells were treated by replacing medium with that containing 7.5 $\mu$m 15d-PGJ$_2$ or ethanol as vehicle control. Total RNA was isolated 8 h after treatment using a Versagene RNA purification kit (Gentra Systems, Minneapolis, MN), and cDNA was synthesized using the iSCRIPT cDNA synthesis kit (Bio-Rad). Gene expression measurements were performed using TaqMan gene expression assays (Applied Biosystems, Foster City, CA) and iQ Supermix (Bio-Rad). Data from real-time quantitative PCR was analyzed using the $2^{-\Delta\Delta Ct}$ relative quantification method as previously described (20). Glyceraldehyde-3-phosphate dehydrogenase transcript level was found to remain unchanged in both vehicle-treated and 15d-PGJ$_2$-treated cells. Hence, it was used for normalization.

**Immunoblotting**—293 cells were seeded onto 6-well culture plates at 1 million cells/well 18 h before treatment with 7.5 $\mu$m 15d-PGJ$_2$ in the growth medium. After a 16-h incubation, cells were washed with ice-cold PBS and collected by centrifugation following trypsinization. Cells were then resuspended in HEPES buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol) supplemented with the Complete protease inhibitor mixture. After a 15-min incubation on ice, Nonidet P-40 was added to the suspension to achieve a final concentration of 0.6%. The suspension was then vortexed and nuclei collected by centrifugation at 13,000 × g.

Nuclear fractions were resuspended in loading buffer containing 1% SDS and boiled for 5 min. Protein extracts were analyzed by SDS-PAGE and electrotransferred to ECL Hybond nylon membrane (Amersham Biosciences). Immunodetection was performed using rabbit Nrf2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) followed by horseradish peroxidase-conjugated goat anti-rabbit serum (Bio-Rad). ECL reagents (Amersham Biosciences) were used for chemiluminescent detection.

**Statistical Analysis**—Non-linear regression computation was conducted using SigmaPlot 9.0 software. Statistical comparison was performed using one-way analysis of variance.

**RESULTS**

15d-PGJ$_2$-inducible Nqo1 Activity Is Blocked in Both Nrf2 Null and rAor Transgenic MEF Cells—Nqo1 is known to be inducible via the Nrf2-mediated pathway (21). To examine whether 15d-PGJ$_2$ induces Nqo1 gene transcription, MEF derived from Nrf2 null and rAor transgenic animals, along with their corresponding wild-type counterparts, were incubated with graded concentrations of 15d-PGJ$_2$ (Fig. 1). The wild-type MEF showed a strong dose-dependent induction of Nqo1 activity following treatment with 15d-PGJ$_2$ ($R^2 > 0.98$). The difference in the magnitudes of responses to 15d-PGJ$_2$ in the wild-type MEF cell lines may reflect the different genetic backgrounds of the cells. The wild-type cells used in Fig. 1A were established from ICR mice, whereas the ones in Fig. 1B were derived from mice of mixed C57BL/6 and DBA/2 background. Interestingly, the inducibility of Nqo1 by 15d-PGJ$_2$ was abrogated in both Nrf2 null and rAor transgenic MEF. The lack of effect in the Nrf2 null cells highlights the importance of Nrf2 in the induction of Nqo1, whereas the loss of inducibility in rAor transgenic cells indicates that overexpression of rAor disrupts 15d-PGJ$_2$ signaling in the Nrf2-inducible pathway.

Overexpression of Rat and Human Aor Blocks Nrf2-mediated Cytoprotective Enzyme Induction in 293 Cells—Previous studies demonstrated that 15d-PGJ$_2$ could form adducts with Keap1 in rat macrophages (6) and promoted nuclear accumulation of Nrf2 in 293 cells (22). To investigate whether overexpression of rat and human Aor blocks 15d-PGJ$_2$-induced, Nrf2-regulated gene transcription, an ARE luciferase reporter gene system was utilized. 293 cells were transfected with an episomal Aor (rat or human)-expressing vector (pCEP4-rAor, pCEP4-human AOR) or a control vector (pCEP4). Transfected cells were then selected by addition of hygromycin to the growth medium. Following selection, cells were co-transfected with a vector containing a 3 × NF-E2-driven luciferase sequence and a vector constitutively expressing Renilla luciferase. Cells were then incubated with 0.5–10 $\mu$m 15d-PGJ$_2$ as indicated, and luciferase activities were measured 24 h later. The ratios of firefly and
**Aor Attenuates 15d-PGJ₂ Induction of the Nrf2 Pathway**

![Graph showing log [15d-PGJ₂] (μM) vs. fold induction](image)

**FIGURE 2. Induction of the Nrf2 pathway is attenuated in 293 cells overexpressing rat and human Aor.** A, 293 human embryonic kidney cells were transfected either with a blank episomal vector pCEP4 or pCEP4-rAor, a rat Aor expression vector, or pCEP4-human AOR, a human AOR expression vector. Cells were co-transfected with a 3 × NF-E2-driven luciferase pRBGP2 and a constitutive Renilla luciferase expression vector pRL-TK. Luciferase activities were normalized against Renilla luciferase activity. Results are represented as mean ± S.E. from three independent experiments. *, p < 0.01. B, Nqo1 transcript levels were measured via quantitative reverse transcription PCR in wild-type or rat and human Aor-transfected 293 cells 8 h after treatment with 7.5 μM 15d-PGJ₂. C, nuclear extracts from 293 cells treated with 7.5 μM 15d-PGJ₂ were analyzed by SDS-PAGE and examined with Nrf2 antibody (pC, pCEP4; rA, rat Aor; hA, human AOR).

**FIGURE 3. Metabolite of 15d-PGJ₂ completely loses inducer function.** A, 15d-PGJ₂ was incubated with rAor in vitro with or without NADPH. The reactions were extracted with ethyl acetate and reconstituted in ethanol. The ethanol solutions were quantified by UV spectrophotometry. B, 293 wild-type cells transfected with luciferase plasmids were treated with equal molar amounts of 15d-PGJ₂ or metabolite followed by measurements of luciferase activities. Results are represented as mean ± S.E. from three independent experiments.

Renilla luciferase activities are shown in Fig. 2A. 293 cells containing the empty vector displayed a strong dose-dependent response to 15d-PGJ₂ treatment (R² > 0.98), and the concentration required to double the expression of the reporter was less than 2 μM. 15d-PGJ₂ induction of the NF-E2-driven reporter was substantially repressed in both Aor-transfected cells.

D3T is a potent inducer of Nqo1 that activates Nrf2 signaling but is not a substrate for Aor. Expression of the luciferase reporter induced by D3T in the wild-type, rAor-, and human AOR-overexpressing cells reached 8.5-, 8.3-, and 6.6-fold, respectively (n = 3, p > 0.6), indicating Aor overexpression itself did not affect Keap1-Nrf2 signaling.

Induction of native Nqo1 transcripts was also analyzed by quantitative real-time PCR in wild-type and Aor-overexpressing 293 cells after treatment with 15d-PGJ₂. Nqo1 transcript levels were significantly induced in wild-type cells yet showed little change in Aor-overexpressing cells compared with vehicle-treated controls (Fig. 2B). Mechanistically, this may be explained by the changes in Nrf2 nuclear localization (Fig. 2C). Nuclear extracts from wild-type cells showed a significantly higher accumulation of Nrf2 after administration of 15d-PGJ₂, whereas in comparison modest changes were observed in Aor-overexpressing cells. These data further bolstered the results from luciferase assay and indicated that the overexpression of Aor attenuates activation of Nrf2 signaling by 15d-PGJ₂.

**FIGURE 3. Metabolite of 15d-PGJ₂ fails to induce Nrf2-mediated transcription.** To investigate the hypothesis that 15d-PGJ₂ loses its inducibility due to metabolism by Aor, 15d-

PGJ₂ was added to recombinant rAor with and without the co-factor NADPH, followed by measurement of inductive efficacy in the ARE luciferase assay. As shown in Fig. 3A, UV-visible spectrophotometry indicated that most of the 15d-PGJ₂ in the −NADPH reaction was recovered whereas the majority of the 15d-PGJ₂ was metabolized by rAor in the presence of NADPH. Wild-type 293 cells transfected with luciferase reporter were then treated with equal molar amounts of recovered 15d-PGJ₂ or metabolite at the concentrations indicated. 15d-PGJ₂ recovered from the −NADPH reaction effectively activated Nrf2, whereas the metabolite was unable to invoke a response with the ARE luciferase reporter (Fig. 3B). These results indicate that metabolism by Aor deactivates 15d-PGJ₂, thereby blunting activation of Nrf2 signaling.

**In Vitro Metabolism of 15d-PGJ₂ by Aor.** To characterize the metabolite formed from 15d-PGJ₂ by Aor, 10 nmol 15d-PGJ₂ was incubated with 10 μg of recombinant rAor and NADPH (150 μM) in the reaction buffer (0.5 × PBS, 0.01% Triton X-100, 0.1% bovine serum albumin). Reactions were carried out at
37 °C and terminated at 10 and 20 min. 15d-PGJ$_2$ has a maximum absorbance at 306 nm as indicated by the UV-visible spectrum (Fig. 4B). Chromatography monitored at 306 nm showed a time-dependent disappearance of the 15d-PGJ$_2$ peak, which eluted at 9.2 min. Piperine, used as the internal standard during the ethyl acetate extraction, eluted at 7 min (Fig. 4A). The metabolite eluted very close to 15d-PGJ$_2$ itself, and separation was difficult to optimize. Because of the significant absorbance of 15d-PGJ$_2$ at the $\lambda_{max}$ of the metabolite (Fig. 4B), only substrate disappearance was measured instead of product formation for further kinetic calculation. $K_m$ and $k_{cat}$ values were obtained and are listed in Table 1. The $K_m$ for rAor was determined to be 9.6 $\mu$M, which is in accordance with previous studies showing tight binding between Aor and 15-oxo-prostaglandin E$_2$ with a $K_m$ value of 10 $\mu$M (15). $k_{cat}$ is a modest 18.5 min$^{-1}$, much lower that of 2400 min$^{-1}$ for 15-oxo-prostaglandin E$_2$. The lower catalytic activity is likely because of the different position of the C=C double bond in relation to the cofactor NADPH, resulting in a less efficient hydride transfer. In comparison with other Aor substrates, metabolism of 15d-PGJ$_2$ carries modest catalytic rigor and extremely high binding affinity (Table 1).

Electrospray mass spectrometry was used to identify the parent mass of the unknown metabolite. After the reaction reached completion, the metabolite was extracted with ethyl acetate, dried down, and reconstituted in mobile phase. Liquid chromatography electrospray ionization tandem mass spectrometry was conducted on a ThermoFinnigan Deca LCQ mass spectrometer operated in positive ion mode. Two mass units were obtained and are listed in Table 1. The $K_m$ for rAor was determined to be 9.6 $\mu$M, which is in accordance with previous studies showing tight binding between Aor and 15-oxo-prostaglandin E$_2$ with a $K_m$ value of 10 $\mu$M (15). $k_{cat}$ is a modest 18.5 min$^{-1}$, much lower that of 2400 min$^{-1}$ for 15-oxo-prostaglandin E$_2$. The lower catalytic activity is likely because of the different position of the C=C double bond in relation to the cofactor NADPH, resulting in a less efficient hydride transfer. In comparison with other Aor substrates, metabolism of 15d-PGJ$_2$ carries modest catalytic rigor and extremely high binding affinity (Table 1).

Electrospray mass spectrometry was used to identify the parent mass of the unknown metabolite. After the reaction reached completion, the metabolite was extracted with ethyl acetate, dried down, and reconstituted in mobile phase. Liquid chromatography electrospray ionization tandem mass spectrometry was conducted on a ThermoFinnigan Deca LCQ mass spectrometer operated in positive ion mode. Two mass units were obtained and are listed in Table 1. The $K_m$ for rAor was determined to be 9.6 $\mu$M, which is in accordance with previous studies showing tight binding between Aor and 15-oxo-prostaglandin E$_2$ with a $K_m$ value of 10 $\mu$M (15). $k_{cat}$ is a modest 18.5 min$^{-1}$, much lower that of 2400 min$^{-1}$ for 15-oxo-prostaglandin E$_2$. The lower catalytic activity is likely because of the different position of the C=C double bond in relation to the cofactor NADPH, resulting in a less efficient hydride transfer. In comparison with other Aor substrates, metabolism of 15d-PGJ$_2$ carries modest catalytic rigor and extremely high binding affinity (Table 1).

Electrospray mass spectrometry was used to identify the parent mass of the unknown metabolite. After the reaction reached completion, the metabolite was extracted with ethyl acetate, dried down, and reconstituted in mobile phase. Liquid chromatography electrospray ionization tandem mass spectrometry was conducted on a ThermoFinnigan Deca LCQ mass spectrometer operated in positive ion mode. Two mass units were obtained and are listed in Table 1. The $K_m$ for rAor was determined to be 9.6 $\mu$M, which is in accordance with previous studies showing tight binding between Aor and 15-oxo-prostaglandin E$_2$ with a $K_m$ value of 10 $\mu$M (15). $k_{cat}$ is a modest 18.5 min$^{-1}$, much lower that of 2400 min$^{-1}$ for 15-oxo-prostaglandin E$_2$. The lower catalytic activity is likely because of the different position of the C=C double bond in relation to the cofactor NADPH, resulting in a less efficient hydride transfer. In comparison with other Aor substrates, metabolism of 15d-PGJ$_2$ carries modest catalytic rigor and extremely high binding affinity (Table 1).

Electrospray mass spectrometry was used to identify the parent mass of the unknown metabolite. After the reaction reached completion, the metabolite was extracted with ethyl acetate, dried down, and reconstituted in mobile phase. Liquid chromatography electrospray ionization tandem mass spectrometry was conducted on a ThermoFinnigan Deca LCQ mass spectrometer operated in positive ion mode. Two mass units were obtained and are listed in Table 1. The $K_m$ for rAor was determined to be 9.6 $\mu$M, which is in accordance with previous studies showing tight binding between Aor and 15-oxo-prostaglandin E$_2$ with a $K_m$ value of 10 $\mu$M (15). $k_{cat}$ is a modest 18.5 min$^{-1}$, much lower that of 2400 min$^{-1}$ for 15-oxo-prostaglandin E$_2$. The lower catalytic activity is likely because of the different position of the C=C double bond in relation to the cofactor NADPH, resulting in a less efficient hydride transfer. In comparison with other Aor substrates, metabolism of 15d-PGJ$_2$ carries modest catalytic rigor and extremely high binding affinity (Table 1).
phosphatidylinositol 3 kinase and, in turn, the MAPK (mito-
gen-activated protein kinase) kinase cascade (3–5,40). Inter-
estingly, it was also found that 15d-PGJ2, an analog of 15d-
PGJ2, inhibits isopeptidase activity in ubiquitin-mediated pro-
teasomal degradation, crippling proteasomal function (41). Given the close resemblance between the structures of 15d-
PGJ2 and 12-PGJ2, it is reasonable to postulate that 15d-PGJ2 may also inhibit isopeptidase, adding another dimension to the role of 15d-PGJ2 in up-regulation of the Nrf2 pathway. Mechanistically, the involvement of 15d-PGJ2 in these signaling pathways is likely due to its ability to form protein adducts with critical signaling molecules, consequently inducing conformational changes in regulators of these pathways. Moreover, the discovery that 15d-PGJ2 is an irreversible ligand for PPARγ pointed to the possibility that its role as PPARγ agonist is also due to its ability to form protein adducts (42). Interestingly, 15d-PGJ2 showed equal potency in activating PPARγ-dependent pathways when compared with other PPARγ agonists displaying much lower $K_d$ values, such as thiazolidinediones that have a $K_d$ as low as 40 nM (43). This is likely because the covalent conjugation of 15d-PGJ2 with PPARγ receptor results in an irreversible conformational change that causes the receptor to remain in a consistently “active” state. In addition, there also could be a synergistic effect among the multiple signaling pathways that can be evoked by 15d-PGJ2.

15d-PGJ2 belongs to the family of cyclopentenones that contain an $\alpha,\beta$-unsaturated ketone moiety furnishing a highly electrophilic center ready to attack susceptible mac-
romolecules. Two such moieties are present within 15d-
PGJ2, providing two reactive carbons at the 9 and 13 posi-
tions, with C9 located on the cyclopentenone ring. Both of these carbon atoms are able to carry out Michael addition with thiol groups as illustrated by Perez-Sala et al. (16). The authors demonstrated the presence of cysteine adducts formed on c-Jun at C9 and C13 of 15d-PGJ2 in cell lysates as well as in cultured HeLa cells. Itoh et al. (6) also observed direct alkylation between Keap1 and 15d-PGJ2 leading to
activation of Nrf2 pathway, indicating such covalent binding is the key factor for inducer properties of 15d-PGJ_2. Between the two carbon centers, C9 displays a higher chemical reactivity than C13 (16). However, when the 9,10 endocyclic double bond was eliminated, as in the molecule 9,10-dihydro-15d-PGJ_2, a considerable amount of chemical reactivity remained (44). In fact, 9,10-dihydro-15d-PGJ_2 carries equal potency to 15d-PGJ_2 at activating peroxisome-proliferator response element-driven luciferase activity (34), indicating that C12,13 double bond is critical in activating the PPARγ pathway. This observation also concurs with previous research demonstrating that the cyclopentenone ring alone was not sufficient to induce a comparable response to 15d-PGJ_2. Kim et al. (45) reported that, in MCF-7 breast cancer cells, 10 μM 15d-PGJ_2 was able to incur a strong induction of heme-oxygenase-1; however, concentrations of 2-cyclopenten-1-one or PGA2 up to 30 μM failed to induce a detectable change in expression. Both of these analogs contain an α,β-unsaturated ketone moiety on the cyclopentenone ring. Straus et al. (46) also showed that up to 50-fold more cyclopentenone was needed to achieve the same extent of inhibition of NFκB DNA binding ability by 15d-PGJ_2. Interestingly, on the contrary, 9,10-dihydro-15d-PGJ_2 was unable to repress EGFR expression as did 15d-PGJ_2 in human oral squamous carcinoma cells (5). Collectively, these results suggest that alkylation by these two reactive centers might lead to differential outcomes downstream. Thus far, glutathione conjugation, which occurs preferentially at C9 position (47), has been considered the major route of 15d-PGJ_2 deactivation. In this study, however, we demonstrated that 15d-PGJ_2 can also be deactivated by alkenal/one oxi-doreductase. Aor hydrogenates 15d-PGJ_2 at the 12,13 position, thus eliminating one reactive center and subsequently attenuating its ability to activate Nrf2 signaling. Although both the C9 and C13 constitute the potent inducer activity of 15d-PGJ_2, it is likely that Aor and glutathione could share the burden of 15d-PGJ_2 deactivation, leading to attenuation in different downstream pathways. Given the importance of the 12,13 position for PPARγ activation and inhibition of NFκB signaling, it is reasonable to suspect that Aor might blunt the effects of 15d-PGJ_2 on these pathways as well.

Unlike PGJ_2 and PGD_2, its physiological predecessors, 15d-PGJ_2 binds to prostaglandin D receptor (DP) with a >100-fold lower affinity; thus 15d-PGJ_2 was not considered to act through DP to exert its physiological effects (48). Recently, 15d-PGJ_2 was found to bind to a membrane-spanning, G-protein-coupled receptor, CRTH2, with affinity in the nM range, resulting in a decreased concentration of intracellular cAMP (48, 49). This observation certainly adds additional complexity to the network of pathways that are affected by 15d-PGJ_2. However, in our study, neither the PPARγ inhibitor GW9662 nor the CRTH2 antagonist Ramatroban was able to block 15d-PGJ_2 activation of the Nrf2 pathway (data not shown). Thus, it appears that Nrf2 activation by 15d-PGJ_2 is more likely to be the result of direct alkylation on Keap1 instead of an indirect effect through PPARγ or CRTH2 signaling.

Limited work has been published on the effects of 15d-PGJ_2 in vivo primarily due to the difficulty in accurately measuring its concentrations in biological fluids. However, an increased amount of immunoreactive 15d-PGJ_2 has been detected in spinal cord sections of amyotrophic lateral sclerosis patients (50) and carrageenan-activated rat macrophages (6), as well as specimens from atherosclerotic arterial tissue (1). The cloning of prostaglandin transporters (51) suggests that cellular concentrations of prostaglandins, including PGD_2, might far exceed those of the inter-cellular fluids. Shibata et al. (1) reported the non-enzymatic conversion of PGD_2 to 15d-PGJ_2 in a serum-containing medium and, at an even higher rate, in a serum-free medium. Considering 15d-PGJ_2 acts intracellularly, in contrast to other prostaglandins that act principally as extracellular mediators, it is reasonable to assume that the concentration of 15d-PGJ_2 would be substantially higher than that reported in the extracellular fluids. In addition, because 15d-PGJ_2 is a lipophilic molecule originating from membrane lipid metabolism, it could accumulate in hydrophobic cellular membranes, thereby leading to a higher localized concentration. It should also be noted that, when used in cultured cell systems, the high abundance of serum protein present in the medium could considerably reduce the actual concentrations of 15d-PGJ_2 that reach the intracellular space. Although the k_m for Aor reduction of 15d-PGJ_2 is modest, the K_m is quite low, at 9.6 μM, which allows Aor to constantly operate at high capacity.

Previously, Aor has been found to exhibit leukotriene B_4 dehydrogenase activity (13) as well as α,β-unsaturated ketone reductase activity (15). In addition, Aor was found to be highly inducible by the dithiolethione family of chemopreventive agents (14). All of these observations define Aor as an antioxidative enzyme aiding in global defense mechanisms against harmful chemicals generated from lipid peroxidation, inflammation, and environmental stresses. Therefore, it is intriguing to find that Aor could negatively regulate inducible function of 15d-PGJ_2, currently regarded as a potent endogenous anti-inflammatory agent among other functions. This certainly adds to the complexity of the roles Aor could play in cell physiology. Interestingly, sequence analysis of Aor from guinea pig, porcine, rabbit, human, and rat revealed a conserved motif featuring the sequence PPGPXXXPE between amino acids 250–260. This motif agrees with the consensus SH3 domain recognition site, further supported by additional residues in + and – positions from the conserved sequence (24). The possibility of a SH3 domain-containing protein that could potentially interact and modulate Aor activity awaits further investigation. In addition, it is of interest to examine the transcriptional regulation of Aor with regard to NFκB signaling as well as the Nrf2 pathway. Preliminary analysis of the Aor promoter region in the mouse genome reveals multiple copies of ARE 4 kb upstream from the start of the second exon. Collectively, further study is needed to improve our understanding of the multiplicity of the interacting pathways up-regulated by 15d-PGJ_2 and the role that Aor could play in modulating this regulation.

Acknowledgments—We thank Dr. John Groopman for helpful discussion regarding mass spectrometry and Patrick Dolan for assistance with tissue culture. We also thank Alana Rivera for critically reading the manuscript.

September 8, 2006 • Volume 281 • Number 36

JOURNAL OF BIOLOGICAL CHEMISTRY 26251
