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Observation of Two Modes of Inhibition of Human Microsomal Prostaglandin E Synthase 1 by the Cyclopentenone, 15-Deoxy- $\Delta^{12,14}$ -Prostaglandin J₂

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Abstract

Microsomal prostaglandin E synthase 1 (MPGES1) is an enzyme that produces the pro-inflammatory molecule PGE₂. Effective inhibitors of MPGES1 are of considerable pharmacological interest for the selective control of pain, fever and inflammation. The isoprostane, 15-deoxy- $\Delta^{12,14}$ -PGJ₂ (15d-PGJ₂), a naturally occurring degradation product of prostaglandin D₂, is known to have anti-inflammatory properties. In this paper we demonstrate that 15d-PGJ₂ can inhibit MPGES1 by covalent modification of residue C59 and by non-covalent inhibition through binding at the substrate (PGH₂) binding site. The mechanism of inhibition is dissected by analysis of the native enzyme and the MPGES1 C59A mutant both in the presence of glutathione (GSH) and glutathione sulfonate (GSO₃[−]). The location of inhibitor adduction and non-covalent binding was determined by MS³ sequencing and with backbone amide H/D exchange mass spectrometry. The kinetics, regiochemistry, and stereochemistry of the spontaneous reaction of GSH with 15d-PGJ₂ were determined. The question of whether the anti-inflammatory properties of 15d-PGJ₂ are due to inhibition of MPGES1 is discussed.

Prostaglandins (PGs) are signaling molecules derived from arachidonic acid (Figure 1) that possess a wide range of biological activities, exerting their functions by binding to G-protein-coupled receptors. For example, prostaglandin E₂ (PGE₂) binds to E-prostanoid (EP) receptors 1–4, eliciting responses involving gastric mucosal integrity, fertility, immune modulation, and inflammation.^{1–4} Similarly, prostaglandin D₂ (PGD₂) promotes vasodilation and is also involved in the regulation of body temperature and physiological sleep by binding to the D-prostanoid (DP) receptors 1 and 2.^{5–7}

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ASSOCIATED CONTENT

Supporting Information. Eighteen pages including Figure S1, MS fragmentation analysis of L-Cys adduct with 15d-PGJ₂; Figures S2-S4, NMR spectra of 9-(S-glutathionyl)-15d-PGJ₂; Table S1, chemical shifts and coupling constants from the NMR spectra; Figure S5, MS³ fragmentation of peptide 49–59 from MPGES1 modified with 15d-PGJ₂ and Figures S6-S15, H/D exchange kinetic profiles including the amplitudes and rate constants for all peptides in MPGES1 and MPGES1 C59A in the presence and absence of 15d-PGJ₂ and Figure S16, the peptic peptide map of MPEGS1. This material is free of charge via the internet at <http://pubs.acs.org>.

AUTHORS CONFLICT OF INTEREST

All authors declare that they have no financial or other conflicts of interest associated with this work.

Dehydration of PGE₂ and PGD₂ gives rise to a subclass of prostaglandins known as cyclopentenone PGs (cyPGs).⁸ One member of this subclass is 15-deoxy-^{12, 14}-prostaglandin J₂ (15d-PGJ₂), arises from the spontaneous dehydration of PGD₂ (Figure 1) initially forming PGJ₂. PGJ₂ then isomerizes to form ¹²-PGJ₂, which, after the spontaneous loss of an additional molecule of water, results in the formation of 15d-PGJ₂.⁹ The biological attributes of this particular cyPG are interesting and wide-ranging. Although it has a much lower affinity for its corresponding DP receptors, it has been reported to selectively bind to peroxisome proliferator-activated receptor (PPAR) with an EC₅₀ value in the low micromolar range, which may impart the reported anti-inflammatory signaling properties associated with 15d-PGJ₂.¹⁰ For a recent and excellent review of the chemistry and biological activities of 15d-PGJ₂ and other isoprostane eicosanoids the reader is referred to Milne et al.¹¹

The chemical properties of 15d-PGJ₂ appear to be dominated by the electrophilic, α,β -unsaturated carbonyl group on the cyclopentenone ring that results in the formation of adducts with cellular nucleophiles via Michael addition at C-9.¹² The nucleophiles commonly include cysteine-containing molecules, such as glutathione and cellular proteins.¹³ In principle, 1,4-addition at C-13 or 1,6-addition at C-15 could also occur, but they have not been observed. For a comprehensive review of the interaction of electrophilic lipids with cellular nucleophiles, particularly proteins, the reader is directed to Stamatakis and Perez-Sala.¹⁴

The pro-inflammatory signaling molecule PGE₂ is synthesized in a glutathione (GSH) dependent isomerization reaction catalyzed by microsomal prostaglandin E synthase 1 (MPGES1) as shown in Figure 1. PGE₂ is a well-known mediator of pain, fever, and inflammation.¹⁵ Most current anti-inflammatory therapies rely on the inhibition of cyclooxygenase (COX) (see Figure 1) by non-steroidal anti-inflammatory drugs (NSAIDs) or COX-2 selective inhibitors (coxibs), which decrease the concentration of PGH₂ and hence that of PGE₂. However these treatments, which alter the concentrations of several prostaglandins and thromboxanes, can have adverse cardiovascular effects.¹⁶ The MPGES1 enzyme, which is induced under inflammatory conditions, is functionally coupled to COX-2 and represents an alternative therapeutic target for the treatment of inflammatory diseases.^{17,18} As a consequence, MPGES1 is currently under investigation as a target for direct therapeutic intervention.

One known naturally occurring inhibitor of MPGES1 is 15d-PGJ₂. In 2002 Quraishi et al. reported that MPGES1 was inhibited by 15d-PGJ₂ with an IC₅₀ value of 0.3 μ M.¹⁹ In addition, the authors noted that increasing the substrate concentration (PGH₂) failed to rescue enzyme activity suggesting that the inhibition is not simply competitive but might involve allosteric binding of the inhibitor or the formation of a covalent adduct. The inhibition of MPGES1 is another possible route for the observed anti-inflammatory properties of 15d-PGJ₂. The exact mechanism of inhibition and its physiological relevance has remained an enigma.

The chemical and physical properties of 15d-PGJ₂ raise several very interesting questions about the possible mechanisms of inhibition of MPGES1. Is the enzyme covalently adducted by the inhibitor? Is the enzyme inhibited competitively by 15d-PGJ₂? Is the enzyme inhibited by a GSH adduct of 15d-PGJ₂? Does the enzyme catalyze the addition of GSH to 15d-PGJ₂?

In this paper we describe a series of biochemical and mass spectroscopy (MS) experiments, which indicate that the inhibition of MPGES1 by 15d-PGJ₂ can occur by covalent adduction of the enzyme at C59 with a stoichiometry of one adduct per subunit in the trimer or by

simple competitive inhibition of the C59A mutant protein. We demonstrate that the molecular footprints of the two modes of inhibition are quite distinct, as revealed by backbone amide hydrogen/deuterium (H/D) exchange kinetics. The equilibrium constant for the reaction of GSH with 15d-PGJ₂ to form the 9-(S-glutathionyl)-15d-PGJ₂ adduct is 3800 M⁻¹. In addition, the enzyme does not appear to catalyze the formation of this adduct. The NaBH₃CN-reduced adducts, 9-(S-glutathionyl)-11-hydroxy-15d-PGJ₂, are not effective inhibitors of the enzyme. The kinetic and biochemical results suggest that 15d-PGJ₂ is an effective multimode-inhibitor of MPGES1 *in vitro*. The question as to whether 15d-PGJ₂ has special significance *in vivo*, perhaps as an anti-inflammatory molecule, is discussed.

MATERIALS AND METHODS

Materials

Buffer salts and common chemicals were of the highest quality commercially available. Detergents were from Affymetrix Anatrace, Santa Clara, CA. 15d-PGJ₂, PGH₂, and malondialdehyde were obtained from Cayman Chemical, Ann Arbor, MI. The MPGES1 enzyme with GSH or glutathione sulfonate (GSO₃⁻) bound was expressed and purified as previously described.²⁰

Expression and Purification of the C59A Mutant of MPGES1

The gene encoding the native human MPGES1 enzyme with a C-terminal hexahistidine tag was contained in a pET-21b vector. With standard PCR, cysteine 59 was mutated to alanine using the forward primer, GGC CCC CAG TAT GCC AGG AGT GAC CCC, and the reverse primer, GGG GTC ACT CCT GGC ATA CTG GGG GCC. The resulting enzyme is hereafter referred to as MPGES1 C59A. *E. coli* cell culture and enzyme preparation utilizing ultracentrifugation, Ni²⁺-affinity chromatography, and sulfopropyl strong cation exchange chromatography were performed as previously described²⁰ with the exception that, dithiothreitol (DTT) was not included in either the last chromatography step or the final dialysis. Instead, these buffers were purged with argon.

Preparation of 9-(S-glutathionyl)-11-hydroxy-15d-PGJ₂ and 11-hydroxy-15d-PGJ₂

The 9-(S-glutathionyl)-11-hydroxy-15d-PGJ₂ adduct of 15d-PGJ₂ was prepared by adding 40 μL of a 25 mM solution of 15d-PGJ₂ in DMSO to 160 μL of 6.3 mM GSH in 100 mM KH₂PO₄ (pH 7.0) and incubating at room temperature for one hour. The adduct was then reduced by the addition of 10 μL of a 1 M suspension of NaBH₃CN in THF and storing overnight at 4 °C. The reaction was diluted ten-fold in 20 mM Tris (pH 8.0). The product was purified by diethylaminoethyl (DEAE) weak anion exchange chromatography, washing with 20 mM Tris (pH 8.0) and eluting with 100 mM KH₂PO₄ (pH 7.5) containing 1 M KCl, 1% glycerol, and 1% CHAPS. The 11-hydroxy-15d-PGJ₂ was prepared as above without the reaction with GSH.

Preparation of 9-(S-glutathionyl)-15d-PGJ₂

The addition of GSH to 15d-PGJ₂ was initiated by the addition of 1.5 mL of 10 mM GSH in 100 mM KH₂PO₄ (pH 7.0) to 500 μg of 15d-PGJ₂ at 40 °C for 30 minutes. The reaction was stopped by the addition of 100 μL of 70% H₂SO₄, after which the sample was applied to a 6 cc Oasis HLB solid-phase extraction cartridge that was previously activated with methanol and hydrated with 50 mM ammonium acetate (pH 3). The sample was washed with 50 mM ammonium acetate (pH 3) and eluted with methanol. After evaporation under nitrogen, the sample was reconstituted in 15% CH₃CN and 0.01% formic acid and stored on dry ice. It was subsequently purified by HPLC, monitoring at 308 nm, using a Beckman 5 μ, 80 Å C18 column (4.6 mm x 25 cm), and eluted at 1 mL/min, with a gradient of 15% to 95% CH₃CN

and 0.01% formic acid over a 30-minute period. After collecting the 9-(S-glutathionyl)-15d-PGJ₂ peak at approximately 20 minutes on dry ice, the sample was dried under vacuum and stored desiccated at -20 °C.

Structure of 9-(S-glutathionyl)-11-hydroxy-15d-PGJ₂

The structure of the glutathionyl-adduct of 15d-PGJ₂ was determined by LC-MS/MS, as well as by 2D NMR spectroscopy. For MS analysis, the borohydride-reduced GSH adduct was prepared as described above. In addition, a cysteine adduct was prepared as described above, replacing GSH with equimolar L-cysteine. Analysis was performed by reverse-phase LC-MS/MS, using a Phenomenex Synergi 2.5 μ , Hydro-RP, 100 Å, (100 $\times$ 2.00 mm) C18 column, eluted at 0.2 mL/min with a gradient of 15% to 95% CH₃CN and 0.01% formic acid over a 30 min period. Ions of m/z 300 to 1300 were detected on a ThermoFinnigan TSQ triple-quadrupole mass spectrometer using positive electrospray ionization. The PG adduct was identified with a mass corresponding to the monoisotopic mass of GSH or cysteine plus the monoisotopic mass of 15d-PGJ₂, and the site of adduction was determined by MS/MS fragmentation.

The diastereoselectivity of the reaction of GSH with 15d-PGJ₂ was determined by 2D NMR spectroscopy at high field. NMR data were acquired either using a 22.1 T Bruker magnet equipped with a Bruker AV-III console or a 14.9 T Bruker magnet equipped with a Bruker AV-III console. All spectra were acquired in 3 mm NMR tubes using a Bruker 5 mm TCI cryogenically cooled NMR probe. Chemical shifts were referenced internally to CD₃OD (3.30 ppm) or D₂O (4.70 ppm), which also served as the ²H lock solvents. For 1D ¹H NMR, typical experimental conditions included 32K data points, 13 ppm sweep width, a recycle delay of 1.5 seconds and 32 scans. For samples acquired in D₂O, water suppression using pre-saturation was implemented in order to reduce the signal of residual H₂O. For 2D ¹H-¹H COSY and DQF-COSY, experimental conditions included 2048 $\times$ 512 data matrix, 13 ppm sweep width, recycle delay of 1.5 seconds and 4 scans per increment. The data was processed using squared sine-bell window function, displayed in either the magnitude mode (COSY) or absolute intensity mode (DQF-COSY). Similar experimental parameters were used to acquire 2D ¹H-¹H nuclear Overhauser effect (NOESY) experiments which were acquired with a mixing time of 400 ms. The data was processed using a $\pi/2$ shifted squared sine window function displayed in absolute intensity mode. Multiplicity-edited HSQC experiments were acquired using a 1024 $\times$ 256 data matrix, a J(C-H) value of 145 Hz which resulted in a multiplicity selection delay of 34 ms, a recycle delay of 1.5 seconds and 16 scans per increment along with GARP decoupling on ¹³C during the acquisition time (150 ms). The data was processed using a $\pi/2$ shifted squared sine window function and displayed with CH/CH₃ signals phased positive and CH₂ signals phased negative.

Kinetics of the Spontaneous Reaction of GSH with 15d-PGJ₂

The kinetics of the approach to equilibrium were determined at 25 °C under pseudo-first-order conditions with a fixed concentration of 40 μ M 15d-PGJ₂ and a variable excess concentration of GSH ranging between 0.5 to 5.0 mM in 100 mM KH₂PO₄ (pH 7.0). Reactions were initiated by the addition of 10 μ L of a 4.0 mM stock solution of 15d-PGJ₂ in 20 mM KH₂PO₄ (pH 7.0) to 990 μ L of GSH in the same buffer. The formation of the glutathionyl-adduct was observed spectrophotometrically as an exponential decrease in absorbance at 250 nm. The concentration dependence of k_{obs} was used to determine the rate constants for the forward (k_1) and reverse (k_{-1}) reactions from; $k_{\text{obs}} = k_1[\text{GSH}] + k_{-1}$.

In addition, the equilibrium constant for formation (K_f) of 9-(S-glutathionyl)-15d-PGJ₂ was calculated by determining the final concentrations of reactants and products at equilibrium under four different initial reactant concentrations. Two reactions were initiated by the

addition of 25 μL of 2 mM 15d-PGJ₂ stock solution in 100 mM KH₂PO₄ (pH 7) to 475 μL of 105 μM or 211 μM GSH in the same buffer, giving an initial 15d-PGJ₂ concentration of 100 μM and initial concentrations of GSH of 100 μM or 200 μM , respectively. Two additional reactions were initiated by the addition of 50 μL of the same 15d-PGJ₂ stock solution to 450 μL of 111 or 222 μM GSH, giving a 15d-PGJ₂ concentration of 200 μM and GSH concentrations of 100 or 200 μM , respectively. Each reaction mixture was incubated at 4 °C overnight. After equilibrating to 22 °C for one hour the samples were analyzed by HPLC, monitoring the absorbance at 316 nm, using a Phenomenex Synergi 2.5 μm , Hydro-RP, 100 Å, C18 column (100 $\times$ 2.00 mm) and eluting at 0.2 mL/min with a gradient of 15% to 95% CH₃CN and 0.05% trichloroacetic acid over a 30 min period.

Enzyme Activity Assays

The catalytic conversion of PGH₂ to PGE₂ was verified by the previously described method,¹⁸ and the detection of PGE₂ by LC-MS/MS was performed as previously published.²¹ Reactions were initiated at 0 °C by the addition of 100 μL of a 100 nM enzyme stock solution in reaction buffer [100 mM KH₂PO₄ (pH 7.5) containing 2.5 mM GSH, 1% glycerol and 1% CHAPS] to 6.7 μL of 75, 150, or 300 μM PGH₂ in acetone. After a one-minute incubation on ice, unreacted PGH₂ was decomposed to malondialdehyde (MDA) and 12-(S)-hydroxy-8,10-*trans*-5-*cis*-heptadecatrienoic acid (12-HHT) by the addition of 400 μL of 25 mM FeCl₂ in 50 mM citric acid (pH 3) which included 0.5 μM 11-PGE₂ as an internal standard. After solid-phase extraction, the resulting prostaglandins were reconstituted in 5% CH₃CN and analyzed by reversed-phase LC-MS/MS, with a Phenomenex Synergi 2.5 μm , Hydro-RP, 100 Å C18 column (100 $\times$ 2.00 mm) eluting at a flow rate of 0.2 mL/min with 32% CH₃CN and 0.1% formic acid in water. Single-reaction monitoring (SRM) of the transition *m/z* 351 to 271 was utilized for PGE₂ detection on a ThermoFinnigan TSQ triple-quadrupole mass spectrometer using negative electrospray ionization. To verify inhibition by 15d-PGJ₂, the procedure was repeated with enzyme pre-incubated for one hour on ice with 100 μM 15d-PGJ₂.

The inhibition of MPGES1 by 15d-PGJ₂ was determined with a method adapted from a previously described technique.²² The enzyme, complexed with GSO₃⁻, was combined with the inhibitor by addition of 10 μL of solutions of 15d-PGJ₂ ranging in concentrations from 0.1 μM to 5 mM in reaction buffer (lacking GSH) to 90 μL of a 1.1 μM enzyme solution in reaction buffer, giving a final enzyme concentration of 1 μM and a final 15d-PGJ₂ concentration range of 0.01 to 500 μM . An additional incubation was initiated by the addition of 40 μL of a 5 mM stock solution of 15d-PGJ₂ in reaction buffer (lacking GSH) to 60 μL of 1.7 μM enzyme, giving final concentrations of 2 mM and 1 μM , respectively. After a one-hour incubation on ice, 2.5 μL of a 100 mM stock solution of GSH in 100 mM KH₂PO₄ (pH 7.0) were added to the pre-incubated protein, mixed and incubated at 0 °C for five minutes. The reaction was initiated by the addition of 100 μL of each sample to 5 μL of 100 μM PGH₂ in acetone and incubated at 22 °C for one minute. Un-reacted PGH₂ was decomposed to MDA and 12-HHT by the addition of 200 μL of 50 mM FeCl₂ in 500 mM KH₂PO₄ (pH 2). To form a fluorescent complex of MDA in solution, 500 μL of 15 mM thiobarbituric acid (TBA) in 80 mM KH₂PO₄ (pH 2.0) was added to the reaction mixture and heated to 80 °C for thirty minutes. Precipitate was removed by centrifugation, and the resulting fluorescent MDA-TBA complex was detected with a Horiba Fluorolog fluorescence spectrometer, tuned to excitation and emission wavelengths of 530 nm and 550 nm, respectively. The same method was used to examine the inhibition of MPGES1 by 11-hydroxy-15d-PGJ₂ and 9-(S-glutathionyl)-11-hydroxy 15d-PGJ₂.

Covalent Modification of MPGES1 by 15d-PGJ₂

The site of adduction of the enzyme by 15d-PGJ₂ was determined by LC-MS/MS sequencing. The covalent modification was initiated by the addition of 2 μ L of 25 mM 15d-PGJ₂ in dimethyl sulfoxide (DMSO) to 100 μ L of 1 mg/mL (60 μ M) purified native enzyme in 50 mM KH₂PO₄ (pH 7) containing 300 mM KCl, 7.5% glycerol, 1% CHAPS, and either 1 mM GSH or 1 mM GSO₃⁻. After an overnight incubation at 4 °C, the Michael-adduct was reduced with NaBH₃CN, as described above. For MS analysis, 10 μ L of the pre-incubated protein was diluted five-fold in H₂O at 22 °C, after which 50 μ L of ice-cold 100 mM KH₂PO₄ (pH 2.3) was added, dropping the pH to 2.5. The protein was then digested on ice for five minutes by the addition of 2 μ L of 10 mg/mL (290 μ M) pepsin in H₂O. The resulting peptides were separated by HPLC at 0 °C on a Phenomenex Jupiter 5 μ , 300 Å C18 column (50 $\times$ 1.00 mm) eluting at 0.3 mL/min with a 30-minute gradient of 2–50% CH₃CN and 0.4% formic acid. Ions of m/z 300 to 1500 were detected on a ThermoFinnigan TSQ triple-quadrupole mass spectrometer using positive electrospray ionization. Peptides containing the reduced adduct were identified as those with a mass shift corresponding to the monoisotopic mass of reduced 15d-PGJ₂, and were verified by MS/MS sequencing.

In order to determine the regiochemistry of enzyme adduction on 15d-PGJ₂, the procedure from the previous paragraph was repeated on a ThermoFinnigan LTQ ion trap mass spectrometer in the positive electrospray ionization. The peptide identified as containing the PG adduct was subject to MS³ fragmentation, selecting for the y-ion containing the adducted cysteine-59 residue.

Analysis of Backbone Amide H/D Exchange Kinetics

The methods of the H/D exchange assay and kinetic analysis were performed as previously described.^{20,23} The analysis was performed on the native enzyme, as well as the C59A mutant, in complex with either GSH or GSO₃⁻, and in the presence of 500 μ M 15d-PGJ₂. Pre-incubation of the enzyme with the inhibitor was initiated by the addition of 4 μ L of 25 mM 15d-PGJ₂ in DMSO to 200 μ L of 1 mg/mL (60 μ M) purified native enzyme or C59A mutant in 50 mM KH₂PO₄ (pH 7.0) containing 300 mM KCl, 7.5% glycerol, 1% CHAPS, and either 1 mM GSH or 1 mM GSO₃⁻, and was incubated overnight at 4 °C. Deuterium incorporation was initiated by a five-fold dilution of 10 μ L of the pre-incubated protein solution in D₂O at 22 °C. The incorporation was then quenched by a two-fold dilution in ice-cold 100 mM KH₂PO₄ (pH 2.3). The protein was then digested on ice for five minutes by the addition of 2 μ L of 10 mg/mL (290 μ M) pepsin in H₂O. The resulting peptides were separated by reversed-phase HPLC at 0 °C on a Phenomenex Jupiter 5 μ , 300 Å, C18 column (50 $\times$ 1.00 mm), and eluted at a flow rate of 0.3 mL/min with a fifteen-minute gradient of 2–50% CH₃CN and 0.4% formic acid. The elution conditions differ from those previously reported²⁰ in order to resolve the peptide 49–59. In doing so, however, it was no longer possible to resolve peptide 49–58, a peptide previously utilized to analyze the structural impact of competitive inhibitor binding. Scans of m/z 300 to 1500 were utilized for peptide detection on a ThermoFinnigan TSQ triple-quadrupole mass spectrometer using positive electrospray ionization. Deuterium incorporation was observed as a shift in the centroid of the ion envelope (average mass) for each peptide.

RESULTS

Kinetics of the Spontaneous Reaction of 15d-PGJ₂ with GSH

The spontaneous reaction of GSH with 15d-PGJ₂ (Scheme 1) was previously documented¹² but not with respect to its kinetics. The kinetics of the approach to equilibrium for the reaction at pH 7.0 and 25 °C, determined under pseudo-first-order conditions with [GSH] [15d-PGJ₂], gave rate-constants $k_1 = 0.75 \pm 0.02 \text{ M}^{-1} \text{ s}^{-1}$ and $k_{-1} = (2.0 \pm 0.7) \times 10^{-4} \text{ s}^{-1}$ for

the forward and reverse reactions, respectively. An equilibrium constant for the formation of the adduct can be estimated from $K_f = k_1/k_{-1} \approx 3800 \pm 1000 \text{ M}^{-1}$. Analysis by HPLC of reaction mixtures of GSH and 15d-PGJ₂ at various concentrations gave a similar value of $K_f = 1700 \pm 200 \text{ M}^{-1}$.

Regiochemistry of the Spontaneous Reaction of 15d-PGJ₂ with GSH and L-Cys

The reaction of 15d-PGJ₂ with GSH or L-Cys resulted in one major peak on analysis by HPLC. Further analysis by LC-MS/MS fragmentation revealed C-9 to be the predominant site of Michael addition on 15d-PGJ₂ by both GSH and L-Cys, in agreement with what was previously reported for GSH.¹² However, both of these adducts proved to be labile under the ESI conditions in the mass spectrometer. Reduction of the adduct with NaBH₃CN (Scheme 1) provided a stable product for more definitive analysis by MS/MS (Figure S1, Supporting Information). This analysis provided positive evidence for adduction at C-9 but no evidence for adduction at C13 or C-15. The observation of a single major species by HPLC and the absence of any MS/MS evidence for addition at C-13 or C-15 suggested that the major site of adduction was at C-9.

Diastereoselectivity of the Reaction of GSH with 15d-PGJ₂

An earlier paper¹² suggested that the stereochemistry of addition of GSH to 15d-PGJ₂ occurred on the *si*-face (bottom) of the 9–10 double bond resulting in (*9S*)-S-glutathionyl-15d-PGJ₂. Our examination of molecular models, however, indicated that the approach of the nucleophile from the *si*-face was considerably more hindered and less likely than addition from the top (*re*-face). Given that, we revisited the structure of the adduct with high-field NMR spectroscopy.

A detailed NMR analysis of the purified adduct at 600 MHz and 900 MHz in D₂O and CD₃OD were consistent only with the addition of GSH from the *re*-face resulting in the (*9R*)-S-glutathionyl-15d-PGJ₂ diastereomer. The NMR assignments for this molecule were established using 2D ¹H-¹H COSY/DQF-COSY and NOESY along with 2D ¹H-¹³C HSQC experiments. The stereochemistry of the glutathione adduct was determined by analysis of the H7 (2.36, 2.27 ppm), H8 (3.06 ppm), H9 (4.57 ppm), and H10 (2.81, 3.05 ppm) protons. In the 1D NMR spectrum in CD₃OD, H9 appears as two doublets, which strongly suggests coupling between two protons and not three. These doublets were assigned to coupling between H9 to the two protons on C10 and confirmed in the 2D COSY. The COSY shows two correlations between H9 and the two H10 protons and no correlation between H9-H8. The NOESY spectrum also revealed correlations between the C10 protons and H9 but no evidence of a correlation between H9-H8. The lack of a coupling between H8-H9 can be explained if the bond angle between these two protons approaches 90°, which is unique to the (*9R*)-S-glutathionyl-15d-PGJ₂ diastereomer.

On switching the solvent to D₂O changes were observed in the chemical shifts for the H7 (2.41, 2.45 ppm), H8 (3.17 ppm), H9 (4.75 ppm), and H10 (3.03, 3.20 ppm) protons. A triplet was observed for H9 in D₂O, which is again consistent with coupling to two protons (H10). Results from both 2D COSY and DQF-COSY showed coupling between H9 to H10 but no evidence of coupling between H8-H9. This was further supported by the 2D NOESY

experiment, which also failed to show any correlation between H8-H9. NMR spectra of 9-GS-15d-PGJ₂ are provided in the Supporting Information (Figure S2–S4 and Table S1).

Covalent Modification of MPGES1 by 15d-PGJ₂

The original work of Quraishi et al.¹⁹ suggested that the inhibition of MPGES1 by 15d-PGJ₂ might be covalent in nature. An examination of the three-dimensional structure of the protein determined by Jegerschöld et al.²⁴ indicated that there were three buried cysteine residues and one (C49) on the surface. LC-MS/MS sequencing of the native MPGES1 enzyme covalently modified by the 15d-PGJ₂ and digested with pepsin revealed that C59 of the peptide 49–59 was the specific and exclusive site of PG adduction. An estimate of the ratio of the adducted to un-adducted peptide suggested that there were about 0.2 adducts/subunit. When the adduct was stabilized by reduction with NaBH₃CN analysis of the TIC chromatographs of MPGES1 complexed with GSH revealed that approximately 80–90% of the enzyme is modified under the experimental conditions of the H/D exchange assay, while the MPGES1•GSO₃[−] complex appeared to be fully adducted.

The regiochemistry of the protein adduct was determined by MS³ experiments on the peptic peptide containing C59. The peptide identified as containing the 15d-PGJ₂ adduct (residues 49–59) was subjected to MS³ fragmentation selecting for the y-ion of the adducted C59 residue. The results, shown in Figure S5 (Supporting Information), provide evidence only for adduction at C-9 of 15d-PGJ₂, as was found with the GSH adduct. However, the absence of evidence for addition at C-13 of C-15 of 15d-PGJ₂ does not strictly rule out its occurrence.

Catalytic Properties of MPGES1 and Its C59A Mutant

The catalytic and physical properties of the MPGES1 were observed as previously described²¹. The C59A MPGES1 protein exhibited a catalytic activity toward PGH₂ that was indistinguishable from that of the native enzyme. Neither the native enzyme nor the C59A mutant catalyzed the addition of GSH to 15d-PGJ₂.

Inhibition of MPGES1 by 15d-PGJ₂

The native enzyme was inhibited by 15d-PGJ₂ with an IC₅₀ value of 0.6 μM, (Figure 2A) in good agreement with results previously reported¹⁹. Inasmuch as the concentration of the enzyme in the assay is ≈ 1 μM the value of 0.6 μM is the upper limit of the true IC₅₀ and that covalent modification close to complete. Moreover, increasing the substrate concentration failed to rescue the activity of native MPGES1 suggesting that the inhibition was not competitive (Figure 3A). In contrast, the inhibition of the C59A mutant of MPGES1 was reversible with increasing concentrations of PGH₂ (Figure 3B) with an IC₅₀ value of 12 μM (Figure 2B). The inhibition of the native enzyme utilizing 11-OH-15d-PGJ₂ as an inhibitor was similar to that of the C59A mutant, having IC₅₀ values of 11 μM and 16 μM, respectively (Figure 2). Neither enzyme was appreciably inhibited by 9-(S-glutathionyl)-11-hydroxy-15d-PGJ₂.

H/D Exchange MS of Native MPGES1 and MPGES1 C59A

The backbone H/D exchange kinetics of the native MPGES1 enzyme, complexed with GSH, and in the presence or absence of 15d-PGJ₂ were compared. Kinetic plots for some selected peptides are shown in Figure 4. A complete set of kinetic plots for all peptides along with the amplitudes and rate constants for the fits of the data are provided in the Supporting Information (Figures S6 to S15). These data reveal that peptides within proximity of, or containing, the residue C59 (peptides 37–54, 49–59, and 60–68) (Figure 5A) displayed decreased deuterium incorporation rates when adducted with 15d-PGJ₂. This was not

unexpected, inasmuch as 15d-PGJ₂ is adducted to C59. In addition to these changes, peptides within the α -helical core of the protein (peptides, 78–83, 104–107, 124–129, 130–132, 133–140, and 141–152) also displayed significant differences in H/D exchange behavior (Figures 4C, 4D, 5A). On further inspection, it was obvious that these regions were similar to those previously identified as constituting the hydrophobic substrate binding cleft by H/D exchange experiments of MPGES1•GSH in the presence of inhibitors competitive for PGH₂ binding.²⁰

To further explore the possibility that MPGES1 binds 15d-PGJ₂ in its substrate-binding site, the above experiments were repeated with the MPGES1 C59A mutant. The H/D exchange kinetics of the C59A mutant in complex with GSH are essentially the same as those of the native enzyme as shown in Figure 4. However, the H/D exchange kinetics of MPGES1 C59A in the presence of GSH and 15d-PGJ₂ are remarkably different. Here regions within close proximity of A59 display no significant difference in deuterium incorporation rates. Regions within the CHAPS micelle (18–23, 78–83, 104–107, 124–129, 130–132, 133–140, and 141–152), however, do display changes (Figure 5B), which are associated with the non-covalent binding of 15d-PGJ₂ to the substrate-binding site.

In order to eliminate contributions of the spontaneous adduction of 15d-PGJ₂ to the cofactor, GSH, the H/D exchange kinetic analysis of native MPGES1, as well as MPGES1 C59A complexed with GSO₃[−] were performed in the absence and presence of 15d-PGJ₂ (Figures 6 and 7). Here, peptide 37–54 (Figure 7A) still displays an overall decrease in deuterium incorporation rates for the native enzyme, whereas peptides 49–59 and 60–68 no longer display this large change in kinetics. This may be due to the fact that GSO₃[−] binding distorts the structural conformation of MPGES1¹⁹, complicating the data interpretation. Regions within the detergent micelle (peptides 78–83, 104–107, 130–132, and 141–152) still exhibit substantial changes in H/D exchange kinetics, suggesting that the enzyme binds 15d-PGJ₂ within in the substrate binding site.

DISCUSSION

The Chemistry of 15d-PGJ₂

The formation of 15d-PGJ₂ and its reactions with cellular nucleophiles has been studied by numerous investigators. The regiochemistry of the reaction of GSH with 15d-PGJ₂ appears to occur predominantly at C-9 as previously reported by others.^{12, 26} This is also shown to be true for the adduction of MPEGS1 at C59. The stereoselectivity of the spontaneous reaction was reported give the (9*S*)-(S-glutathionyl)-15d-PGJ₂ diastereomer.¹² In contrast, our NMR results at 600 MHz and 900 MHz in D₂O and CD₃OD suggest that the nucleophile adds to the least hindered face of the 9–10 double-bond to give the (9*R*)-(S-glutathionyl)-15d-PGJ₂ diastereomer.

The chemistry and kinetics of the reaction of GSH with 15d-PGJ₂ is a key element to understanding the cellular concentration of the cyclopentenone and its potential anti-inflammatory activity. The spontaneous reaction with GSH is relatively slow (ca 3×10^{-3} s^{−1}) at pH 7, 25 °C and physiological concentrations of GSH (ca 4 mM). Once formed, the adduct has a half-life of about one hour. It is also clear that at chemical equilibrium in aqueous solution virtually all 15d-PGJ₂ is in the form of the 9-S-glutathionyl-adduct. The rates of formation of 9-S-glutathionyl-15d-PGJ₂ and its penultimate precursor PGJ₂ are also known to be accelerated, *in vitro*, by soluble glutathione transferases.^{23, 26} However the efficiency of the enzyme-catalyzed addition of GSH, at least to PGJ₂, appear to be rather low with $k_{\text{cat}}/K_{\text{M}}$ values of 100 to 300 M^{−1} s^{−1}.²⁶ The exact role of GSH transferases in the metabolic disposition PGJ₂ and 15d-PGJ₂ is not known.

Inhibition of MPGES1 by 15d-PGJ₂

The anti-inflammatory properties of 15d-PGJ₂ may be associated with its inhibition of MPGES1. As defined in this work, there are two mechanisms of inhibition; the covalent modification of MPGES1 at C59 and the competitive inhibition revealed by the experiments with the C59A mutant. It is also clear that the enzyme is not inhibited by (9R)-S-glutathionyl-15d-PGJ₂ and that the enzyme does not catalyze the formation of this molecule. It is highly unlikely that a non-covalent, competitive inhibition of MPGES1 by 15d-PGJ₂ (IC₅₀ ≈ 12 μM) has any biological relevance *in vivo*. The concentration of free 15dPGJ₂ in the cell, though unknown, is likely to be well below the micro-molar range.²⁷ The most robust inhibition of the enzyme is via the covalent modification of C59 with an IC₅₀ 0.6 μM. It is important to point out this IC₅₀ is an upper limit given the low sensitivity of the inhibition assay.

The effects of the modification of C59 on the H/D exchange behavior of the backbone could suggest a possible physical mechanism for the covalent inhibition of the enzyme. The substrate of MPGES1, PGH₂, is generated on the luminal side of the membrane and the product is thought to be released from the cytosol face.^{28–30} The exact pathways for entry and egress, however, are not clear. One can imagine entry of the substrate from the luminal face of the enzyme and dissociation of the product from the cytosolic side, or the lateral diffusion of the substrate from the membrane into the active site and the lateral release of PGE₂ into the membrane and subsequent release to the cytosol. The predominant effect of the covalent modification of MPGES1 by 15-dPGJ₂ on the H/D exchange behavior of the backbone is a decreased rate of exchange at the cytosolic ends of helices TMI and TMII and the cytosolic loop that connects them (Figure 5). Cysteine 59 is located at the junction of the cytosolic loop and TMII. If the decrease in H/D exchange rates reflects a reduced flexibility of the egress channel for the product, then the inhibitory effect of the covalent modification of C59 could be reasonably ascribed to blockage of product release.

In contrast, the competitive inhibition of MPGES1 by non-covalent inhibitors that appear to occupy the substrate binding site often increase the H/D exchange kinetics of the cytosolic loop between TMI and TMII.²⁰ This observation suggests that the physical mechanism of inhibition by competitive inhibitors and the covalent inhibition by 15d-PGJ₂ are quite different. That is to say, the competitive inhibitors restrict the access substrate to the active site while adduction by 15d-PGJ₂ may damp the flexibility of the product release channel or alter its conformation, preventing either product formation or release.

Biological Consequences of 15d-PGJ₂

Inasmuch as the concentration of 15d-PGJ₂ in the cell is not known, it is not possible to conclude if the covalent modification of MPGES1 occurs to any significant extent *in vivo*. In addition to the cellular concentration of 15d-PGJ₂, there are other issues that are not fully understood. For example, the kinetics and stereochemistry of its reaction with C59 with 15d-PGJ₂ have not been evaluated. Given the physical properties of the molecule, the location of 15d-PGJ₂ is likely to be concentrated in the membrane where the enzyme is also located. Moreover, it is not known if the efficiency of soluble or membrane-bound GSH transferases is sufficient to contribute to the clearance of 15d-PGJ₂ by catalyzing the formation of the GSH adduct.

Although potential biological effects of 15d-PGJ₂, such as those cited in this paper and elsewhere¹¹ are interesting, the significance of all these findings is complicated by what we do not know. The biological effects of 15d-PGJ₂ formed *in vivo* are controversial inasmuch as the formation, concentration, location, and metabolic disposition of the molecule are poorly understood.¹⁰ The concentration of 15d-PGJ₂ in human urine has been estimated to

be in the low pM range, suggesting that its level is insufficient to support biological activity.²⁷ However, the level in urine may not reflect local cellular concentrations that could greatly affect its bioactivity.

Finally, the transformation of PGD₂ to 15d-PGJ₂ appears to occur by spontaneous chemistry that is unregulated. This fact alone would suggest that 15d-PGJ₂ may not play a regulatory role as an anti-inflammatory molecule. That does not mean, however, that it could not play a role under pathological conditions.

Conclusions

The results reported in this paper demonstrate that 15d-PGJ₂ can act as an effective covalent inhibitor of MPGES1 *in vitro* and define, from a chemical and physical standpoint, the interaction of the inhibitor with the enzyme. A structure of the adducted enzyme would greatly facilitate the understanding of the physical mechanism of inhibition. The currently available biological data do not preclude an anti-inflammatory role for 15d-PGJ₂ *in vivo*. However the gaps in our knowledge of the *in vivo* concentration of 15d-PGJ₂, its kinetic disposition and homeostasis are too large to conclude that it plays a direct role in the biochemistry of inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

9-GS-15d-PGJ₂	9-(S-glutathionyl)-15-deoxy- ^{12,14} -prostaglandin J ₂
9-GS-11-OH-15d-PGJ₂	9-(S-glutathionyl)-11-hydroxy-15-deoxy- ^{12,14} -prostaglandin J ₂
11-OH-15d-PGJ₂	11-hydroxy-15-deoxy- ^{12,14} -prostaglandin J ₂
12-HHT	12-(S)-hydroxy-8,10- <i>trans</i> -5- <i>cis</i> -heptadecatrienoic acid
15d-PGJ₂	15-deoxy- ^{12,14} -prostaglandin J ₂
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate
COX	cyclooxygenase
coxib	COX-2 selective inhibitor
cyPG	cyclopentenone prostaglandin
DEAE	diethylaminoethyl weak anion exchange

DMSO	dimethyl sulfoxide
DP	D-prostanoid receptor
DTT	dithiothreitol
EP	E-prostanoid receptor
GSH	glutathione
GSO₃⁻	glutathione sulfonate
H/D exchange	hydrogen/deuterium exchange
MDA	malondialdehyde
MPGES1	microsomal prostaglandin E synthase 1
MS	mass spectrometry
NSAID	non-steroidal anti-inflammatory drug
PG	prostaglandin
PPAR	peroxisome proliferator-activated receptor
SRM	single reaction monitoring
TBA	thiobarbituric acid

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Figure 1.

Pathway for the enzyme-catalyzed formation of PGE_2 and PGD_2 from PGH_2 . The formation of PGJ_2 , $\Delta^{12}\text{-PGJ}_2$, and $15d\text{-PGJ}_2$ from PGD_2 is thought to occur through a series of spontaneous reactions.

Figure 2.

Inhibition of the isomerase activity of native MPGES1 (panel A) and the C59A mutant (panel B) by 15d-PGJ₂ (black) 11-OH-15d-PGJ₂ (blue) and 9-GS-11-OH-15d-PGJ₂. The IC₅₀ values, 95% confidence range, and Hill coefficients are given in the parentheses for MPGES1; [15d-PGJ₂ (0.6 μM, 0.5 – 0.8 μM, 0.91)], [11-OH-15d-PGJ₂ (11 μM, 9 – 13 μM, 0.84) and 9-GS-11-OH-15d-PGJ₂ (1 mM)] and for C59A MPGES1; [15d-PGJ₂ (12 μM, 11 – 14 μM, 0.81)], [11-OH-15d-PGJ₂ (16 μM, 13 – 19 μM, 0.79) and 9-GS-11-OH-15d-PGJ₂ (1 mM)].

Figure 3.

(A) Assay of native and 15d-PGJ₂-modified MPGES1 and (B) the C59A mutant in the absence and presence of 100 μM 15d-PGJ₂, at three different concentrations of the substrate, PGH₂.

Figure 4.

Selected H/D exchange kinetic profiles of the native (black) and C59A mutant (blue) of MPGES1 in complex with GSH in the absence of 15d-PGJ₂ and the native (red) and the C59A mutant (green) enzyme in the presence of 15d-PGJ₂. Data for the GSH and GSO₃⁻ complexes of the native enzyme (except for peptide 49–59) are from reference 20 and are shown for comparative purposes. The amplitudes and rate constants for the fits of the data can be found in Supporting Information.

A

B

Figure 5.

(A) Stereoview of H/D exchange results mapped to the secondary structural elements in the native protein and (B) in the C59A mutant. Representation of the three-dimensional structure of MPGES1 was derived from PDB file 3DWW.²⁴ A single active site is composed of trans-membrane helices Ia and IIa from one subunit and helices IIb, IIIb, and IVb from the adjacent subunit, with GSH shown in stick representation. The blue and orange peptides indicate regions with decreased and increased rates of exchange, respectively, in the presence of 15d-PGJ₂.

Figure 6.

Selected H/D exchange kinetic profiles of the native (black) and C59A mutant (blue) of MPGES1 in complex with GSO_3^- in the absence of 15d-PGJ₂ as well as the native enzyme (red) and the C59A mutant (green) in the presence of 15d-PGJ₂. Data for the GSH and GSO_3^- complexes of the native enzyme (except for peptide 49–59) are from reference 20 and are shown for comparative purposes. The amplitudes and rate constants for the fits of the data can be found in Supporting Information.

Figure 7.

(A) Stereoview of H/D exchange results mapped to the secondary structural elements in the native protein and (B) in the C59A mutant in complex with GSO_3^- , where the position of GSH in the native protein is shown in stick representation. The three-dimensional structure of MPGES1 was derived from PDB file 3DWW.²⁴ A single active site is composed of trans-membrane helices Ia and IIa from one subunit and helices IIb, IIIb, and IVb from the adjacent subunit. The blue and orange peptides indicate regions with decrease and increased rates of exchange, respectively, in the presence of 15d-PGJ₂.

Scheme 1.