The Cyclopentenone Prostaglandin 15d-PGJ$_2$ Inhibits the NLRP1 and NLRP3 Inflammasomes

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Inflammasomes are cytosolic protein complexes that respond to diverse danger signals by activating caspase-1. The sensor components of the inflammasome, often proteins of the nucleotide-binding oligomerization domain–like receptor (NLR) family, detect stress, danger stimuli, and pathogen-associated molecular patterns. We report that the eicosanoid 15-deoxy-Δ12,14–PGJ2 (15d-PGJ2) and related cyclopentenone PGs inhibit caspase-1 activation by the NLR family leucine-rich repeat protein (NLRP)1 and NLRP3 inflammasomes. This inhibition was independent of the well-characterized role of 15d-PGJ2 as a peroxisome proliferator receptor-γ agonist, its activation of NF erythroid 2–related factor 2, or its anti-inflammatory function as an inhibitor of NF-κB. Instead, 15d-PGJ2 prevents the autoproteolytic activation of caspase-1 and the maturation of IL-1β through induction of a cellular state inhibitor to caspase-1 proteolytic function. The eicosanoid does not directly modify or inactivate the caspase-1 enzyme. Rather, inhibition is dependent on de novo protein synthesis. In a mouse peritonitis model of gout, using monosodium urate crystals to activate NLRP3, 15d-PGJ2 caused a significant inhibition of cell recruitment and associated IL-1β release. Furthermore, in a murine anthrax infection model, 15d-PGJ2 reversed anthrax lethal toxin-mediated NLRP1-dependent resistance. The findings reported in this study suggest a novel mechanism for the anti-inflammatory properties of the cyclopentenone PGs through inhibition of caspase-1 and the inflammasome.

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The PGs are lipid signaling molecules derived from arachidonic acid that have diverse functions. Production of PGs depends on the action of the cyclooxygenase (COX) enzymes of which there are two isoforms. COX-1 is expressed constitutively and predominately produces prostanoids with housekeeping functions. COX-2 is induced following inflammatory stimuli and is the dominant source of PGs during the inflammatory response. Most PGs exert their effects through activation of specific transmembrane G protein–coupled receptors. These receptors activate a varied set of intracellular signaling pathways that lead to diverse biological activities (3). Many PGs have demonstrated proinflammatory effects, but anti-inflammatory effects have been reported for cyclopentenone PGs. Cyclopentenone PGs contain a highly reactive α,β-unsaturated carbonyl that can form covalent attachments with thiol groups on proteins, thus altering their function (4). These PGs inhibit proinflammatory NF-κB activity through covalent modification of IKK as well as direct inhibition of NF-κB binding to DNA (5–7). They also upregulate and activate anti-inflammatory heat shock proteins (HSPs) such as HSP70 (8).

15-Deoxy-Δ12,14–PGJ2 (15d-PGJ2) is the best studied anti-inflammatory PG. It is formed as a dehydration product of PGD2 (9) and was the first identified endogenous ligand of the nuclear receptor peroxisome proliferator–activated receptor γ (PPARγ) (10, 11). 15d-PGJ2 also activates the transcription factor NF erythroid 2–related factor 2 (NRF2) through formation of adducts with the NRF2 inhibitor KEAP1 (12). Both PPARγ and NRF2 are known to have anti-inflammatory roles (13, 14). It is through these actions that this lipid mediator can alter expression of cytokine, chemokine, and proinflammatory genes (15). A number of studies have shown that 15d-PGJ2 has therapeutic potential in animal models of inflammatory disease (for review, see Ref. 15).

In this study, we report on a novel mechanism for the anti-inflammatory actions of 15d-PGJ2 and related cyclopentenone PGs. Our studies show that these PGs are potent inhibitors of the anthrax lethal toxin (LT) activation of the NLR family leucine-rich repeat protein (NLRP)1 inflammasome and nigericin-mediated...
activation of the NLRP3 inflammasome. These PGs inhibit inflammasome-mediated activation of caspase-1 and, as a consequence, prevent maturation and release of IL-1β, both in vitro and in vivo, in a manner independent of effects on NF-κB. Our findings confirm the role of these lipid mediators as anti-inflammatory agents and support their development as therapeutic agents for the treatment of inflammatory diseases.

Materials and Methods

Reagents

15d-PGJ2, Cay10410 (9,10-dihydro-15d-PGJ2), PGD2, PGE2, PGF2α, rosiglitazone, and T0070097 were purchased from Cayman Chemical (Ann Arbor, MI). PGα2, PGβ2, 4-cyclo pentene-1,3-dione, 2-cyclo pentenone, cycloheximide, actinomycin D, indomethacin, buthionine sulfoximine, and N-acetylcysteine and uric acid were from Sigma-Aldrich (St. Louis, MO). Cyclopentanone and cyclopentenone were obtained from Tokyo Chemical Industry (Portland, OR). Structures of these prostanooids are shown in Table I. Nigericin, lactacystin, and ultrapure LPS were purchased from Calbiochem (San Diego, CA).

Toxins

Protective Ag (PA), lethal factor (LF), and FP59 were purified from Bacillus anthracis as described previously (16, 17). LPf is a fusion protein of the PA binding domain of LF to the ADP-ribosylation domain of Pseudomonas aeruginosa exotoxin A (18, 19). LF-Fla, a toxin also delivered by PA, is a similar fusion of the first 254 aa of LF to full-length flagellin from Legionella pneumophila (gift of Dr. Russell Vance, University of California at Irvine, Berkeley, CA) (20). FlaTox is the combination of LF-Fla and PA. LT is the combination of LF and PA. Concentrations of LT correspond to the concentration of each toxin component (i.e. 1 μg/ml LT is 1 μg/ml LF plus 1 μg/ml PA). Concentrations of FlaTox correspond to the concentration of LF-Fla. Concentration of PA was always twice that of LF-Fla in FlaTox experiments (i.e., 1 μg/ml FlaTox is 2 μg/ml PA plus 1 μg/ml LF-Fla).

B. anthracis spores were prepared from the nonencapsulated, toxigenic B. anthracis Ames 35 (A35) strain (21) by growing the bacteria on sporulation agar at 37°C for 1 d followed by 5 d at 30°C, and inspection by microscopy to verify >95% sporulation. Spores were purified from plates by four rounds of centrifugation and sterile water washes, followed by two heat treatments at 70°C for 30 min (to kill any vegetative bacteria). Spore quantification was performed using a Petroff Hauser counting chamber (Hauser Scientific, Horsham, PA) and verified by dilution plating.

Cell culture

RAW264.7 cells and L929 mouse fibroblast cells were grown in DMEM supplemented with 10% FBS, 10 mM HEPES, and 50 μg/ml gentamicin (all purchased from Life Technologies, Grand Island, NY). Selected studies used lower amounts of FBS as indicated in the figure legends. Mouse bone marrow was cultured in complete DMEM (as above) supplemented with 30% L929 cell–conditioned supernatant and grown 7–10 d to allow time for differentiation to bone marrow–derived macrophages (BMDMs).

Animal studies

Mice were used as source of bone marrow. BALB/cJ (harbouring Nlrp1b<sup>−/−</sup> and LT-responsive macrophages), C57BL/6J (harbouring Nlrp1b<sup>−/−</sup>), and mice on the Nlrp1b<sup>−/−</sup> background (N10) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice deleted for NLRP3 have been previously described (22). C57BL/6N(Tac Nlrp1b<sup>−/−</sup>) congenic mice carrying the LT-responsive Nlrp1b<sup>−/−</sup> have been previously described (23). These mice are resistant to spore infection relative to their congenic C57BL/6N(Tac Nlrp1b<sup>−/−</sup>) counterparts due to LT-mediated activation of the NLRP1 inflammasome (23). COX-1 knockout mice on the C57BL/6NTac background were a gift from Katrin Mayer-Barber (National Institute of Allergy and Infectious Diseases, Bethesda, MD). These mice were backcrossed (four generations) in our laboratory to the C57BL/6NTac Nlrp1b<sup>−/−</sup> congenic mice carrying the LT-responsive Nlrp1b<sup>−/−</sup> allele.

For the mouse uric acid–induced peritonitis studies (used as a model for the arthritic disease gout) (24), C57BL/6J mice were injected i.p. with 125 μg 15d-PGJ2 or vehicle (10% DMSO in PBS) at 5 min prior and 4 h after administration of monosodium urate (MSU) crystals. MSU crystals were prepared by crystallization of uric acid as previously described (25) and injected into mice (4 mg/250 μl PBS, i.p.). At 6 h after MSU administration, mice were euthanized and peritoneal lavage was performed with PBS (6 ml/mouse). Infiltrating cells were counted following erythrocyte lysis using ACK buffer (Life Technologies). IL-1β measurements in lavage fluids were made using an ELISA kit from R&D Systems (Minneapolis, MN).

For sensitization of anthrax spore–resistant NLRP1b<sup>−/−</sup>expressing mice, 15d-PGJ2 or vehicle was administered to BALB/cJ, C57BL/6J, C57BL/6NTac Nlrp1b<sup>−/−</sup> or C57BL/6N(Tac Nlrp1b<sup>−/−</sup>) mice (100 μg, 200 μl, s.c., at 5 min and 1 h after spore infection). Animals were infected with 2 × 10<sup>7</sup> spores (200 μl, s.c.) and monitored for 7 d postinfection for signs of malaise or death.

All animal experiments were performed in strict accordance with guidelines from the National Institutes of Health and the Animal Welfare Act, approved by the Animal Care and Use Committee of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

Cytotoxicity assays

RAW264.7 cells and BALB/cJ (LT-responsive) BMDMs were grown in 96-well plates to 90% confluence and preincubated with various drugs or vehicle at a range of doses or times (as described in the figure legends). Cells were then incubated with LT or medium. Cell viability was assessed by MTT (Sigma-Aldrich) as previously described (26). In selected experiments, cell death was assessed by staining cells with 5 μM propidium iodide (Sigma-Aldrich) in medium without phenol red. Fluorescence was measured on a Wallac 1420 Victor 3V (PerkinElmer, Waltham, MA) plate reader with excitation at 530 nm and emission at 615 nm.

MEK, caspase-1, and IL-1β cleavage

RAW264.7 cells and BMMDMs were exposed to LPS (1 μg/ml) and then various drugs or vehicle (at doses and timing indicated in the figure legends) prior to addition of inflammasome activators (LT, FlaTox, or nigericin, at indicated doses). Cells were then lysed in RIPA buffer containing LF inhibitor PT-168541-1 (gift of Alan Johnson, Panthera Biopharma) and processed for Western blotting using primary Abs against MEK1 (444942, Calbiochem), IL-1β (AF-401-NA, R&D Systems), MEK3 (sc-959), and caspase-1 p10 (sc-514, Santa Cruz Biotechnology, Santa Cruz, CA) as previously described (26). Secondary Abs used in these studies were anti-goat infrared dye (800CW) (Rockland Immunochemicals, Gilbertsville, PA) and anti-rabbit infrared dye (800CW) (LI-COR Biosciences, Lincoln, NE). Immunoblots were visualized with the Odyssey infrared imaging system (LI-COR Biosciences).

In vitro caspase-1 assay

LPS (1 μg/ml for 2 h) was used to induce pro–IL-1β as a substrate for recombinant caspase-1. Approximately 10<sup>6</sup> cells were lysed per milliliter sucrose buffer (250 mM sucrose, 10 mM HEPES [pH 7.3]) by passage through a 29-gauge needle. Lysates of LPS-treated RAW264.7 cells were incubated with 1 U active recombinant mouse caspase-1 (MBL International, Woburn, MA) per 50 μl lysis in the presence or absence of 15d-PGJ2 or Boc-Asp(OBzI)-chloromethylketone (Anascap, San Jose, CA) for 3 h at 37°C. In other experiments cells were first pretreated with 15d-PGJ2 for 1 h prior to preparation of lysates. Caspase-1–mediated cleavage of IL-1β was analyzed by Western blotting. Because 15d-PGJ2 is an inhibitor of the NF-κB cascade (5–7), and thus could impact LPS-mediated upregulation of IL-1β, LPS priming was always performed prior to 15d-PGJ2 treatment in each experiment.

Evaluation of caspase-1 sequestration in a high m.w. complex

Sucrose buffer lysates of 15d-PGJ2–treated or heat-shocked (42°C) RAW264.7 cells were incubated with 1 U active recombinant mouse caspase-1 (MBL International, Woburn, MA) per 50 μl lysis in the presence or absence of 15d-PGJ2 or Boc-Asp(OBzI)-chloromethylketone (Anascap, San Jose, CA) for 3 h at 37°C. In other experiments cells were first pretreated with 15d-PGJ2 for 1 h prior to preparation of lysates. Caspase-1–mediated cleavage of IL-1β was analyzed by Western blotting. Because 15d-PGJ2 is an inhibitor of the NF-κB cascade (5–7), and thus could impact LPS-mediated upregulation of IL-1β, LPS priming was always performed prior to 15d-PGJ2 treatment in each experiment.

Evaluation of 15d-PGJ2 conjugation to NLRP3

C57BL/6J wild-type or NLRP3-deficient BMDMs were stimulated with LPS (1 μg/ml, 4 h) to induce upregulation of NLRP3. Cells were then incubated with 15d-PGJ2 or biotinylated 15d-PGJ2 (Cayman Chemical) (50 μM, 1 h). Sucrose buffer lysates were made in the presence of complete protease inhibitor mixture (Roche Diagnostics, Indianapolis, IN). Approximately 200 μg cell lysate was mixed with 5 μg anti-NLRP3 Ab (MAB7578, R&D Systems) and placed on a rotary shaker at 4°C for 2 h. Protein A/G agarose slurry (20 μl, Santa Cruz Biotechnology) was added and the incubation was continued overnight. Bound immune complexes were washed with PBS four times prior to elution of proteins in 2× SDS loading buffer. Biotinylated adducts were detected with IR680-conjugated streptavidin (LI-COR Biosciences) by Western blotting.
Results

Cyclopentenone PGs require an α,β-unsaturated carbonyl to prevent NLRP1-dependent pyroptosis

Anthrax LT contains a protease that cleaves the N terminus of rodent NLRP1 proteins, leading to caspase-1 activation (28–30). Activation of caspase-1 leads to a rapid form of cell death known as pyroptosis (for review, see Ref. 31). LT activation of the NLRP1 inflammasome in selected inbred rodent strains that harbor LT-responsive alleles leads to pyroptosis, whereas strains with resistant NLRP1 alleles can undergo apoptosis (32–35). Our earlier studies showed that heat shock inhibits the inflammasome through sequestration of procaspase-1 in a large complex (27), preventing LT-mediated pyroptosis. We tested a number of cyclopentenone PGs that induce HSPs such as HSP70 (36, 37) along with cyclopentanone PGs for the ability to inhibit the NLRP1 inflammasome-dependent pyroptosis of LT-sensitive macrophages. All the cyclopentenone PGs tested inhibited the well-characterized NLRP1-dependent LT-induced pyroptosis. Interestingly, most of the cyclopentanone PGs that were tested failed to inhibit (Fig. 1A, Table I). The exception was the cyclopentanone PG PGD2, which had low inhibitory activity (Fig. 1A, Table I); however, it has been shown that this PG undergoes nonenzymatic dehydration to the cyclopentenone PG PGJ2 in aqueous media (38), and many of the purported physiological effects of PGD2 are actually mediated by PGJ2 and its downstream dehydration products such as 15d-PGJ2 (39).

The restriction of inhibitory activity to only those PGs with cyclopentenone rings suggested a requirement for an α,β-unsaturated carbonyl. We further evaluated this requirement by examining the inhibitory activity of cyclopentene, cyclopentanone, 2-cyclopentenone, and various structural analogs (Table I). Cyclopentene and cyclopentanone, which have no α,β-unsaturated carbonyls, had no effect on LT-induced pyroptosis. However, 2-cyclopentenone, which has one carbonyl, did demonstrate inhibitory activity. Furthermore, 4-cyclopentene-1,3-dione, which has two α,β-unsaturated carbonyls, had a lower EC50 than did 2-cyclopentenone. Finally, the 15d-PGJ2 analog CAY10410, which is identical to 15d-PGJ2 except for the absence of the α,β-unsaturated bond in the ring, had no inhibitory activity.

15d-PGJ2 inhibited LT-induced pyroptosis of both RAW264.7 cells (Fig. 1A) and BMDMs (Fig. 1B) with an EC50 in the micromolar (20–40 μM) range. Reducing the amount of FBS to 1% or lower decreased the EC50 by up to 10-fold (Supplemental Fig. 1A). This finding is in agreement with studies showing that electrophilic cyclopentenone PGs such as 15d-PGJ2 are rapidly inactivated by FBS in cell culture medium (40).

15d-PGJ2 does not inhibit LT cleavage of MEKs

To determine whether 15d-PGJ2 protects against LT pyroptosis by inhibiting toxin translocation to the cytosol or enzymatic activity, we examined the cleavage of the cytosolic MEK substrates of LT. LT rapidly cleaves the N terminus of multiple MEK proteins, and this cleavage can be monitored by loss of an epitope (MEK1) and by altered mobility of the MEK3 protein (41, 42). We found that cleavage of the MEK proteins was uninhibited by treatment of the cells with 15d-PGJ2, thus confirming that, in the presence of the drug, active LT translocated to the cytosol (Fig. 1C). Thus 15d-PGJ2 does not impact the binding, endocytosis, translocation, or activity of LF.

15d-PGJ2 inhibits cytokine processing of multiple inflammasomes

Inflammasome activation causes rapid autoproteolysis of caspase-1, which leads to processing and secretion of the proinflammatory cytokine IL-1β (1). 15d-PGJ2 inhibits NF-κB phosphorylation by IkB as well as binding of NF-κB to DNA (5–7). NF-κB signaling is important for priming of the inflammasome. For all studies of inflammasome activation and cytokine processing, cells were primed with LPS before exposure to 15d-PGJ2 to avoid any NF-κB signaling inhibitory effects that could lead to differences in priming and IL-1β levels. Using this method, no differences in inflammasome priming, indicated by pro–IL-1β levels, were observed in PG-treated cells in any experiments (as demonstrated by all Western blots). 15d-PGJ2 inhibited caspase-1 autoproteolysis (monitored by generation of the p10 fragment) as well as maturation and secretion of IL-1β in LT-treated cells at a step subsequent to NF-κB signaling (Fig. 2A). Early studies have established that MEK signaling pathways that led to NF-κB activation (41, 42) are inactivated by LT, and, furthermore, inhibitors of NF-κB such as BAY11-7082, BAY11-7085, SN-50, and knockdown of p65 have previously been shown to have no effect on LT-mediated activation of NLRP1 (data not shown). Thus, the inhibitory effects of LT on caspase-1 were independent of effects on NF-κB. Interestingly, application of the drug up to 45 min after LT caused significant inhibition of cytokine processing, further confirming an
15d-PGJ2 is capable of inhibiting multiple inflammasomes with diverse activating stimuli.

**15d-PGJ2 induces a cellular state inhibitory to caspase-1**

Given the ability of this compound to inhibit multiple inflammasomes, we hypothesized that 15d-PGJ2 could inhibit caspase-1 directly. The α,β-unsaturated carbonyl makes cyclopentenone PGs highly electrophilic and capable of conjugation to cysteine residues on various proteins, altering their functions (4). Caspase-1, a cysteine protease, contains catalytically important thiol groups that could be modified. However, using recombinant active caspase in

### Table I. EC$_{50}$ values of structural variants of 15d-PGJ2

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC$_{50}$ ($\mu$M)</th>
<th>Structure</th>
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<tr>
<td>PGF2α</td>
<td>&gt;1000</td>
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<tr>
<td>Cyclopentenone</td>
<td>&gt;1000</td>
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<tr>
<td>Cyclopentanone</td>
<td>&gt;1000</td>
<td><img src="image" alt="structure" /></td>
</tr>
<tr>
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</tr>
<tr>
<td>4-cyclopenten-1,3-dione</td>
<td>~60</td>
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<tr>
<td>CAY10410</td>
<td>&gt;1000</td>
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$^a$RAW264.7 cells were treated 30 min with variable concentrations of compound. Cells were challenged with LT (1 μg/ml, 1.5 h) and cell viability was measured by MTT staining. At least two experiments represented by a single shown study were used to generate an EC$_{50}$ value for each compound’s ability to inhibit LT-induced pyroptosis.

FIGURE 2. 15d-PGJ2 inhibits caspase-1 autoproteolysis and cytokine maturation of multiple inflammasomes. BALB/cJ BMDMs were primed with LPS (1 μg/ml, 2 h). Cells were then exposed to 15d-PGJ2 (50 μM, 30 min) followed by (A) LT (1 μg/ml), (B) nigericin (50 μM), or (C) FlaTox (1 μg/ml) for indicated times. Western blotting of cell lysates and culture supernatants was performed with Abs against the p10 fragment of caspase-1 or IL-1β. The asterisk (*) indicates a cross-reactive protein band that acts as an internal equal loading control. Results shown in these Western blots are representative of between 4 and up to 16 similar experiments per treatment.
activity was induced by 15d-PGJ2 in intact cells. a cellular state or protein that could inhibit caspase-1 enzymatic activity was induced by 15d-PGJ2 in intact cells.

Inflammasome inhibition by 15d-PGJ2 is dependent on protein translation

We previously reported that both heat shock and arsenical compound inhibition of inflammasome activation did not require de novo protein translation (27, 43). In the case of heat shock, pro-caspase-1 is trapped in a high m.w. complex that can be centrifuged away from the cytosolic cellular fraction (27). We found that 15d-PGJ2, a potent inducer of heat shock proteins (8), did not induce sequestration of caspase-1 into a high m.w. complex (Supplemental Fig. 2).

In contrast to the response to both heat shock and arsenical compounds, the transcription inhibitor actinomycin D and the translation inhibitor cycloheximide reversed the protective effects of 15d-PGJ2 in a dose-dependent manner (Fig. 4A, 4B). This requirement for transcription further supported the fact that 15d-PGJ2 manifested its inhibitory effects on the inflammasome in a manner independent of the eicosanoid’s inhibitory effect on NF-κB transcription. Similar results were found upon an even more complete enzymatic inhibition of translation using FP59, a fusion protein of an ADP-ribosylating enzyme that targets elongation factor 2 (Fig. 4C). Furthermore, inhibition of translation partially restored cytokine processing in 15d-PGJ2–treated cells (Fig. 4D). This evidence indicated that inflammasome inhibition by 15d-PGJ2 required de novo protein translation, either to induce a cytoprotective protein or to maintain a certain threshold level of a caspase-1 inhibitory protein with normally rapid turnover (the levels of which diminish after inhibition of protein synthesis). Interestingly, inhibition of NLRP3-mediated cytokine processing was not affected by cycloheximide treatment (data not shown), suggesting that the lipid mediator could have multiple inhibitory mechanisms.

Inhibition is independent of PPARγ, NRF2, or COX-1

15d-PGJ2 is a ligand of the nuclear receptor PPARγ (10, 11), which is known to have anti-inflammatory roles (14). We hypothesized that the eicosanoid could mediate its effects through this receptor. However, the PPARγ agonist rosiglitazone was unable to inhibit LT-induced pyroptosis (Fig. 5A). Furthermore, despite loss of the α,β-unsaturated bond in the ring, the 15d-PGJ2 analog CAY10410 retains the ability to activate PPARγ (46). This drug does not protect cells from LT-induced pyroptosis (Table I). Similarly, these compounds were unable to prevent LT-induced (Fig. 5B) or nigericin-induced (data not shown) cytokine processing. Additionally, the PPARγ inhibitor T0070907 did not reverse the ability of 15d-PGJ2 to inhibit the inflammasome (Fig. 5C, 5D). Taken together, these data suggest that PPARγ signaling and transcriptional processes downstream of this receptor are not required for the effects of 15d-PGJ2 on the inflammasome.

15d-PGJ2 also activates the cap’n’collar family transcription factor NRF2 (12) by binding to cysteine residues of the NRF2 repressor KEAP1. NRF2 controls the expression of a large and diverse group of cytoprotective and anti-inflammatory genes (13), which we hypothesized could be inhibitory to inflammasome function. However, this seemed unlikely as NRF2 is stabilized and activated by proteasome inhibition (47–50), and treatment of macrophages with the proteasome inhibitor lactacystin does not prevent nigericin-based NLRP3-dependent cytokine processing (data not shown and Ref. 35). We tested the effects of 15d-PGJ2 in BMDMs lacking NRF2 and found that the drug was capable of inhibiting nigericin-induced cytokine processing, indicating that the effects of this eicosanoid on the inflammasome did not involve this transcription factor (Fig. 5E).

Recent studies have linked inflammasome activation with release of a variety of lipid mediators described as an “eicosanoid storm” (20). We hypothesized that modulation of this prostanoid release by exogenous 15d-PGJ2 could alter inflammasome function. However, 15d-PGJ2 inhibited LT-mediated pyroptosis and cytokine processing independent of COX-1, a central enzyme in the PG biosynthetic pathway (Fig. 6). Additionally, the cyclooxygenase inhibitor indomethacin did not reverse 15d-PGJ2–mediated protection from pyroptosis (Supplemental Fig. 3A).

Glutathione modulation does not alter inhibition

Cyclopentenone PGs form conjugates with cellular glutathione that can alter the physiological effectiveness of the PG (51). Increasing the levels of cellular glutathione using the glutathione precursor N-acetylcysteine or decreasing cellular levels of glutathione using the glutathione synthesis inhibitor buthionine sulfoximine had no effect on the protection of cells from LT-induced pyroptosis by 15d-PGJ2 (Supplemental Fig. 3B, 3C).

Cyclopentenone PGs are also potent inducers of intracellular reactive oxygen species (52), and oxidative stress is a potential mechanism of inflammasome regulation (53). However, the failure of N-acetylcysteine, a potent antioxidant, to alter 15d-PGJ2 protection suggests that induction of reactive oxygen species does not
play a role in the effects of 15d-PGJ2 on the inflammasome. Furthermore, alterations of oxidative state by 15d-PGJ2 are usually coupled with direct modification of proteins. We used a biotin-labeled 15d-PGJ2 analog coupled with immunoprecipitation and did not find any evidence that 15d-PGJ2 directly bound to NLRP3 (data not shown), suggesting that a novel inhibitory mechanism is operating that does not involve covalent adduct formation to NLRP3.

**FIGURE 4.** 15d-PGJ2-mediated inhibition of the NLRP1 inflammasome requires protein synthesis. (A) BALB/cJ BMDMs or (B) RAW264.7 cells were incubated with variable concentrations of cycloheximide or actinomycin D for 1 h. Cells were then exposed to 15d-PGJ2 (50 μM, 30 min) followed by LT (1 μg/ml, 1.5–2 h). (C) RAW264.7 cells were treated with 10 ng/ml PA with or without 100 ng/ml FPS9 for 2 h. PA receptor saturation in this cell type occurs at doses 20-fold higher than that used here (69). Thus, sufficient receptor remained on the cell surface to mediate LT intoxication. Unbound toxin was then washed from the cells, and the cells were incubated with 15d-PGJ2 and LT as above. Cell viability was measured by MTT staining. Each point was assayed in triplicate. Shown results are for a representative of two identical experiments. In (C), error bars represent SEM. (D) BALB/cJ BMDMs were treated with LPS (1 μg/ml, 3 h). Cells were then allowed to rest in LPS-free media for 1 h before cycloheximide treatment (2 μg/ml, 1 h). 15d-PGJ2 (50 μM, 30 min) followed by LT (1 μg/ml, 75 min) were then applied. Cell lysates were analyzed for IL-1β maturation by Western blot. The asterisk (*) indicates a cross-reactive protein band that acts as an internal equal loading control. Western blot results are representative of three similar experiments.

**FIGURE 5.** 15d-PGJ2 inflammasome inhibition is independent of PPARγ and NRF2. (A) RAW264.7 cells were incubated with variable concentrations of PPARγ agonist rosiglitazone for 1.5 h. Cells were exposed to LT (1 μg/ml, 2 h) and cell viability was assessed by MTT staining. (B) BALB/cJ BMDMs were primed with LPS (1 μg/ml, 2 h) followed by 15d-PGJ2, CAY10410, or rosiglitazone (50 μM, 30 min) and LT (1 μg/ml, 75 min). IL-1β in cell lysates and supernatants was assessed by Western blot. (C) RAW264.7 cells were treated with variable concentrations of PPARγ antagonist T0070907 for 1 h before treatment with 15d-PGJ2 (50 μM, 30 min). Following LT treatment (1 μg/ml, 2 h), cell viability was assessed by MTT staining. (D) RAW264.7 cells were primed with LPS (1 μg/ml, 2 h) followed by treatment with T0070907 (30 μM, 1 h). Cells were then incubated with 15d-PGJ2 (50 μM, 30 min) and then with nigericin (50 μM, 30 min). Cell lysates and culture supernatants were analyzed for IL-1β maturation by Western blot. In (A) and (C), each condition was assayed in duplicate. Curves are representative of at least two similar experiments. The asterisk (*) indicates a cross-reactive protein band that acts as an internal equal loading control.
15d-PGJ2 inhibits the inflammasomes in mice

To investigate the effects of 15d-PGJ2 in vivo, we used two models. The MSU-induced peritonitis model used to replicate the inflammation associated with gout requires NLRP1 activation and caspase-1-dependent release of IL-1β for cell recruitment (24). We used this model in mice treated with vehicle or 15d-PGJ2 to assess the relative cell numbers recruited to the peritoneum and inhibited by the PG treatment (Fig. 7A). Similarly, the IL-1 recruitment of cells to the peritoneum that was significantly inhibited by the PG treatment (Fig. 7A). Similarly, the IL-1

FIGURE 6. 15d-PGJ2 inflammasome inhibition is independent of COX-1. (A) BMDMs from Cox1−/− (on the C57BL/6NTac Nlrp1bS/S background) mice were primed with LPS (1 μg/ml, 2 h). Cells were then incubated with 15d-PGJ2 (50 μM, 30 min) followed by LT (1 μg/ml, 75 min). Cell lysates were analyzed for IL-1β–dependent control of IL-1β. (B) mature IL-1β. (C) BMDMs were exposed to varying concentrations of 15d-PGJ2 for 30 min. Cells were challenged with 1 μg/ml LT and viability was measured by MTT staining at 1.5 h after LT. Each condition was assayed in triplicate. Curves are representative of two or more similar experiments. The asterisk (*) indicates a cross-reactive protein band that acts as an internal equal loading control.

15d-PGJ2 inhibition of the inflammasomes required for the compound’s effects suggests that direct modification of caspase-1 or NLRP3. The finding that de novo protein translation but independent of the PPAR pathway are responsible for the anti-inflammatory effects of these PGs.

Discussion

In this study, we show that cyclopentenone PGs inhibit activation of caspase-1 and maturation of IL-1β by the NLRP1 and NLRP3 inflammasomes. This inhibition is shown to be independent of the drugs’ inhibitory effects on NF-κB signaling and transcription and not due to interference with inflammasome-activating stimuli or direct modification of caspase-1. Inflammasome inhibition occurred instead through induction of a cellular state inhibitory to caspase-1 enzymatic activity. This cellular state was dependent on de novo protein translation but independent of the PPARγ or NLRP1 signaling pathways often associated with the actions of these PGs. Our results suggest that a novel mechanism and transcription pathway are responsible for the anti-inflammatory effects of these PGs.

There is substantial evidence that the reactive α,β-unsaturated carbonyl group is necessary for many of the biological activities of cyclopentenone PGs (4). The unsaturated carbonyl imparts a reactive electrophilic character to carbon 6 of the molecule. This allows the compound to form adducts with cellular thiols such as glutathione or cysteine residues on proteins (4). The α,β-unsaturated carbonyl appears to be necessary for inflammasome inhibition, as PGs lacking the cyclopentenone ring were inactive. Similarly, 2-cyclopentenone, but not the related compounds cyclopentene and cyclopentanone, was able to inhibit the inflammasome. This requirement for a chemically reactive center suggests that the mechanism of inflammasome inhibition is through conjugation to exposed thiol residues on a key inflammasome regulatory component. However, we found no evidence of direct inactivation of caspase-1 or NLRP3. The finding that de novo protein synthesis is required for the compound’s effects suggests that direct modification of the inflammasome is not likely, but we cannot rule out the possibility of 15d-PGJ2–mediated modification of an unknown yet important inflammasome component. Our finding that inhibition of NLRP3 was not dependent on protein synthesis suggests that the mechanism of 15d-PGJ2 inhibition differs across inflammasomes. This may be due to the varied biochemical mechanisms of inflammasome activation. NLRP1, for example, is activated in rodent macrophages through direct proteolytic cleavage by LT (28–30). NLR family caspase-1 recruitment domain–containing protein 4, in contrast, acts as an adapter for a number of NAIP proteins that directly bind to flagellin and flagellin-like proteins (44, 45), and NLRP3 responds to a wide range of danger signals through an as yet unresolved mechanism (1).

PPARγ and NLRP2 are the primary transcriptional pathways activated by 15d-PGJ2 (15), and we hypothesized that one of them may play a role in the ability of this PG to inhibit the inflammasome. 15d-PGJ2 was the first identified endogenous ligand of PPARγ, and many of the biological effects of this PG result from PPARγ activation (10, 11). However, the compound also induces PPARγ-independent reactions. In fact, prior studies have demonstrated that 15d-PGJ2 has anti-inflammatory effects on macrophages deficient in PPARγ (54), and we found that inflammasome inhibition is also independent of PPARγ. A recent study demonstrated that NRF2-deficient BMDMs are impaired in inflammasome activation event required for IL-1β release.

zation to spore infection and a susceptibility comparable to mice harboring the Nlrp1bR/R allele (Fig. 7C, 7D). Because the effects of LT on susceptibility to spore are uniquely independent of NF-κB signaling, the 15d-PGJ2 effects in this model, unlike the MSU cell recruitment model, are likely to be on a downstream inflammasome activation event required for IL-1β release.
was used to assess impact of 15d-PGJ2 on the NLRP3 inflammasome. A well-established mouse monosodium urate induced peritonitis model was utilized. Control mice were injected with vehicle (10% DMSO in PBS) at 5 min prior and 4 h after administration of MSU crystals. Control mice were injected with vehicle (PBS, i.p.) and after 6 h infiltrating cells were counted.

**FIGURE 7.** 15d-PGJ2 inhibition of the inflammasome in mice. (A and B) A well-established mouse monosodium urate induced peritonitis model was used to assess impact of 15d-PGJ2 on the NLRP3 inflammasome in vivo. C57BL/6J mice (n = 9/group) were injected i.p. with 125 μg 15d-PGJ2 or vehicle (10% DMSO in PBS) at 5 min prior and 4 h after administration of MSU crystals. Control mice were injected with vehicle (n = 4) or drug (n = 7) alone. MSU crystals were injected into mice (4 mg/250 μl PBS, i.p.) and after 6 h infiltrating cells were counted. (A) and IL-1β levels assessed (B). The p values (unpaired t test) comparing the MSU (plus vehicle) groups to MSU (plus 15d-PGJ2) groups are <0.0001 in both panels. (C and D) Anthrax spore-resistant NLRP1bR indicating BALB/cJ mice or genetically susceptible C57BL/6J mice injected with B. anthracis spores. (D) Spore-sensitive NLRP1bR-expressing C57BL/6J (n = 2) and C57BL/6NTac Nlrp1bR;R (n = 4) mice infected with the same spore dose served as controls. (C and D) Anthrax spore-resistant NLRP1bR-expressing BALB/cJ mice or genetically susceptible C57BL/6J mice injected with B. anthracis spores. (D) The p value comparing spore-treated, spore-infected BALB/cJ mice to the 15d-PGJ2-treated infected BALB/cJ group or infected C57BL/6J mice is <0.0007. There is no significant difference between the infection susceptibility for drug-treated BALB/cJ mice and genetically susceptible C57BL/6J mice injected with B. anthracis spores. (D) The p value comparing spore-treated, spore-infected C57BL/6NTac Nlrp1bR;R mice to 15d-PGJ2–treated infected group is 0.0065. The p value comparing spore-infected BALB/cJ mice to the 15d-PGJ2–treated infected group is 0.007. There is no significant difference between the infection susceptibility for drug-treated BALB/cJ mice and genetically susceptible C57BL/6J mice injected with B. anthracis spores.
induced fever, spinal cord injury, chronic obstructive pulmonary disease, colitis, myocarditis, pancreatitis, and arthritis, has been reported (15). The mechanism of action of the prostanooid in these diverse inflammatory models may not be uniform. For example, troglitazone, a synthetic PPARγ ligand, was equally effective as 15d-PGJ2 at improving adjuvant-induced arthritis (67), whereas the protective effect of 15d-PGJ2 in a carrageenan-induced acute lung injury model was found to be NRF2-dependent (68). The inflammasome is known to play a role in a number of the diseases for which 15d-PGJ2 has shown therapeutic effects (1), suggesting that this compound may be acting through its ability to inhibit IL-1β processing via the inflammasome. In this study, in addition to establishing the inhibitory effects of 15d-PGJ2 in a NLRP3-dependent peritonitis model, we were also able to cause a striking reversal of NLRP1 and caspase-1–dependent resistance of mice to anthrax spore infection by treatment with a single dose of the PG. These findings demonstrate that the anti-inflammatory effects of 15d-PGJ2 in vivo may be partially mediated through its impact on caspase-1 and the inflammasome pathways.

In conclusion, we demonstrate that the cyclopentenone PGs inhibit the inflammasome. Most studies to date have explained the anti-inflammatory effects of 15d-PGJ2 in vivo may be partially mediated through its modulation of IL-1β release through inhibition of the inflammasome.

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Disclosures
The authors have no financial conflicts of interest.

References

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Supplemental Figure 1.  15d-PGJ2 is inactivated by FBS and inhibits the inflammasome at late timepoints. (A) Balb/cJ BMDMs were treated with variable concentrations of 15d-PGJ2 (30 min) in the presence of 10, 1, or 0 % FBS. Cells were challenged with LT (1 µg/mL, 2 h) and cell viability measured by MTT staining. (B) 15d-PGJ2 (50 µM) was applied to RAW264.7 cells at various timepoints before (-) or after (+) LT treatment. Cell viability was assessed at 2 h post-LT challenge by MTT staining. Each condition was assayed in duplicate in an experiment. Shown curves are representative of at least two or more experiments. In (B), error bars represent standard error of the mean. (C) RAW264.7 cells were primed with LPS (1 µg/mL, 2 h). 15d-PGJ2 (50 µM) was applied to cells at various timepoints before (-) or after (+) LT treatment. Cell lysates made at 1.5 h post-LT intoxication were analyzed for IL-1β maturation by Western blot.
Supplemental Figure 2. The mechanism of inflammasome inhibition by 15d-PGJ2 differs from that of heat shock. RAW264.7 cells were incubated with 15d-PGJ2 (50 µM) or heat shocked (42°C) for 1.5 h. Sucrose lysates were separated by centrifugation at 10,000 x g for 10 min at 4°C. Cytosolic (C) and membrane (M) fractions were analyzed for caspase-1 content by Western blot.
Supplemental Figure 3. Cyclooxygenase or glutathione manipulation has no effect on 15d-PGJ2-mediated protection from pyroptosis. RAW264.7 cells or Balb/cJ BMDMs were treated with variable concentrations of (A) indomethacin (4 h), (B) buthionine sulfoximine (2 h), or (C) N-acetylcysteine (2 h). 15d-PGJ2 (50 µM, 30 min) was added before LT (1 µg/mL, 2 h) challenge. Cell viability was analyzed by MTT staining, and cell death was analyzed by propidium iodide staining. Each condition was assayed in triplicate. Shown results are representative of two or more similar experiments.