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 inhibitory 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 production. 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 predominantly 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. 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. These PGs inhibit proinflammatory NF-κB activity through covalent modification of IKK as well as direct inhibition of NF-κB binding to DNA. They also upregulate and activate anti-inflammatory heat shock proteins (HSPs) such as HSP70.

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–activator 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 (NLRP1) 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 T0070907 were purchased from Cayman Chemical (Ann Arbor, MI). PGα1, PGα2, 4-cyclopentene-1,3-dione, 2-cyclopentenone, cycloheximide, actinomycin D, indomethacin, buthionine sulfoximine, and N-acetylcysteine and uric acid were from Sigma-Aldrich (St. Louis, MO). Cyclophilin and cyclophilin were obtained from Tokyo Chemical Industry (Portland, OR). Structures of these prostanoids 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 antracis as described previously (16, 17). LFm is a fusion protein of the PA binding domain of LF to the ADP-ribosylation domain of Pseudomonas aeruginosa exotoxin A (18, 19). LFn-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 Berkeley, Berkeley, CA) (20). FlaTox is the combination of LFn-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 LF is 1 µg/mL PA plus 1 µg/mL LF). Concentrations of FlaTox correspond to the concentration of LFn-Fla. Concentration of PA was always twice that of LFn-Fla in FlaTox experiments (i.e., 1 µg/mL Flax is 2 µg/mL PA plus 1 µg/mL Flax).

B. antracis spores were prepared from the nonencapsulated, toxigenic B. antracis Ames 35 (A35) strain (21) by growing the bacteria on sporulation agar at 37°C for 5 d at 50°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.

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 7–12 d 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

Succrose buffer lysates of 15d-PGJ2-treated or heat-shocked (42°C) RAW264.7 cells (as a positive control for caspase-1 sequestration) (27) were centrifuged at 10,000 × g for 10 min at 4°C. Western blotting for caspase-1 was performed on the supernatant and pellet.

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.

Cyclopentenone PGs inhibit the inflammasome

For sensitization of anthrax spore–resistant NLRP1b-expressing mice, 15d-PGJ2 or vehicle was administered to BALB/cJ, C57BL/6J, C57BL/ 6Nlac15d-PGJ2, or C57BL/6lac15d-PGJ2 mice (100 µg, 200 µl, s.c., at 5 min and 1 h after spore infection). Animals were infected with 2 × 107 A35 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 pretreated 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 BMDMs were exposed to LPS (1 µg/ml) and then various drugs or vehicle (at doses and times 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 (catalogue no. 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 101 cells were lysed per milliliter succrose 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 lysate in the presence or absence of 15d-PGJ2 or Boc-Asp(OBzI)-chloromethylketone (Anspec, 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.

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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 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 inhibited 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 IκB 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
effect independent of inhibition of NF-κB signaling. Inhibition of pyroptosis by 15d-PGJ2 required earlier application of the drug (Supplemental Fig. 1B, 1C). Importantly, the ability of 15d-PGJ2 to inhibit the inflammasome was not limited to NLRP1 or linked to pyroptosis. 15d-PGJ2 also inhibited cytokine processing and secretion in response to treatment with nigericin, an ionophore that activates the NLRP3 inflammasome (Fig. 2B). 15d-PGJ2 demonstrated only modest inhibition of the NLR family caspase-1 recruitment domain–containing protein 4/NLR family apoptosis inhibitory protein 5 inflammasome after stimulation with FlaTox (Fig. 2C). However, previous work has similarly found that FlaTox is more difficult to inhibit (43), possibly due to its ligand binding–based mechanism of activation (44, 45). These data indicate that 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

![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.](http://www.jimmunol.org/)

**Table I. EC₅₀ values of structural variants of 15d-PGJ₂**

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<th>Compound</th>
<th>EC₅₀ (µM)</th>
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*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 EC₅₀ value for each compound’s ability to inhibit LT-induced pyroptosis.
a cell lysate assay where 15d-PGJ2 was added to lysates, we found no evidence of direct caspase-1 inhibition (Fig. 3A). Interestingly, however, lysates made from cells pretreated with 15d-PGJ2 were inhibitory to recombinant caspase-1 (Fig. 3B). This indicated that 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 synthesis (27, 43). In the case of heat shock, procaspase-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-kB 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 1). 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 prostanooid 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

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**FIGURE 3.** 15d-PGJ2 does not inhibit caspase-1 enzymatic activity directly but induces a cellular state inhibitory to caspase-1. (A) RAW264.7 cells were primed with LPS (1 μg/ml, 2 h). Sucrose lysates were incubated with recombinant active caspase-1 (1 U/50 μl, 3 h, 37°C) in the presence or absence of 15d-PGJ2 at indicated concentrations or positive control caspase-1 inhibitor Boc-Asp(OBzl)-chloromethylketone (400 μM). (B) RAW264.7 cells were primed with LPS (1 μg/ml, 2 h) followed by treatment with 15d-PGJ2 (50 μM, 1 h). As above, sucrose lysates were incubated with recombinant active caspase-1. The asterisk (*) indicates a cross-reactive protein band that acts as an internal equal loading control. Western blot results are representative of at least three similar experiments.
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 FP59 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.
mice treated with 15d-PGJ2 (Fig. 7B). To test the effects of the associated levels of secreted IL-1β assessing the relative cell numbers recruited to the peritoneum and inhibited by the PG treatment (Fig. 7A). Similarly, the IL-1β 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 NRF2 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 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 NRF2 signaling and transcription 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 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).

**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 NRF2 signaling and transcription 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.

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type BMDMs, this difference is far less pronounced than previously reported. In striking contrast, treatment with 15d-PGJ2 potently inhibits inflammasome function in a manner independent of NRF2.

The inducible cyclooxygenase COX-2 is thought to have a dual role in inflammation, both establishing the initial inflammatory response and leading to its resolution at later times. COX-2 is rapidly induced during inflammation and contributes to acute inflammation through production of proinflammatory signaling molecules (56). However, COX-2 induction during late stages of inflammation leads to production of anti-inflammatory PGs such as 15d-PGJ2 (57). These PGs along with additional lipid mediators called lipoxins, resolvins, and protectins thus constitute important endogenous molecules in the resolution of inflammation (58). In vivo concentrations of 15d-PGJ2 are thought to be in the nanomolar range (59). Thus, one may question the physiological relevance of the micromolar concentrations necessary for inflammasome inhibition in vitro. However, it has been demonstrated that upon dilution into culture media containing FBS, most (97–99%) of the PG is inactivated and does not enter cells (40).

Thus, the levels actually available for the cells in vitro are likely at physiological concentrations. Additionally, it can be postulated that systemic measurements of free PG in vivo are an underestimate of true levels found within localized pockets of inflammation or in the cell vicinity, giving the high reactivity of these eicosanoids with serum and intracellular proteins (60).

Exogenous administration of 15d-PGJ2 also has efficacy as an anti-inflammatory treatment (15). In the present study we show that 15d-PGJ2 can inhibit MSU-mediated neutrophil recruitment in an NLRP3-dependent inflammatory peritonitis model. Owing to the reactivity and resulting instability of cyclopentenone PGs, most experimental studies rely on administration of relatively high doses of 15d-PGJ2. Thus, alternative delivery mechanisms for the PG may increase its therapeutic potential. Loading of polyglycolide nanocapsules with the PG increased serum availability and improved the anti-inflammatory activity of 15d-PGJ2 in a peritonitis model (61). Retroviral overexpression of the PG synthase responsible for 15d-PGJ2 production has also been successful in a murine air-pouch model of acute inflammation, as well as in bleomycin-induced lung injury and scleroderma models (62–64). Synthetic derivatives of 15d-PGJ2 with similar biological activities have also been reported (65, 66).

Administration as an anti-inflammatory treatment in animal models of inflammation, including ischemic brain injury, LPS-panels. (C and D) Anthrax spore–resistant NLRP1bR/R-expressing BALB/cJ (n = 5) and C57BL/6NTac Nlrp1bR/R (n = 5) mice were treated with 15d-PGJ2 (100 μg, 200 μl s.c., at 5 min and 1 h after spore infection) or with vehicle and infected with 2 × 10^7 A35 spores (C) or 4 × 10^7 A35 spores (D). Spore-sensitive NLRP1bR/R-expressing C57BL/6J (n = 5) and C57BL/6NTac Nlrp1bR/R (n = 4) mice infected with the same spore dose served as controls. For (C), the p value comparing vehicle-treated, spore-infected BALB/cJ mice to the 15d-PGJ2–treated infected BALB/cJ group or infected C57BL/6J mice is <0.007. There is no significant difference between the infection susceptibility for drug-treated BALB/cJ mice and genetically susceptible C57BL/6J mice infected with B. anthracis spores. For (D), the p value comparing vehicle-treated, spore-infected C57BL/6NTac Nlrp1bR/R to 15d-PGJ2–treated infected group is 0.0065. The p value comparing vehicle-treated, spore-infected C57BL/6NTac Nlrp1bR/R mice to similarly infected C57BL/6NTac Nlrp1bR/R mice is 0.00429. The log-rank test was used for assessment of all p values in (C) and (D).
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 NFR2-dependent (68). The inflammasome is known to play a role in a number of the diseases 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 NLRP1L and caspase-1–dependent resistance of mice to anthrax spore inoculation 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 by its effects on transcriptional pathways such as PPARγ, NFR2, and NF-κB. We suggest that a full explanation of the anti-inflammatory effects of 15d-PGJ2 must include its modulation of IL-1β release through inhibition of the inflammasome.

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Disclosures

The authors have no financial conflicts of interest.

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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.