THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 280, NO. 42, pp. 35562–35570, October 21, 2005

Cyclopentenone Isoprostanes Inhibit the Inflammatory Response in Macrophages*[S]

Received for publication, May 2, 2005, and in revised form, July 27, 2005 Published, JBC Papers in Press, August 12, 2005, DOI 10.1074/jbc.M504785200

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Although both inflammation and oxidative stress contribute to the pathogenesis of many disease states, the interaction between the two is poorly understood. Cyclopentenone isoprostanes (IsoPs), highly reactive structural isomers of the bioactive cyclopentenone prostaglandins PGA_2 and PGJ_2, are formed non-enzymatically as products of oxidative stress *in vivo*. We have, for the first time, examined the effects of synthetic 15-A_2- and 15-J_2-IsoPs, two groups of endogenous cyclopentenone IsoPs, on the inflammatory response in RAW264.7 and primary murine macrophages. Cyclopentenone IsoPs potently inhibited lipopolysaccharide-stimulated IκBα degradation and subsequent NF-κB nuclear translocation and transcriptional activity. Expression of inducible nitric-oxide synthase and cyclooxygenase-2 were also inhibited by cyclopentenone IsoPs as was nitrite and prostaglandin production ($IC_{50} \sim 360$ and 210 nM, respectively). 15-J_2-IsoPs potently activated peroxisome proliferator-activated receptor γ (PPARγ) nuclear receptors, whereas 15-A_2-IsoP did not, although the anti-inflammatory effects of both molecules were PPARγ-independent. Interestingly 15-A_2-IsoPs induced oxidative stress in RAW cells that was blocked by the antioxidant 4-hydroxy-TEMPO (TEMPOL) or the mitochondrial uncoupler carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone. TEMPOL also abrogated the inhibitory effect of 15-A_2-IsoPs on lipopolysaccharide-induced NF-κB activation, inducible nitric-oxide synthase expression, and nitrite production, suggesting that 15-A_2-IsoPs inhibit the NF-κB pathway at least partially via a redox-dependent mechanism. 15-J_2-IsoP, but not 15-A_2-IsoP, also potently induced RAW cell apoptosis again via a PPARγ-independent mechanism. These findings suggest that cyclopentenone IsoPs may serve as negative feedback regulators of inflammation and have important implications for defining the role of oxidative stress in the inflammatory response.

Oxidative stress has been implicated in the pathogenesis of a variety of disease states, including atherosclerosis, cancer, and neurodegeneration (1–4). Oxidation and inflammation go hand in hand as activated inflammatory cells such as macrophages produce reactive oxygen species (ROS)[2] via NADPH oxidase and other enzymes, causing substantial oxidant injury to surrounding tissue (5, 6). However, the interaction between oxidative stress and inflammation is poorly understood.

In macrophages, the inflammatory response is largely controlled through regulation of the transcription factor nuclear factor κ-B (NF-κB). NF-κB exists primarily in the cytosol as a p50/p65 heterodimer complexed with its inhibitor protein IκBα. Activation of the IκB kinase (IκK) complex by numerous stimuli, including exposure to the bacterial endotoxin lipopolysaccharide (LPS), leads to the phosphorylation of IκBα, causing its dissociation from the NF-κB heterodimer and subsequent degradation by the proteasome. Loss of IκBα reveals a nuclear localization sequence on the NF-κB heterodimer that allows its rapid translocation to the nucleus and facilitates transcription of proinflammatory proteins (7, 8). NF-κB is a redox-sensitive transcription factor that can be either activated or inhibited by oxidative stress depending on cell type and circumstance (9, 10).

Excessive production of ROS leads to oxidation of membrane polyunsaturated fatty acids, yielding a multitude of lipid peroxidation products. One such family of products is the isoprostanes (IsoPs), prostaglandin-like molecules produced by free radical-mediated peroxidation of arachidonic acid (11, 12). The measurement of IsoPs has emerged as arguably the most accurate and reliable index of oxidative stress *in vivo* (13). IsoP levels are elevated in many tissues exposed to chronic inflammation, including postmortem brain samples from patients with Alzheimer disease (14), atherosclerotic plaques from individuals with peripheral atherosclerosis (15), and synovial fluid from rheumatic joints (16). Furthermore injection of LPS into the mouse brain causes significant increases in IsoP levels, which can be abrogated by genetic deletion of the NF-κB p50 subunit or the NF-κB-responsive gene inducible nitric-oxide synthase (iNOS) (17, 18), demonstrating a link between NF-κB activation and IsoP formation.

Several classes of IsoPs containing distinct prostane ring configurations arise from a common endoperoxide radical intermediate following arachidonate oxidation (Fig. 1). Our group has previously described the formation of a class of IsoPs containing an electrophilic α,β-unsaturated cyclopentenone ring known as cyclopentenone or A_2/J_2 IsoPs (19). These compounds react rapidly with thiol-containing molecules such as GSH and cellular proteins by Michael addition. We have also shown definitively that these compounds are formed *in vivo* and that their

* This work was supported by National Institutes of Health Grants GM15431 and DK48831 and a medical student research grant from the Pharmaceutical Research and Manufacturers of America (PhRMA) Foundation (to E. S. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental data.

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[2] The abbreviations used are: ROS, reactive oxygen species; IsoP, isoprostane; LPS, lipopolysaccharide; NF-κB, nuclear factor κ-B; IκBα, inhibitor κ-B α; IκK, IκB kinase; COX-2, cyclooxygenase-2; iNOS, inducible nitric-oxide synthase; PPARγ, peroxisome proliferator-activated receptor γ; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; DAPI, 4′,6-diamidino-2-phenylindole; PG, prostaglandin; BAF, Boc-aspartyl (OMe)-fluoromethyl ketone; Boc, *t*-butoxycarbonyl; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; TNFα, tumor necrosis factor-α; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MOPS, 4-morpholinepropanesulfonic acid; HNE, 4-hydroxynonenal; ANOVA, analysis of variance.

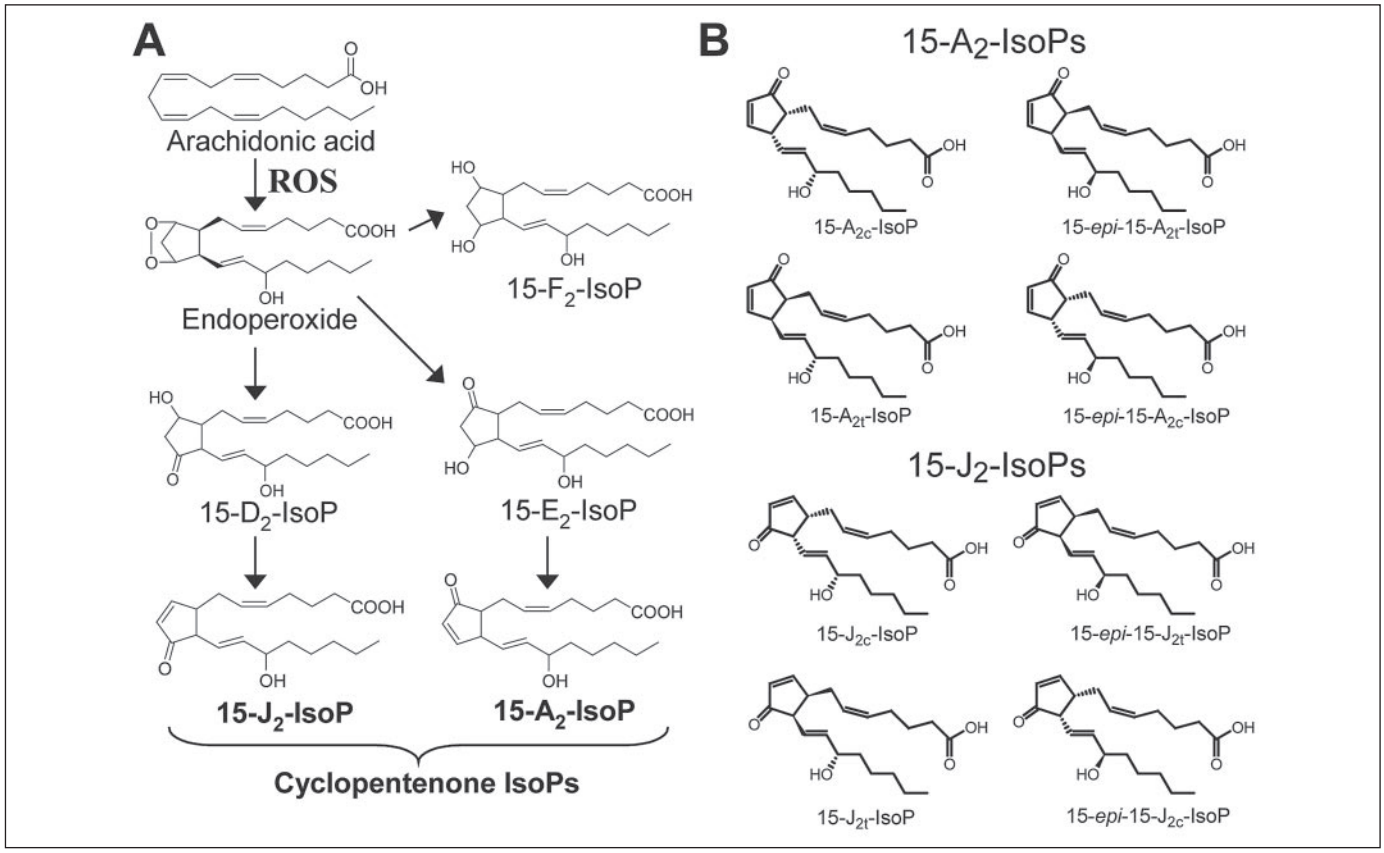

FIGURE 1. **Formation of cyclopentenone isoprostanes.** *A*, oxidation of arachidonic acid yields an unstable endoperoxide intermediate, which can be reduced to yield F_2-IsoPs or rearrange to form D- and E-ring IsoPs, which spontaneously dehydrate to form J- and A-ring (cyclopentenone) IsoPs. Only 15-series isomers are shown for simplicity, although three other regioisomers are formed, and stereochemistry is not indicated (13). *B*, structures of the four 15-A_2-IsoP and four 15-J_2-IsoP stereoisomers contained in the racemic IsoP mixtures used in these studies.

levels are elevated 22-fold under conditions of oxidative injury in rat liver (19). The biological effects of cyclopentenone IsoPs, however, are essentially unexplored.

Importantly A_2/J_2-IsoPs are isomeric to the cyclooxygenase-derived cyclopentenone prostaglandins (PGs), and two cyclopentenone IsoP isomers formed abundantly *in vivo*, 15-A_2-IsoP and 15-J_2-IsoP, are isomeric to PGA_2 and PGJ_2 (Fig. 1*A*), respectively, differing only in the *cis* orientation of their side chains with respect to the prostane ring, whereas PGs have *trans* configurations (20). Cyclopentenone PGs, along with the PGJ_2 metabolite 15-deoxy-$\Delta^{12,14}$-PGJ_2, have demonstrated a wide variety of potent biological actions, including anti-inflammatory, antiviral, and both cytotoxic and cytoprotective effects (21). The anti-inflammatory effects of cyclopentenone PGs are likely due to inhibition of NF-κB signaling through impairment of IκK activity (22, 23), perturbation of NF-κB DNA binding activity (24), activation of PPARγ nuclear receptors (25), or induction of the anti-inflammatory proteins heat shock protein 70 (26, 27) or heme oxygenase-1 (28). PGA_2 has also been shown previously to inhibit iNOS transcription and NF-κB activation in a PPAR-independent manner in LPS-stimulated microglia (29).

Given the abundant formation of IsoPs during inflammation and the potent immunomodulatory effects of the structurally similar cyclopentenone PGs, we sought to evaluate the biological effects of cyclopentenone IsoPs in a model of inflammation. Importantly the stereochemical differences between IsoPs and PGs often leads to divergent biological effects (30); thus the bioactivity of A_2-IsoPs cannot necessarily be inferred from that of PGA_2. The recent synthesis of both 15-A_2-IsoPs and 15-J_2-IsoPs (31, 32) allowed us to examined the effects of cyclopentenone IsoPs on the inflammatory response in LPS-stimulated RAW 264.7 murine macrophage cells and primary murine macrophages for the first time. The biological action of cyclopentenone IsoPs on macrophages represents a novel interaction between oxidative stress and inflammation, two processes central to many disease states.

EXPERIMENTAL PROCEDURES

Reagents—15-A_2-IsoPs and 15-J_2-IsoPs were obtained by total synthesis (31, 32) and were each applied as a racemic mixture of four stereoisomers (see Fig. 1*B*). Cyclopentenone IsoPs were stored in ethyl acetate at −80 °C until immediately before an experiment at which time they were dried under nitrogen and resuspended in ethanol. IsoPs were added to culture medium immediately before its addition to cells, and because 15-A_2-IsoPs rapidly react with albumin (19), serum-free medium was used. LPS (from *Salmonella* Minnesota Re 595), 4-hydroxy-TEMPO (TEMPOL), *N*-acetylcysteine, and carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) were from Sigma. The PPARγ antagonists GW9662 and T0070907 were from Cayman Chemical (Ann Arbor, MI). Boc-aspartyl (OMe)-fluoromethyl ketone (BAF) was from Enzyme Systems Products (Dublin, CA). The NF-κB inhibitor BAY 11-7082 was from Biomol Research Laboratories (Plymouth Meeting, PA). Primary antibodies to IκBα, iNOS, cyclooxygenase-2 (COX-2), p65, and actin were from Santa Cruz Biotechnology (Santa Cruz, CA), and Hsc70 and ERK1/2 antibodies were from Cell Signaling Technologies (Beverly, MA).

Cell Culture—RAW 267.4 murine macrophage cells were obtained from ATCC (Manassas, VA), and BV-2 murine microglial cells were a generous gift of Dr. Thomas Montine (University of Washington). Cells

were grown in DMEM containing 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, and 100 mg/ml streptomycin and plated on 12-well plates or 100-mm tissue culture dishes 24 h before experimentation. No cells older than passage 10 were used as RAW cells showed highly variable responses at higher passage numbers. PC3 prostate carcinoma and LT234 colon carcinoma cells were from ATCC.

Primary Bone Marrow Macrophages and Macrophage Cell Lines—Primary macrophages and macrophage cell lines were generated using bone marrow from mice expressing a luciferase construct driven by the NF-κB-dependent portion of the human immunodeficiency virus, type 1, long terminal repeat (33). Bone marrow cavities from femurs were flushed with phosphate-buffered saline (PBS) using a 27-gauge needle. The cells were then pelleted at 1000 rpm for 5 min, and red blood cells were lysed in 1 ml of NH_4Cl lysis solution (Sigma). After 5 min, 4 ml of PBS was added, and cells were pelleted as above and resuspended in DMEM supplemented with 10% fetal bovine serum. Macrophages were cultured in DMEM, 10% fetal bovine containing 10% L-medium for 4–5 days to obtain adherent, differentiated macrophages.

Cell Treatment—Cells were rinsed twice with serum-free DMEM before treatment, and all treatments were carried out in serum-free DMEM containing penicillin and streptomycin. Cells were preincubated with IsoPs for 30 min prior to stimulation with LPS, and both IsoPs and LPS were present throughout the stimulation period in all experiments. Subsequently medium was removed and immediately stored at −80 °C. For experiments using inhibitors or antioxidants, cells were preincubated for 30 min with inhibitor and then for 30 min with inhibitor plus IsoPs prior to LPS addition. Appropriate vehicle controls (ethanol, 0.5%) were carried out in all cases.

Measurement of Nitrite—Nitrite, a stable breakdown product of nitric oxide, was measured in cell medium using the Griess reaction. Briefly 100 μl of conditioned medium was mixed with 50 μl each of Griess reagent R1 and R2 (Cayman Chemical) in a 96-well plate, and absorbance was measured at 540 nm. Quantitation was achieved by comparison with sodium nitrite standards in DMEM.

Tumor Necrosis Factor-α (TNFα) Quantification—TNFα levels were quantified in conditioned cell medium using a Quantikine mouse TNFα enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) following the manufacturer's instructions.

MTT Assay—Cell viability was assessed by measuring MTT reduction (34). Following IsoP treatment, cells were rinsed and incubated in 0.5 mg/ml MTT reagent dissolved in DMEM for 2 h. Cells were then permeabilized by addition of acidified (0.1 N HCl) isopropanol, and absorbance was measured at 595 nm.

Western Blotting—Total protein extract was prepared as described previously (35). Equal protein concentrations were separated using Criterion Tris-HCl gels (Bio-Rad). Proteins were then transferred to polyvinylidene difluoride membranes (Amersham Biosciences) and blocked for 1 h at room temperature with Odyssey blocking buffer (LiCor Biosciences, Lincoln, NE). Membranes were then incubated overnight in primary antibodies diluted in blocking solution. Membranes were washed in PBS containing 0.1% Tween 20 and then incubated in Alexa 800-conjugated fluorescent secondary antibodies (Molecular Probes, Eugene, OR) for 1 h at room temperature. Protein bands were visualized using an Odyssey infrared imaging system and software (version 1.2, LiCor Biosciences). Densitometry was performed using Scion Image software (version 4.0.2, Scion Corp., Frederick, MD).

F_2-Isoprostane Assay—Total F_2-IsoPs were measured in combined medium and cell pellet as described previously (12). Briefly cells were scraped directly into their conditioned medium and then collected by centrifugation. The cell pellet was resuspended in 0.5 ml of methanol containing 0.005% butylated hydroxytoluene, sonicated, and then subjected to chemical saponification using 15% KOH to hydrolyze bound F_2-IsoPs. The conditioned medium was then added back to the cell lysate and adjusted to pH 3 followed by addition of 1 ng of $^4H_2$-labeled 15-F_{2t}-IsoP internal standard. Free F_2-IsoPs were then purified by C_{18} and silica Sep-Pak extraction and thin layer chromatography and analyzed as pentafluorobenzyl ester, trimethylsilyl ether derivatives via gas chromatography, negative ion chemical ionization mass spectrometry.

PPARγ Reporter Assay—PC3 cells were transiently transfected with 150 ng/ml of full-length PPARγ, 150 ng/ml of $(PPRE)_3$-TK-luciferase, and 2 ng/ml pRL-SV40 plasmids using FuGENE 6 at a lipid:DNA ratio of 4:1. Transfections were performed in RMPI 1640 cell medium supplemented with 10% charcoal-stripped fetal bovine serum. After 4 h, cells were treated either 0.1% vehicle (Me_2SO) or the indicated concentration of 15-J_2-IsoP and 15-A_2-IsoP. The PPARγ antagonist GW9662 (5 μM) was applied 2 h prior to IsoP exposure. After 24 h, cells were harvested in 1× luciferase lysis buffer. Relative light units from firefly luciferase activity were determined using a luminometer and normalized to the relative light units from *Renilla* luciferase using the Dual Luciferase kit (Promega).

In separate experiments, LT234 cells were transiently co-transfected with 0.3 μg of USA-thymidine kinase-luciferase reporter and 0.4 μg of PPARγ (ligand binding domain)-GAL4 chimera and then treated with 15-A_2-IsoPs for 16 h at which time luminescence was quantified as above.

Immunofluorescence Microscopy—For immunofluorescence staining, RAW cells were grown on glass coverslips. Following exposure to 15-A_2-IsoPs or vehicle, cultures were fixed in 10% formaldehyde for 20 min, rinsed with PBS, permeabilized with 0.1% Triton X-100, and blocked for 1 h with 8% bovine serum albumin diluted in PBS. Coverslips were then incubated overnight at 4 °C in rabbit anti-p65 (1:100) primary antibody in 1% bovine serum albumin. Cells were then washed in PBS for a total of 20 min and incubated in Cy-2-labeled secondary antibodies for 1 h. Cells were then washed again and stained with 1.4 μM 4′,6-diamidino-2-phenylindole (DAPI) for 10 min followed by further washes. Coverslips were mounted on microscope slides, and fluorescence was visualized with a Zeiss Axioplan microscope.

NF-κB Reporter Assay—NF-κB transcriptional activity was assessed using a reporter plasmid containing the human immunodeficiency virus long terminal repeat 36-base pair enhancer (containing a total of eight NF-κB binding sites) upstream of the herpes simplex virus minimal thymidine kinase promoter driving expression of a green fluorescent protein-luciferase fusion protein (fusion protein from Clontech). DNA was mixed with Superfect liposome reagent (Qiagen, Valencia, CA) in serum-free medium, incubated at room temperature for 15 min, supplemented with serum-containing medium, and then added to cells and incubated for 4 h. The DNA/liposome solution was removed, and the cells were washed once with PBS. Serum-containing medium was then added to the cells and placed at 37 °C overnight. Experiments were performed the next day. Primary macrophages were obtained from transgenic mice expressing this construct and were not transfected. Following treatment, cells were scraped at select time points in Lysis Buffer (Promega). The cell debris was pelleted by centrifugation at 13,000 rpm for 5 min, and 20 μl of the sample was used to perform a standard luciferase assay using a luminometer.

Northern Blotting—Total cellular RNA was isolated from cells by TRI reagent (Molecular Research Center, Cincinnati, OH) following the manufacturer's protocol. Five micrograms of total RNA was fractionated with a MOPS-formaldehyde-agarose gel and transferred to Hybond N1 membrane (Amersham Biosciences). Following UV cross-

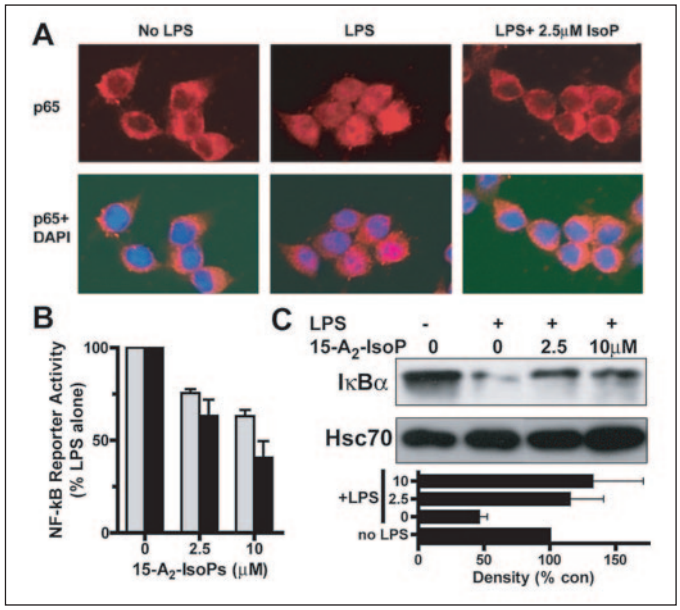

FIGURE 2. **15-A_2-IsoPs inhibits LPS-induced NF-κB activation.** *A*, RAW cells were pretreated with 15-A_2-IsoPs for 30 min, then stimulated with vehicle or 1 μg/ml LPS for 30 min, then fixed, and subjected to immunofluorescence microscopy following staining with NF-κB p65 antibody (*red*) and DAPI to visualize nuclei (*blue*). *B*, RAW cells were transiently transfected with an NF-κB luciferase reporter plasmid (*gray bars*), or primary macrophages were obtained from NF-κB reporter transgenic mice (*black bars*). Cells were pretreated with 15-A_2-IsoPs for 30 min and then stimulated with 1 μg/ml LPS for 4 h at which time luciferase production was assessed by measurement of luminescence. Data are presented as percent luciferase production of cells treated with LPS alone. *C*, RAW cells were pretreated with 15-A_2-IsoPs for 30 min, then stimulated for 30 min with 1 μg/ml LPS, and then harvested. IκBα levels were determined by Western blot. Hsc70 is shown as a loading control. Blots are representative of four independent experiments. Densitometry data represent the mean ± S.E. from those experiments. *con*, control.

linking, the blots were prehybridized for 30 min at 42 °C in Hybrisol I (Intergen Co., Purchase, NY), hybridized using ^{32}P-labeled cDNA in the same buffer at 42 °C, washed, and subjected to autoradiography. Briefly an 860-bp Northern blot probe for COX-2 (generously provided by Dr. Sanjoy Das, Vanderbilt University) was generated by restriction endonuclease digestion of an 1156-bp COX-2 cDNA fragment with EcoRI and PstI.

RESULTS

IκBα Degradation and NF-κB Translocation and Transcriptional Activity Are Inhibited by 15-A_2-IsoPs—To explore the effect of cyclopentenone IsoPs on the inflammatory response, we examined the effect of 15-A_2-IsoPs on NF-κB activation in LPS-stimulated RAW 264.7 macrophages. Following LPS exposure, translocation of NF-κB p50/p65 heterodimers to the nucleus is required for NF-κB-mediated transcription and can be inhibited by cyclopentenone PGs (36). As shown in Fig. 2*A*, treatment of RAW cells with LPS induced a marked translocation of NF-κB p65 subunit into the nucleus. Application of 15-A_2-IsoPs blocked this translocation. We quantified NF-κB-dependent transcriptional activity using RAW cells transiently transfected with an NF-κB reporter plasmid. LPS stimulation of these cells induced a 3-fold increase in luciferase production after 6 h that was inhibited dose-dependently by co-application of 15-A_2-IsoPs (Fig. 2*B*).

The effects of cyclopentenone IsoPs on NF-κB-dependent transcriptional activity were next examined in primary bone marrow-derived macrophages obtained from transgenic mice expressing an NF-κB-responsive luciferase reporter construct. 15-A_2-IsoPs inhibited NF-κB reporter activity dose-dependently in LPS-stimulated in these primary macrophages to a degree similar to that seen in RAW cells, demonstrating that these effects are not unique to RAW cells (Fig. 2*B*).

Phosphorylation and subsequent degradation of IκBα is required for NF-κB translocation to the nucleus and can be blocked by cyclopentenone PGs (22, 37). Thus, we examined by Western blot the degradation of IκBα in LPS-stimulated RAW cells treated with 15-A_2-IsoPs. Stimulation of RAW cells with LPS led to a marked degradation of IκBα within 30 min, whereas co-treatment with 15-A_2-IsoPs inhibited LPS-induced IκBα degradation in a dose-dependent manner (Fig. 2*C*). Densitometry revealed that treatment with 15-A_2-IsoPs actually stabilized IκBα to greater-than-control levels (Fig. 2*C*), suggesting that these molecules inhibit basal IκBα degradation. Thus, our results demonstrate that 15-A_2-IsoPs inhibit IκBα degradation and subsequent NF-κB translocation and transcriptional activity.

15-A_2-IsoPs Inhibit Nitrite Production and iNOS Induction in LPS-stimulated RAW 264.7 and Primary Macrophages—Activation of NF-κB drives transcription of a diverse array of NF-κB-responsive genes. In LPS-stimulated macrophages, iNOS and COX-2 are two proinflammatory gene products that are NF-κB-responsive (38, 39). To determine the downstream effects of NF-κB inhibition by 15-A_2-IsoPs, we assessed iNOS protein expression and activity (as determined by production of nitrite, a stable metabolite of nitric oxide) in LPS-stimulated RAW 264.7 macrophages. In unstimulated cells, neither iNOS protein nor nitrite was detectable (data not shown). Stimulation of RAW cells with LPS alone led to marked expression of iNOS protein, an effect that was blocked in a dose-dependent manner by 15-A_2-IsoPs (Fig. 3*A*). 15-A_2-IsoP treatment also caused a dose-dependent inhibition of LPS-induced nitrite accumulation in cell medium with an IC_{50} of ~360 nM (Fig. 3*B*). LPS-induced nitrite production was similarly inhibited by BAY 11-7082 (3 μM), a specific NF-κB inhibitor, suggesting that blockade of nitrite production is due to NF-κB inhibition (data not shown). 15-A_2-IsoPs also inhibited LPS-induced nitrite production and expression of the proinflammatory cytokine TNFα in primary bone marrow-derived macrophages (supplementary data).

To determine whether the effects of 15-A_2-IsoPs are shared by all A_2/J_2-IsoPs, we examined the effects of 15-J_2-IsoPs on LPS-stimulated RAW cells. 15-J_2-IsoPs inhibited nitrite production with potency similar to that of 15-A_2-IsoPs (Fig. 3*F*), suggesting that these two compounds share similar biology. We also observed that 4-hydroxynonenal (HNE), an abundant lipid peroxidation product also containing an α,β-unsaturated carbonyl capable of forming Michael adducts, inhibited nitrite production in LPS-stimulated RAW cells with slightly less potency then either 15-A_2- or 15-J_2-IsoPs (Fig. 3*F*). This observation is in keeping with previous reports that HNE can inhibit the NF-κB pathway (40, 41) and suggests that this is a common action of lipid peroxidation products containing reactive α,β-unsaturated carbonyls.

LPS-induced COX-2 Induction and Prostaglandin Production Are Blocked by 15-A_2-IsoPs—We next assessed the ability of 15-A_2-IsoPs to modulate COX-2 induction and PG production in LPS-stimulated RAW264.7 macrophages. LPS-induced COX-2 mRNA and protein levels were also decreased in cells treated with 15-A_2-IsoPs (Fig. 3, *C* and *D*). 15-A_2-IsoPs blocked production of PGD_2 in a dose-dependent manner with an IC_{50} of ~210 nM (Fig. 3*E*).

Metabolism by Glutathione Adduction Renders 15-A_2-IsoPs Biologically Inactive—Cyclopentenone IsoPs are rapidly metabolized in cells via enzymatic conjugation to GSH (42). We hypothesized that the bioactivity of 15-A_2-IsoPs is dependent on the reactivity of the cyclopentenone ring and should be abrogated by conjugation to GSH. To test this hypothesis, GSH-15-A_2-IsoP conjugates were prepared by incubation of 15-A_2-IsoPs with excess GSH and bovine liver glutathione transferase followed by purification by high pressure liquid chromatography (42). The GSH-15-A_2-IsoP conjugates, which can no longer adduct cellular

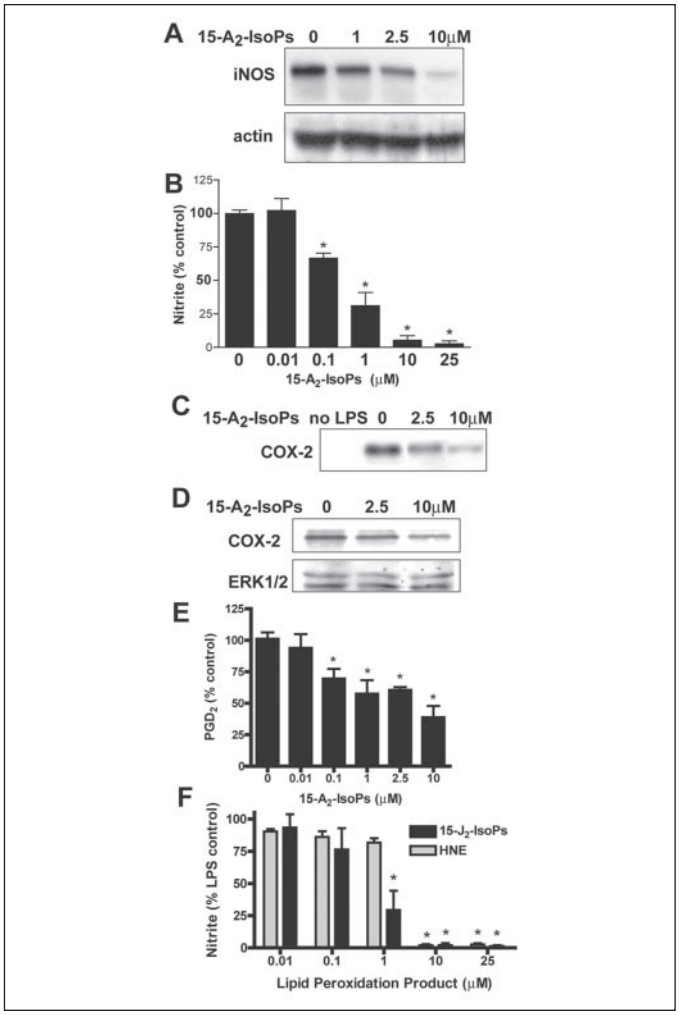

FIGURE 3. **15-A_2-IsoPs inhibit iNOS COX-2 expression in LPS-stimulated RAW 264.7 macrophages.** In all experiments, RAW 264.7 murine macrophages were pretreated with 15-A_2-IsoPs for 30 min and then stimulated with 100 ng/ml LPS in the presence of 15-A_2-IsoPs for 9 h (*A*, *B*, *D*, *E*, and *F*) or 5 h (*C*) at which time conditioned medium and cells were harvested. *A*, 15-A_2-IsoPs inhibit LPS-induced iNOS protein expression as assessed by Western blot. *B*, 15-A_2-IsoPs inhibit LPS-induced production of nitrite, a stable breakdown product of nitric oxide, as quantified in conditioned medium by Griess assay. No iNOS or nitrite was detectable in unstimulated cultures. 15-A_2-IsoPs inhibit LPS-induced production of COX-2 mRNA (*C*) as analyzed by Northern blot after 5 h, COX-2 protein by Western blot (*D*), and PGD_2 as quantified in conditioned medium by gas chromatography/mass spectrometry (*E*). *F*, cells were preincubated for 30 min with 15-J_2-IsoPs or HNE and then stimulated with 100 ng/ml LPS in the presence of IsoPs or HNE for 9 h at which time nitrite levels were assessed in the conditioned medium. *, $p < 0.05$ *versus* control by one-way ANOVA. *B*, *E*, and *F* represent mean ± S.E. of at least three separate experiments done at least in duplicate. Blots in are representative of at least three (*A* and *D*) or two (*C*) independent experiments.

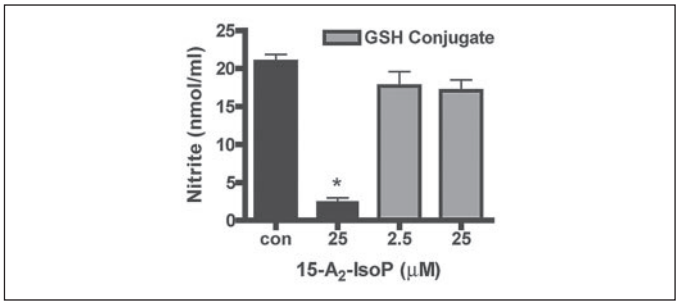

FIGURE 4. **Conjugation to GSH renders 15-A_2-IsoPs inactive.** RAW cells were pretreated with 15-A_2-IsoPs (25 μM) or 15-A_2-IsoP-GSH conjugates (2.5 and 25 μM) for 30 min and then stimulated with 100 ng/ml LPS for 9 h. Nitrite was measured in conditioned medium and expressed as percent control (vehicle-treated) cultures. *, $p < 0.05$ *versus* control by one-way ANOVA.

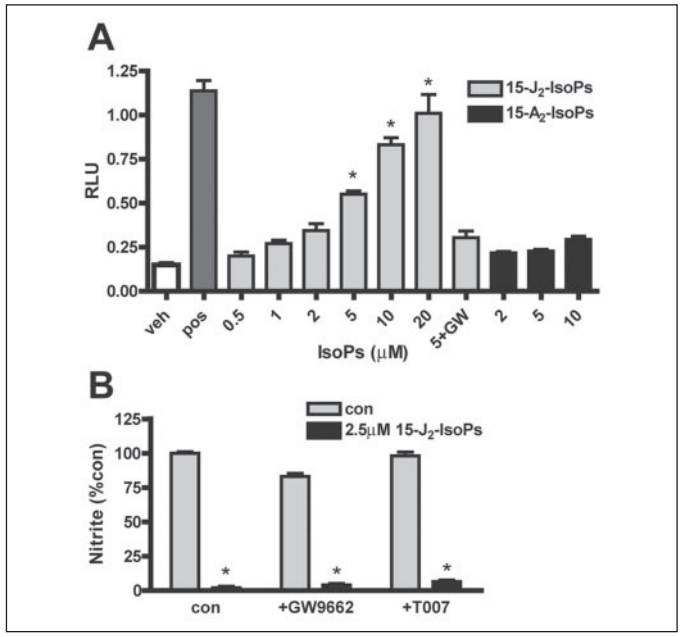

FIGURE 5. **15-J_2-IsoPs, but not 15-A_2-IsoPs, are potent PPARγ activators.** *A*, PC3 cells were transfected with both PPARγ receptor, peroxisome proliferator response element luciferase reporter, and *Renilla* luciferase plasmid constructs. Twenty-four hours later, cells were treated with increasing concentrations of 15-A_2- or 15-J_2-IsoPs or with 1 μM GW7845, a PPARγ agonist (*pos*), for 18 h after which cells were harvested and assayed for luminescence. Some cells were preincubated with GW9662 (5 μM), a PPARγ antagonist, for 2 h prior to exposure to 5 μM 15-J_2-IsoP (*5+GW*). Luminescence was normalized to *Renilla* luciferase luminescence, and data are expressed as relative light units (*RLU*). *B*, RAW cells were preincubated with Me_2SO (*con*), GW9662 (5 μM), or T0070907 (0.5 μM) for 1 h and then with 15-J_2-IsoPs (5 μM) for 30 min and then stimulated with LPS for 10 h. Nitrite was measured in conditioned medium and expressed as percent control (vehicle-treated) cultures. *, $p < 0.05$ *versus* control by one-way ANOVA. *veh*, vehicle; *con*, control.

thiols, failed to inhibit nitrite production in stimulated RAW cells even at concentrations as high as 25 μM (Fig. 4), suggesting that chemical reactivity is crucial to the bioactivity of 15-A_2-IsoPs. Furthermore 15-F_{2t}-IsoP, which lacks the reactive cyclopentenone ring but is otherwise structurally identical to 15-A_2-IsoP, failed to inhibit LPS-induced nitrite production at concentrations up to 25 μM (data not shown), again implicating the cyclopentenone moiety in the bioactivity of 15-A_2-IsoPs.

15-J_2-IsoPs, but Not 15-A_2-IsoPs, Activate PPARγ Nuclear Receptors—The cyclopentenone prostaglandin metabolite 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 is a ligand for the PPARγ nuclear receptor (43, 44), which is thought to mediate some of the anti-inflammatory effects of this molecule (45). To determine whether 15-A_2- and 15-J_2-IsoPs also act as PPARγ agonists, we examined the ability of these molecules to activate a PPARγ luciferase reporter system in PC3 cells. As shown in Fig. 5, 15-J_2-IsoPs potently induced luciferase production in this system with an EC_{50} of ~4 μM, and this activation of PPARγ could be blocked by the specific PPARγ antagonist GW9662 (5 μM). 15-A_2-IsoPs, however, did not significantly activate PPARγ at concentrations as high as 10 μM, whereas GW7845, a specific PPARγ agonist, caused marked induction of luciferase production (Fig. 5*A*). 15-A_2-IsoPs also failed to activate a PPARγ ligand binding domain reporter system at concentrations up to 10 μM (data not shown). Thus, 15-J_2-IsoPs, but not 15-A_2-IsoPs, are novel ligands for the PPARγ nuclear receptor and activate this receptor at concentrations consistent with their anti-inflammatory activity.

This finding suggested that the anti-inflammatory effects of 15-J_2-IsoPs might be PPARγ-dependent as ligation of this receptor has been shown to lead to NF-κB inhibition (45). However, we observed that preincubation of RAW cells with GW9662 (5 μM), which blocks 15-J_2-

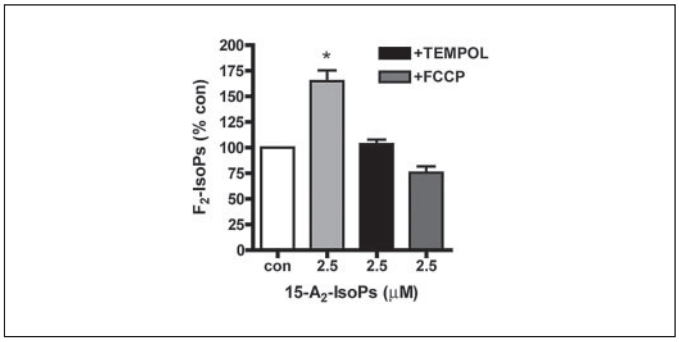

FIGURE 6. **15-A_2-IsoPs promote mitochondria-dependent oxidative stress in RAW cells.** Cells were preincubated with vehicle (0.1% Me_2SO), 1 mM TEMPOL, or 10 μM FCCP for 30 min and then treated with vehicle or 15-A_2-IsoPs for 5 h. TEMPOL and FCCP were present throughout the experiment. F_2-IsoPs were measured in cells and conditioned medium by gas chromatography/mass spectrometry. Data represent the mean ± S.E. from three independent experiments. *, $p < 0.05$ *versus* control by one-way ANOVA. *con*, control.

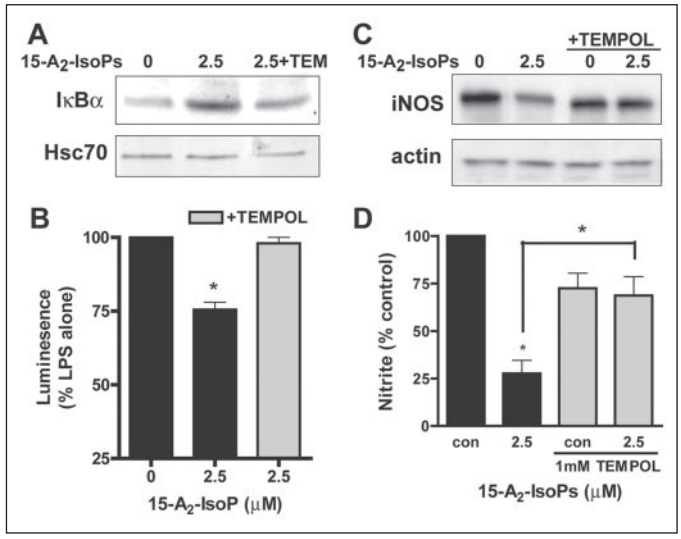

FIGURE 7. **TEMPOL blocks the anti-inflammatory effects of 15-A_2-IsoPs.** In *A–D*, RAW cells were incubated with TEMPOL (1 mM) or vehicle for 15 min, then 15-A_2-IsoPs was added for 90 min, and then cells were stimulated with LPS. *A*, co-application of TEMPOL blocks the ability of 15-A_2-IsoPs to impair LPS-induced IκBα degradation. Cells were harvested after 30 min of LPS stimulation, and IκBα levels were assessed by Western blot. *B*, TEMPOL prevents 15-A_2-IsoP-induced inhibition of NF-κB activation. RAW cells transfected with NF-κB reporter plasmid were harvested after 6 h of LPS stimulation and assayed for luminescence. Data show mean ± S.E. of triplicate determinations from a single experiment that was replicated three times independently with similar results. *C* and *D*, TEMPOL rescues LPS-induced iNOS and nitrite production in 15-A_2-IsoP-treated RAW cells. Cells were stimulated with 100 ng/ml LPS for 10 h and assayed for iNOS (*C*) or nitrite (*D*). Data in *D* represent mean ± S.E. of four independent experiments, each done in triplicate. *, $p < 0.05$ *versus* control by one-way ANOVA. *con*, control; *TEM*, TEMPOL.

IsoP-induced PPARγ activation, or with T0070907 (0.5 μM), a second PPARγ antagonist, had no effect on the ability of these compounds to inhibit LPS-induced nitrite production (Fig. 5*B*), demonstrating that like 15-A_2-IsoPs, 15-J_2-IsoPs inhibit the inflammatory response in a PPARγ-independent manner.

Oxidative Stress Is Increased in RAW Cells by 15-A_2-IsoP Treatment—Several studies have suggested that cyclopentenone PGs can act as inducers of intracellular oxidative stress and that some of the biological activities of these molecules depend on this initial free radical production (46, 47). Thus, we tested the ability of 15-A_2-IsoPs to induce oxidative stress by measuring levels of F_2-IsoPs, structural relatives of the A_2-IsoPs measured as an index of lipid peroxidation (12), in RAW cells treated with 15-A_2-IsoPs. Exposure to 2.5 μM 15-A_2-IsoP, a concentration that exerts potent anti-inflammatory effects, caused a 60% increase in F_2-IsoP levels in RAW cells within 5 h of treatment (Fig. 6), suggesting that 15-A_2-IsoPs induce intracellular ROS production and subsequent lipid peroxidation. The increase in F_2-IsoPs stimulated by 2.5 μM 15-A_2-IsoPs could be completely inhibited by co-treatment with the free radical scavenger TEMPOL (1 mM). To investigate the role of mitochondria, a major intracellular free radical source, in this effect, cells were co-treated with FCCP (10 μM), a compound that collapses the mitochondrial membrane potential and can inhibit mitochondrial ROS production (48). FCCP co-treatment completely eliminated the 15-A_2-IsoP-induced increase in F_2-IsoPs, suggesting that mitochondrial function is required for ROS production following 15-A_2-IsoP treatment (Fig. 6). These data demonstrate that 15-A_2-IsoPs are pro-oxidant molecules that induce ROS production in a mitochondria-dependent manner.

The Antioxidant TEMPOL Attenuates the Anti-inflammatory Effects of 15-A_2-IsoPs—NF-κB activation and transcriptional activity are regulated by oxidative stress in a variety of cell types (9, 49). Thus, we sought to elucidate the potential relationship between 15-A_2-IsoP-induced ROS production and NF-κB inhibition. We thus examined the effect of TEMPOL co-administration on 15-A_2-IsoP-induced impairment of IκBα degradation. Co-application of 1 mM TEMPOL partially rescued LPS-induced IκBα degradation in 15-A_2-IsoP-treated cells (Fig. 7*A*), suggesting that 15-A_2-IsoPs impair IκBα degradation in part via a ROS-dependent mechanism and that scavenging ROS with TEMPOL block this effect of 15-A_2-IsoPs. TEMPOL alone did not alter LPS-induced IκBα degradation (supplemental data). TEMPOL also prevented the inhibition of NF-κB transcriptional activity by 2.5 μM 15-A_2-IsoPs in LPS-stimulated RAW cells, returning LPS-induced NF-κB activation to near control levels (Fig. 7*B*). This result further supports the notion that 15-A_2-IsoP-induced ROS production is important in mediating the anti-inflammatory effects of the molecule. Accordingly TEMPOL co-treatment also prevented 15-A_2-IsoP-induced inhibition of LPS-stimulated iNOS production without increasing iNOS protein expression in cells treated with LPS but without IsoPs (Fig. 7*C*). Similarly we found that although treatment of LPS-stimulated RAW cells with 2.5 μM 15-A_2-IsoPs alone caused a significant reduction in nitrite production, co-treatment with 1 mM TEMPOL blocked this effect, rescuing nitrite production to levels similar to those measured in cells treated with LPS alone (Fig. 7*D*). TEMPOL co-treatment caused a slight decrease in nitrite production in LPS-stimulated cells in the absence of IsoPs but rescued nitrite production in 15-A_2-IsoP-treated cells, showing that this compound does not simply potentiate nitrite production. These results suggest that 15-A_2-IsoPs can inhibit IκBα degradation and subsequent NF-κB activation at least partially via a redox-dependent mechanism. Furthermore we found that the anti-inflammatory effects of 15-A_2-IsoPs could also be partially abrogated by co-application of *N*-acetylcysteine (1 mM, data not shown), a thiol antioxidant, demonstrating that this effect is not unique to TEMPOL.

Although antioxidants could mitigate the anti-inflammatory effects of 15-A_2-IsoPs, this effect was not complete. Furthermore antioxidants were ineffective in preventing the anti-inflammatory effects of higher concentrations (>10 μM) of 15-A_2-IsoPs (data not shown), suggesting that 15-A_2-IsoPs can inhibit LPS-induced inflammation via two mechanisms, one of which is ROS-dependent and one of which is likely due to direct covalent modification of proteins.

15-J_2-IsoPs, but Not 15-A_2-IsoPs, Induce RAW Cell Apoptosis—The induction of macrophage apoptosis is a crucial anti-inflammatory mechanism and could underlie the observed effects of cyclopentenone IsoPs. However, we observed that 15-A_2-IsoPs did not induce RAW cells death at concentrations as high as 25 μM (Fig. 8*A*), demonstrating that their effects are independent of cell toxicity. Conversely 15-J_2-IsoPs were potent inducers of RAW cell death, killing these cells with an LD_{50} of ~3.5 μM (Fig. 8*A*). 15-J_2-IsoPs were equally toxic in the presence or

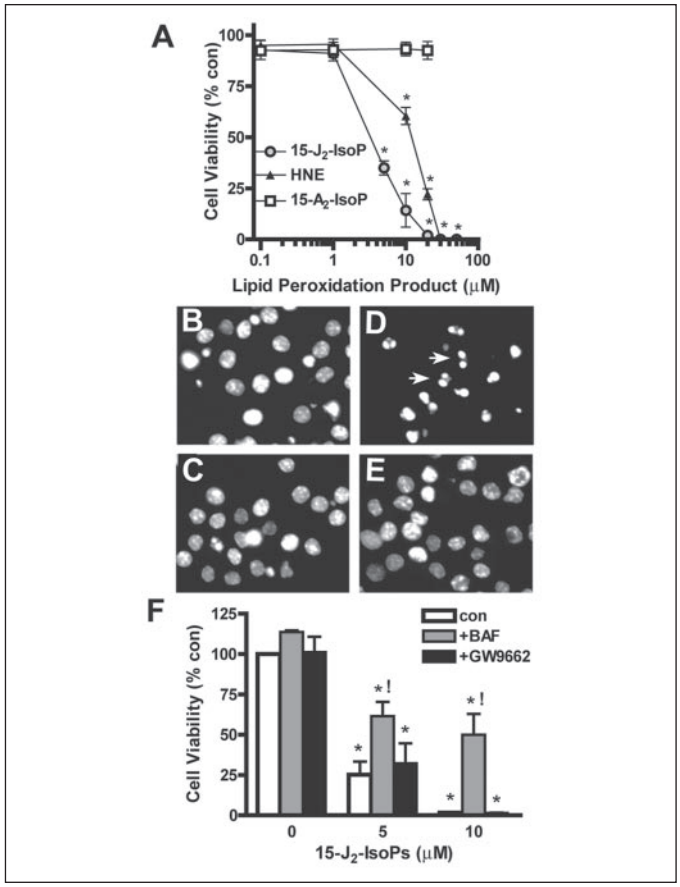

FIGURE 8. **15-J_2-IsoPs induce RAW cell apoptosis via a PPARγ-independent mechanism.** *A*, RAW cells were treated with various lipid peroxidation products for 24 h, and cell viability was assessed by MTT assay. *B–E*, RAW cells were treated with vehicle (*B*), 10 μM 15-A_2-IsoPs (*C*), 5 μM 15-J_2-IsoPs (*D*), or 5 μM 15-J2-IsoPs + the caspase inhibitor BAF (50 μM, *E*) for 24 h, then fixed, and subjected to DAPI nuclear staining and fluorescence microscopy. *Arrowheads* in *D* denote asymmetric chromatin formations indicative of apoptosis. *F*, cells were treated with 5 μM 15-J_2-IsoPs alone or with BAF (50 μM) or the PPARγ antagonist GW9662 (5 μM) for 24 h followed by MTT assay. Cells were preincubated with BAF or GW9662 for 1 h prior to IsoP exposure. *, $p < 0.05$ *versus* control; !, $p < 0.05$ *versus* cells treated with equivalent IsoP alone, both by one-way ANOVA. *con*, control.

absence of LPS (data not shown). As shown in Fig. 8*A*, 15-J_2-IsoPs were also considerably more potent inducers of RAW cell death than HNE ($LD_{50} \sim 14$ μM). DAPI nuclear staining of RAW cells treated for 24 h with 5 μM 15-J_2-IsoPs demonstrated pronounced nuclear condensation and abundant asymmetric chromatin formations, morphological indications of apoptotic cell death (Fig. 8*D*). Nuclei of cells treated with 10 μM 15-A_2-IsoPs, however, appeared similar to control nuclei (Fig. 8, *B* and *C*). Furthermore co-application of the broad spectrum caspase inhibitor BAF (50 μM) provided significant, but not complete, protection from 15-J_2-IsoP-induced RAW cell death (Fig. 8*G*) and prevented nuclear fragmentation (Fig. 8*E*), suggesting that this form of cell death is at least in part apoptotic.

As 15-J_2-IsoPs, but not 15-A_2-IsoPs, both activate PPARγ and induce macrophage apoptosis, we investigated the possibility that 15-J_2-IsoP-induced RAW cell apoptosis is mediated by PPARγ. Pre- and co-incubation of RAW cells with the PPARγ antagonist GW9662 (5 μM) failed to protect cells from death induced by 5 μM 15-J_2-IsoPs (Fig. 8*F*), suggesting that 15-J_2-IsoP toxicity in RAW cells is PPARγ-independent.

Taken together, these results demonstrate that 15-J_2-IsoPs, but not 15-A_2-IsoPs, are potent inducers of RAW cell death via a mechanism that has both caspase-dependent and -independent components. These experiments also suggest that the mechanisms of nitrite inhibition and cell death induction are independent as both 15-A_2- and 15-J_2-IsoPs inhibit nitrite production in RAW cells with similar potency, whereas only 15-J_2-IsoPs induce RAW cell apoptosis, and that both processes are PPARγ-independent.

DISCUSSION

We have provided evidence that cyclopentenone IsoPs, reactive lipid peroxidation products formed *in vivo*, have potent immunomodulatory effects. When administered to LPS-stimulated macrophages, 15-A_2-IsoPs inhibit NF-κB activation via impairment of IκBα degradation. 15-A_2-IsoPs attenuate LPS-induced expression of the NF-κB-responsive proteins TNFα, iNOS, and COX-2 and block nitric oxide and prostaglandin production in a dose-dependent manner at submicromolar concentrations. 15-J_2-IsoPs exert similar effects, suggesting that all cyclopentenone IsoPs might be anti-inflammatory. 15-J_2-IsoPs are also potent ligands for the PPARγ nuclear receptor, whereas 15-A_2-IsoPs are not, although the anti-inflammatory effects of both compounds are PPARγ-independent. 15-A_2-IsoPs promote lipid peroxidation in RAW cells, and the ability of these IsoPs to inhibit NF-κB transcription and iNOS production appears to have a ROS-dependent component as the antioxidant TEMPOL ameliorates these effects to some extent. Furthermore 15-J_2-IsoPs, but not 15-A_2-IsoPs, induce RAW cell apoptosis again by a PPARγ-independent mechanism. These findings show that cyclopentenone products of the IsoP pathway can modulate the inflammatory response in macrophages by several mechanisms.

The transcription factor NF-κB plays a crucial role in the inflammatory response (8) as NF-κB activation can lead to transcription of proinflammatory proteins including iNOS and COX-2 (38, 39). The modulation of NF-κB activity by cyclopentenone PGs has been described thoroughly (21). Several groups have shown that IκK activity is inhibited by cyclopentenone PGs presumably through direct adduction of vulnerable cysteine residues in IκK (22, 36). Our data show that 15-A_2-IsoPs inhibit IκBα degradation likely through inhibition of some upstream signaling event. It should be noted that although 15-A_2-IsoPs completely suppress iNOS expression and nitrite production, they totally inhibit NF-κB reporter activity or COX-2 expression, suggesting that these molecules likely also effect pathways other than NF-κB that contribute to iNOS induction. As IsoPs are produced under different circumstances and often exert much different biological effects than their corresponding PGs (13), these findings have important implications for understanding the biology of lipid oxidation.

Previous work using cyclopentenone PGs (such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2) has suggested that these molecules act via direct covalent adduction to cysteine residues in IκKβ, particularly Cys-179 (22). However, modification of specific cysteine residues in IκKβ, either through direct oxidation (10), nitrosylation (50), or covalent adduction (22), renders the complex inactive. A recent study shows that 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 can induce thiol oxidation of numerous proteins through induction of intracellular ROS production rather than through direct adduction (51). 15-A_2-IsoPs induce ROS production in RAW cells, an effect that can be abrogated by either TEMPOL application or uncoupling of the mitochondrial electron transport chain with FCCP. In support of our findings, several studies have demonstrated increased radical production in cells treated with cyclopentenone PGs (46, 47). 15-A_2-IsoPs are thus byproducts of oxidant injury that in turn promote further oxidative damage by stimulating ROS production, potentially setting in motion a feed-forward oxidation cycle that could rapidly alter the intracellular redox status of macrophages and thereby effect cellular function. Others have suggested that a similar cyclopentenone PG-induced shift in redox state underlies some of the biological actions of

these molecules (51–53). TEMPOL or *N*-acetylcysteine scavenge 15-A_2-IsoP-induced ROS, potentially preventing cysteine oxidation and preserving IκK function, thereby facilitating IκBα degradation (see Fig. 7*A*, *N*-acetylcysteine data not shown). However, future experiments are required to directly examine IκK cysteine oxidation following IsoP exposure. Our data demonstrate a novel indirect oxidative mechanism of NF-κB inhibition that could potentially work in concert with direct covalent adduction to impair NF-κB activation. Indeed cyclopentenone IsoPs can still partially inhibit the inflammatory response in the presence of TEMPOL, particularly at higher concentrations, suggesting that indirect oxidation and covalent adduction mechanisms both contribute to the effect of these compounds.

As products of the IsoP pathway, A_2- and J_2-IsoPs are expected to be formed in equal proportion during oxidative stress and are thus simultaneously present in oxidatively damaged tissue (19). We have shown that like 15-A_2-IsoPs, 15-J_2-IsoPs, cyclopentenone IsoPs that are isomeric to PGJ_2, also inhibit nitrite production in LPS-stimulated RAW cells with similar potency, suggesting that anti-inflammatory properties are shared by all cyclopentenone IsoP isomers. Interestingly 15-J_2-IsoPs also potently induce RAW cell apoptosis, whereas 15-A_2-IsoPs do not. This finding suggests that cyclopentenone IsoP formation could inhibit macrophage function not only via NF-κB inhibition but also through induction of apoptosis, a well described mechanism of inflammatory regulation (54). These results also show that J_2-IsoPs can exert effects distinct from those of A_2-IsoPs and likely modulate unique intracellular targets. Furthermore we have shown that cyclopentenone IsoPs are more biologically potent that HNE, a compound widely considered to be the major toxic product of lipid peroxidation.

Although many of the biological effects of cyclopentenone PGs (particularly J-ring PGs) were initially attributed to the ability of these compounds to activate PPARγ nuclear receptors (43, 44), attention has more recently focused on the PPARγ-independent actions of these molecules, which are thought to be mediated by thiol modification (24, 55, 56). We have demonstrated that 15-J_2-IsoPs are novel and potent activators of PPARγ, whereas 15-A_2-IsoPs are not. However, our observations that the anti-inflammatory effects of 15-J_2-IsoPs cannot be blocked by PPARγ antagonists and that 15-A_2-IsoP bioactivity can be abrogated by conjugation to GSH suggest that the PPARγ-independent effects of these compounds are of primary importance. Nevertheless the finding that 15-J_2-IsoPs can activate this receptor may have important implications for the role of lipid peroxidation in many other cellular processes.

In our studies, cyclopentenone IsoPs exert effects at high nanomolar and low micromolar concentrations. We have detected levels of esterified cyclopentenone IsoPs of 122 ng/g of tissue in CCl_4-treated rat liver (19) and 179 ng/g in oxidized rat brain,[3] which roughly convert to ~364 and ~534 nM, respectively. These measurements are likely significant underestimates of total cyclopentenone levels as they do not account for non-esterified pools of IsoPs or for cyclopentenone molecules derived from eicosapentaenoic acid or docosahexaenoic acid (57). We believe that, taking these considerations into account, the concentrations used in this study have physiological relevance particularly under conditions of severe oxidant injury.

Reactive oxygen species can promote inflammation by increasing NF-κB activation (58, 59), and their generation may be required for normal LPS signaling through Toll-like receptor 4 (60, 61). However, our results and the results of others (40, 41) suggest that products of ROS-mediated lipid peroxidation, such as cyclopentenone IsoPs, can also inhibit the inflammatory response. High levels of oxidative stress have also been shown to impair NF-κB activation by several mechanisms. Oxidation of key cysteine residues in the NF-κB DNA binding site can impair NF-κB transcriptional activity (62). Further upstream, excessive ROS production can inhibit IκBα degradation via impairment of IκK activity (10). Several studies have demonstrated that treatment of cells with the oxidant H_2O_2 can inhibit LPS- or TNFα-induced IκK activity, IκBα degradation, and cytokine production (63–65), suggesting that oxidative stress may exert complex or biphasic effects on the inflammatory response. As inflammation often leads to local oxidative damage (17), these findings would suggest that lipid peroxidation and subsequent formation of cyclopentenone IsoPs could serve as a brake to prevent excessive tissue damage due to inflammation. A similar role has been proposed for cyclopentenone PGs in the enzymatic modulation of inflammation (66). Thus, the degree of oxidant injury and the products generated during an oxidative insult are likely important factors in dictating the ultimate effect of oxidative stress on the inflammatory response. Furthermore as NF-κB, PPARγ, COX-2, and iNOS play important roles not only in inflammation but also in the pathogenesis of many disease states, the biological action of cyclopentenone IsoPs has broad implications for our understanding of the role of oxidative stress in human pathophysiology.

Acknowledgments—We thank Alessio Porta for work on synthesis of 15-A_2-IsoPs, Dr. BethAnn McLaughlin for technical expertise, and Zheyong Yu and Dr. Alan Brash for providing the PC3 cells and the PPARγ reporter construct.

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J. Biol. Chem. 2005, 280:35562-35570.
doi: 10.1074/jbc.M504785200 originally published online August 12, 2005
