

Protein Thiol Modification by 15-deoxy- $\Delta^{12,14}$ -Prostaglandin J₂ Addition in Mesangial Cells: Role in the Inhibition of Pro-inflammatory Genes

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ABSTRACT

The cyclopentenone prostaglandin and PPAR γ agonist 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂) displays anti-inflammatory effects in several experimental models. Direct modification of protein thiols is arising as an important mechanism of cyclopentenone prostaglandin action. However, little is known about the extent or specificity of this process. Mesangial cells (MC) play a key role in glomerulonephritis. In this work, we have studied the selectivity of protein modification by 15d-PGJ₂ in MC, and the correlation with the modulation of several pro-inflammatory genes. MC incubation with biotinylated 15d-PGJ₂ results in the labeling of a distinct set of proteins as evidenced by two-dimensional electrophoresis. 15d-PGJ₂ binds to nuclear and cytosolic targets as detected by fluorescence microscopy and subcellular fractionation. The pattern of biotinylated 15d-PGJ₂-modified polypeptides is readily distinguishable from that of total protein staining or labeling with biotinylated iodoacetamide. 15d-PGJ₂ addition requires the double bond in

the cyclopentane ring. 9,10-Dihydro-15d-PGJ₂, a 15d-PGJ₂ analog that shows the same potency as peroxisome proliferator-activated receptor (PPAR) agonist in MC but lacks the cyclopentenone moiety, displays reduced ability to modify proteins and to block 15d-PGJ₂ binding. Micromolar concentrations of 15d-PGJ₂ inhibit cytokine-elicited levels of inducible nitric-oxide synthase, cyclooxygenase-2, and intercellular adhesion molecule-1 in MC. In contrast, 9,10-dihydro-15d-PGJ₂ does not reproduce this inhibition. 15d-PGJ₂ effect is not blocked by the PPAR γ antagonist 2-chloro-5-nitro-*N*-phenylbenzamide (GW9662). Moreover, compounds possessing an α,β -unsaturated carbonyl group, like 2-cyclopenten-1-one and 2-cyclohexen-1-one, reduce pro-inflammatory gene expression. These observations indicate that covalent modification of cellular thiols by 15d-PGJ₂ is a selective process that plays an important role in the inhibition of MC responses to pro-inflammatory stimuli.

Cyclopentenone prostaglandins (cyPG) are endogenous prostanoids that arise from the dehydration of their parent PG. Thus, dehydration of PGE₂ gives rise to PGA₂, whereas cyPG of the J series, like PGJ₂ and 15-deoxy- $\Delta^{12,14}$ -PGJ₂ (15d-PGJ₂), arise from the dehydration of PGD₂. The generation of 15d-PGJ₂ has been reported to increase under situations associated with COX-2 induction, such as inflamma-

tory processes (Gilroy et al., 1999). 15d-PGJ₂ has been the subject of considerable study because of its identification as a ligand of the transcription factors known as peroxisome proliferator-activated receptors (PPAR), which are involved in the control of lipid metabolism and immune response (Forman et al., 1995). During the course of these studies it has been realized that 15d-PGJ₂ may modulate multiple cellular functions by mechanisms dependent and independent of PPAR.

15d-PGJ₂ displays anti-inflammatory and protective effects against several types of injury both in cellular systems and in animal models (Rovin et al., 2001; Cuzzocrea et al., 2002; Ianaro et al., 2003b; Zingarelli et al., 2003). 15d-PGJ₂

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ABBREVIATIONS: cyclopentenone, 2-cyclopenten-1-one; cyPG, cyclopentenone prostaglandin; PG, prostaglandin; 15d-PGJ₂, 15-deoxy- $\Delta^{12,14}$ -PGJ₂; PPAR, peroxisome proliferator-activated receptor; iNOS, inducible nitric-oxide synthase; MC, mesangial cell; NF- κ B, nuclear factor κ B; AP-1, activator protein 1; COX-2, cyclooxygenase-2; ICAM-1, intercellular adhesion molecule-1; T0070907, 2-chloro-5-nitro-*N*-4-pyridinylbenzamide; TNF, tumor necrosis factor; HRP, Horseradish peroxidase; ECL, enhanced chemiluminescence; IL, interleukin; cyclohexenone, 2-cyclohexen-1-one; DMSO, dimethyl sulfoxide; GW9662, 2-chloro-5-nitro-*N*-phenylbenzamide; DAPI, 4,6-diamidino-2-phenylindole; MALDI-TOF, matrix-assisted laser desorption ionization/time of flight; r.t., room temperature.

has been reported to attenuate the development of acute and chronic inflammation (Cuzzocrea et al., 2002) and to ameliorate the symptoms of septic shock (Zingarelli et al., 2003). In addition, micromolar concentrations of cyPG have been found to inhibit the expression of several pro-inflammatory genes including monocyte chemoattractant protein-1, matrix metalloproteinase-9, or inducible nitric-oxide synthase (iNOS) (Ricote et al., 1998; Reilly et al., 2001; Rovin et al., 2001). Although the pathophysiological importance of these findings is not clear at present, they can be of pharmacological relevance, because the elucidation of the mechanisms involved in the protective effects of cyPG could aid in the identification of potential targets for development of anti-inflammatory strategies. CyPG may modulate multiple cellular processes, including PPAR activation, generation of reactive oxidative species, induction of a heat shock response, and expression of proteins involved in cellular defense mechanisms, such as heme oxygenase (Straus and Glass, 2001). In addition, cyPG can directly modify cellular proteins. CyPG possess an α,β -unsaturated carbonyl group in the cyclopentane ring that can form covalent adducts with free thiols in glutathione or in proteins by Michael addition. This may result in the alteration of cellular redox status and/or in the modulation of protein function. Several proteins have been identified which can be covalently modified by 15d-PGJ₂. Some of these proteins are involved in the modulation of inflammation, including several components of the NF- κ B (Castrillo et al., 2000; Rossi et al., 2000; Straus et al., 2000; Cernuda-Morollón et al., 2001) and AP-1 activation pathways (Pérez-Sala et al., 2003), and proteins involved in the regulation of transcription factor activity by redox changes or electrophiles (Moos et al., 2003; Shibata et al., 2003; Itoh et al., 2004; Levonen et al., 2004). Therefore, the mechanisms operating in a given inflammatory situation may be multiple and depend on several factors like the structure and concentration of the cyPG, the cell type, and the nature of the inflammatory stimuli.

MC play an important role in glomerulonephritis because they are a source for inflammatory mediators, and key players in the production and turnover of extracellular matrix and in the interaction with leukocytes (Mené, 1996). MC express iNOS and cyclooxygenase-2 (COX-2) in response to pro-inflammatory agents, which are responsible for an increased generation of NO and prostaglandins (Rzymkiewicz et al., 1994; Saura et al., 1995). In their activated state, MC can also release cytokines that contribute to glomerular injury and express a variety of chemokines, integrins, and adhesion molecules, like intercellular adhesion molecule-1 (ICAM-1), which play a pivotal role in leukocyte infiltration (Satriano et al., 1997). For these reasons, MC constitutes a relevant cellular model of inflammation. In this study, we illustrate the covalent modification of MC proteins by 15d-PGJ₂ and explore the contribution of this mechanism to the modulation of cytokine-elicited changes in the expression levels of the pro-inflammatory genes iNOS, COX-2, and ICAM-1.

Materials and Methods

Materials. 15-Deoxy- $\Delta^{12,14}$ -prostaglandin J₂ was from Calbiochem-Novabiochem (San Diego, CA) or from Cayman Chemical (Ann Arbor, MI). 9,10-Dihydro-15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (9,10-dihydro-15d-PGJ₂), PGE₂, and T0070907 were from Cayman Chemi-

cal. Rosiglitazone was from Alexis Biochemicals. Recombinant human IL-1 β (5×10^7 U/mg) was from Roche Diagnostics S. L. (Barcelona, Spain). Recombinant human TNF- α was from Serotec (Oxford, UK). Polyclonal anti-iNOS and anti-c-Jun were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-COX-2 antibody was from Oxford Biomedical Research and anti-ICAM-1 was from R&D Systems (Minneapolis, MN). Horseradish peroxidase (HRP) conjugated anti-rabbit immunoglobulins were from DakoCytomation (Glostrup, Denmark). HRP-conjugated streptavidin and enhanced chemiluminescence (ECL) reagents were from Amersham Biosciences (Barcelona, Spain). Cell culture media and supplements were from Invitrogen S.A. (Barcelona, Spain). GelCode Blue Coomassie staining was from Pierce (Rockford, IL). All other reagents used were of the highest purity available from Sigma Chemical Co. (St. Louis, MO).

Cell Culture and Treatments. Rat mesangial cells were obtained as reported earlier (Saura et al., 1995). Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. For experiments, passages 7 to 18 were used. Confluent MC were incubated in serum-free medium for 24 h before experiments. For cytokine stimulation, cells were treated with a combination of 3 ng/ml IL-1 β plus 37 ng/ml TNF- α in serum-free medium without phenol red. PPAR agonists, 2-cyclopenten-1-one (cyclopentenone) and 2-cyclohexen-1-one (cyclohexenone) were dissolved in DMSO and added to cultures 2 h before cytokine stimulation. GW9662 and T0070907 were added in DMSO 30 min before PPAR agonists. Final DMSO concentration was 0.1% (v/v). Cells not treated with agonists received an equivalent volume of DMSO. iNOS activity was estimated from the accumulation of nitrite in the cell medium using the Griess method (Saura et al., 1995). Levels of the protein of interest were assessed by Western blot as described previously (Cernuda-Morollón et al., 2002). The levels of cellular actin were used as a control for intersample variability. None of the compounds used elicited pro-inflammatory gene expression in the absence of cytokines.

Plasmids and Transient Transfections. The PPAR reporter construct p4xAc-Luc, described in (He et al., 1999), was the generous gift of Drs. B. Vogelstein and K. W. Kinzler. To assess PPAR activity, pre-confluent rat mesangial cells were incubated for 3 h in transfection mixture containing 1 μ g of 4xAc-Luc or empty vector (pBV-Luc) and 5 ng of pSG5-Renilla in Opti-MEM medium, in the presence of LipofectAMINE 2000 Reagent (Invitrogen). After a 4-h recovery period in serum-free medium, cells were treated with the indicated agents. The activities of firefly and *Renilla reniformis* luciferases present in cell lysates were measured using a dual luciferase reporter assay system from Promega (Madison, WI). All assays were done in duplicate and results are expressed as the ratio between firefly and *R. reniformis* luciferase activities.

Fluorescence Microscopy. 15d-PGJ₂ biotinylated at the carboxyl group was generously provided by Dr. F. J. Cañada. To visualize the subcellular distribution of 15d-PGJ₂ binding sites, cells were grown on glass coverslips. Subconfluent MC were incubated for 15 min in the presence of 10 μ M biotinylated 15d-PGJ₂ or vehicle (DMSO) in serum-free medium. After incubation, coverslips were washed several times with PBS and cells were fixed by a 15-min incubation with 3.5% formaldehyde and permeabilized by incubation with 0.05% Triton X-100 for 10 min. Coverslips were subsequently washed with PBS, incubated for 20 min with 1% (w/v) bovine serum albumin in PBS, and with 1 μ g/ml Alexa488-streptavidin (Molecular Probes Inc., Eugene, OR) for 30 min. To visualize cell nuclei, coverslips were incubated with 0.2 μ g/ml DAPI (Molecular Probes) for 20 min. After extensive washing, coverslips were allowed to dry and mounted with Fluorsafe (Calbiochem-Novabiochem). Fluorescence was observed with a Zeiss microscope connected to a charge-coupled device camera.

Incorporation of Biotinylated 15d-PGJ₂ into MC Proteins. MC were incubated with biotinylated 15d-PGJ₂ for 2 h in serum-free medium. Total cells lysates were obtained by disrupting cells in 50

mM Tris, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 0.1 mM β -mercaptoethanol, 0.5% SDS containing 2 μ g/ml of each of the protease inhibitors: leupeptin, pepstatin A, and aprotinin. Nuclear and cytosolic extracts were obtained as described previously (Cernuda-Morolón et al., 2001). Protein concentration was determined by the BCA protein assay from Pierce (Rockford, IL). Fifteen microgram of protein from each experimental condition were electrophoresed on 12.5% polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Bedford, MA). For two dimensional electrophoresis, cells were lysed in 20 mM HEPES, pH 7.2, 50 mM NaCl, 1% Nonidet P-40, 0.3% sodium deoxycholate, and 0.1% SDS plus protease inhibitors. Aliquots of cell lysates containing 100 μ g of protein were precipitated with 10% TCA, resuspended in 130 μ l of IEF sample buffer (4% Triton X-100, 2 M thiourea, 7 M urea, 100 mM dithiothreitol, and 2% Bio-lyte ampholytes) and loaded on Ready-Strip IPG Strips (pH 3–10; Bio-Rad) for isoelectric focusing on a Protean IEF cell (Bio-Rad), following the instructions of the manufacturer. For the second dimension, strips were equilibrated and loaded on 15% polyacrylamide SDS gels. Incorporation of biotinylated 15d-PGJ₂ into MC proteins was assessed by Western blot and detection with HRP-conjugated streptavidin and ECL, as described previously (Oliva et al., 2003; Pérez-Sala et al., 2003).

Binding of 15d-PGJ₂ to Proteins in Vitro. The ability of biotinylated 15d-PGJ₂ to form covalent adducts with proteins was explored in vitro by using total cell lysates or recombinant human c-Jun DNA binding domain as a model peptide by a Western blot assay, essentially as described previously by us (Pérez-Sala et al., 2003). The linearity of the detection of incorporated biotin was ensured by using a biotinylated BSA standard (Pierce). The formation of 15d-PGJ₂- or 9,10-dihydro-15d-PGJ₂-c-Jun adducts was assessed by MALDI-TOF mass spectrometry analysis as described previously (Pérez-Sala et al., 2003). In brief, peptides were purified by ZipTip C18 (Millipore, Bedford, MA). The laser desorption/ionization experiments were performed on a BIFLEX III time-of-flight instrument (Bruker-Franzen Analytik, Bremen, Germany) operated in the positive mode. A saturated solution of sinapinic acid in acetonitrile/water (1:2) with 0.1% trifluoroacetic acid was used as the matrix. Equal volumes (0.5 μ l) of the sample solution and the matrix were spotted on the target and air-dried. External calibration was performed, using the protein calibration standard II (Bruker Daltonics, Bremen, Germany), and samples were analyzed in the linear mode.

Statistical Analysis. Results are expressed as mean \pm S.E.M. Statistical analysis was performed with the use of the unpaired two-tailed Student's *t* test or analysis of variance where applicable. Comparisons were considered statistically significant at the *p* < 0.05 level.

Results

15d-PGJ₂ Binds to Multiple Protein Targets in MC.

CyPG may exert their effects by forming covalent adducts with cellular proteins. To assess the extent of protein modification in MC, we analyzed total lysates from cells incubated with 15d-PGJ₂ or biotinylated 15d-PGJ₂ by two-dimensional electrophoresis (Fig. 1). Western blot analysis of the two-dimensional gels showed the presence of several spots corresponding to endogenous biotinylated proteins in the lysates from cells treated with 15d-PGJ₂ (Fig. 1A). In gels from cells treated with biotinylated 15d-PGJ₂, we could detect at least 50 additional spots (Fig. 1B). The same labeling pattern was obtained in several experiments using different batches of cells. Total protein staining revealed over 350 spots. The biotin-positive spots did not coincide with the major proteins detected by Coomassie staining. These observations indicate that biotinylated 15d-PGJ₂ can bind to a defined set of tar-

gets in MC and this binding is determined by factors other than protein abundance.

15d-PGJ₂ Binds to Proteins Distributed in Nuclear and Cytosolic Compartments in MC. To date, a limited number of proteins that can be modified by cyPG have been identified using several experimental systems. To obtain a deeper knowledge of the cellular targets for 15d-PGJ₂ addition, we explored the distribution of the sites for covalent attachment of biotinylated 15d-PGJ₂ in MC by fluorescence microscopy. Incubation of MC in the presence of 10 μ M biotinylated 15d-PGJ₂ led to the incorporation of the modified PG into various cellular structures as detected with fluorescent streptavidin (Fig. 2A). The labeling pattern obtained after incubation with biotinylated 15d-PGJ₂ was clearly different from that of endogenous biotinylated proteins (Fig. 2A, vehicle), which showed a perinuclear distribution typical of mitochondrial localization, as expected for several biotin-dependent carboxylases (Hollinshead et al., 1997). In contrast, biotinylated 15d-PGJ₂ was distributed both in cytoplasmic and nuclear compartments. The position of cell nuclei is evidenced by staining with DAPI. To confirm the presence of both cytoplasmic and nuclear targets for covalent attachment of biotinylated 15d-PGJ₂ in MC, we performed subcellular fractionation. As depicted in Fig. 2B, incubation of MC with the biotinylated PG resulted in the incorporation of biotin into a broad number of polypeptides that could be detected both in nuclear extracts and in the cytosolic fraction. The patterns obtained in both fractions were different, show-

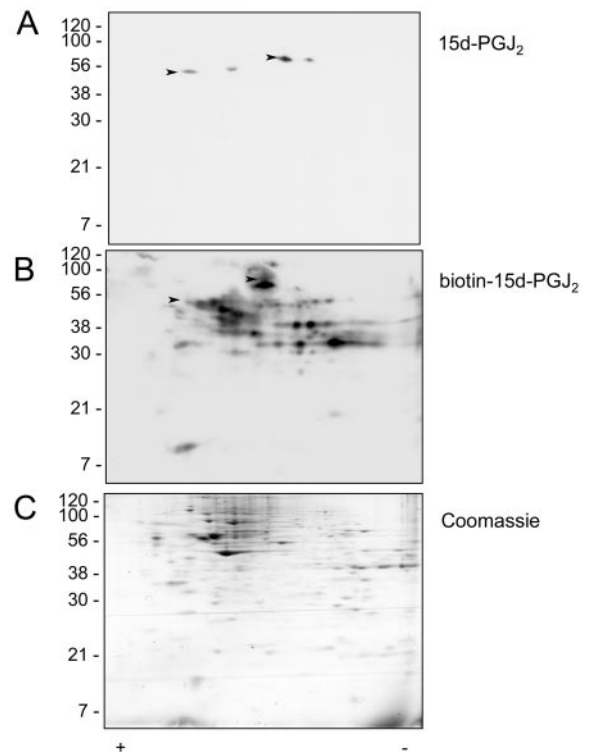


Fig. 1. Modification of MC proteins by biotinylated 15d-PGJ₂. MC were incubated with 5 μ M 15d-PGJ₂ or biotinylated 15d-PGJ₂ for 2 h. Cell lysates containing 100 μ g of protein were analyzed by two-dimensional electrophoresis and Western blot and by detection with HRP-conjugated streptavidin. ECL exposures were carried out for 1 min. The Coomassie staining of a gel run in parallel is shown in C. The position of molecular weight markers is shown on the left and that of the main endogenous biotinylated proteins is marked by arrowheads.

ing the presence of several polypeptides that were enriched in one of the fractions, some of which are marked by arrowheads in Fig. 2B.

Binding of Biotinylated 15d-PGJ₂ to Cellular Proteins Is Selective. We next explored the selectivity of protein modification by biotinylated 15d-PGJ₂. Incorporation of the biotin label into MC polypeptides after incubation of intact cells with biotinylated 15d-PGJ₂ was dose-dependent (Fig. 3A). Several faint bands could be distinguished above the background of endogenous biotinylated proteins after incubation of MC in the presence of 100 nM biotinylated 15d-PGJ₂, whereas micromolar concentrations of the biotinylated PG led to evident protein labeling. 15d-PGJ₂ can bind to purified proteins *in vitro*. We were interested in assessing the ability of 15d-PGJ₂ to modify MC proteins in cell-free extracts. After incubation of MC lysates with biotinylated 15d-PGJ₂, numerous biotin-containing polypeptides could be detected (Fig. 3B), although the intensity of the labeling was lower than when incubating intact cells with the same concentration of biotinylated 15d-PGJ₂. The available evidence suggests that the reaction of 15d-PGJ₂ with protein thiols does not occur randomly but takes place preferentially at specific cysteine residues within given proteins. To further substantiate this point, we compared the labeling pattern obtained by incubation of cell lysates with biotinylated 15d-PGJ₂ and with biotinylated iodoacetamide, a general cysteine-modifying reagent. Incubation of cell lysates with biotinylated iodoacetamide led to the incorporation of biotin into multiple polypeptide bands in a pattern that closely resembled total protein staining (Fig. 3B) and was readily distinguishable from biotinylated 15d-PGJ₂-induced protein modification. These differences were analyzed by image scanning and quantitation of the blots shown in Fig. 3B. The profiles obtained, shown in Fig. 3C, clearly illustrate the lack of coincidence between the main targets for biotinylated 15d-PGJ₂ and biotinylated iodoacetamide incorporation. Taken together, these observations suggest that binding of biotinylated 15d-PGJ₂ occurs at a specific set of cellular proteins and does not correlate with thiol accessibility.

Importance of the Cyclopentenone Moiety in the Modification of Protein Targets by 15d-PGJ₂. We have reported previously that c-Jun is a target for 15d-PGJ₂ addition, both *in vitro* and in intact cells (Pérez-Sala et al., 2003). To explore the structural requirements for protein modifica-

tion by 15d-PGJ₂, we performed an *in vitro* assay using a peptide from human c-Jun as a model of Michael acceptor. This peptide contains two cysteine residues; of them, the one equivalent to cysteine 269 in full-length human c-Jun has been shown to be the preferential site of modification by 15d-PGJ₂ (Pérez-Sala et al., 2003). The formation of adducts between 15d-PGJ₂ and the c-Jun peptide was monitored by MALDI-TOF mass spectrometry (Fig. 4). The control c-Jun peptide showed a peak $m/z = 13,479$, which corresponds to the calculated mass of the construct (Pérez-Sala et al., 2003). As we have reported previously, 15d-PGJ₂ (mass 316.5 Da) readily formed an adduct with c-Jun, as indicated by the appearance of a peak m/z at 13,795. We next explored the behavior of 9,10-dihydro-15d-PGJ₂, a 15d-PGJ₂ analog that lacks the cyclopentenone structure. This analog was designed to retain PPAR γ agonist activity and to be more resistant to metabolism through conjugation with glutathione, which has been proposed to occur across the α,β -unsaturated enone, and more specifically through carbon 9 (Paumi et al., 2003). A peak m/z of 13,802 was detected in the 9,10-dihydro-15d-PGJ₂-treated c-Jun sample (Fig. 4), which is compatible with the formation of an adduct between c-Jun and the 9,10-dihydro analog (expected m/z , 13,798). This suggests that in the absence of the electrophilic carbon in the cyclopentane ring, conjugation can occur through the electrophilic carbon at position 13. However, this analog is less efficient than 15d-PGJ₂ at forming a Michael adduct with c-Jun, as estimated from the relative intensity of the corresponding peaks. As expected, PGE₂, which does not possess an unsaturated carbonyl group, did not bind to the c-Jun peptide.

We next compared the performance of the various prostanooids by using a Western blot-based competition assay. As it is shown in Fig. 5A, incubation of the c-Jun fragment with biotinylated iodoacetamide resulted in the incorporation of the biotin label as detected by Western blot and detection with HRP-conjugated streptavidin. Preincubation of the c-Jun construct with 15d-PGJ₂ clearly reduced labeling with biotinylated iodoacetamide ($49 \pm 3\%$ inhibition, average \pm S.E.M. of three assays). 9,10-Dihydro-15d-PGJ₂ was much less effective than 15d-PGJ₂ ($15 \pm 7\%$ reduction), and PGE₂ was virtually ineffective. In addition, we observed that the binding of biotinylated 15d-PGJ₂ to c-Jun *in vitro* was blocked by the presence of an excess of nonbiotinylated 15d-PGJ₂ ($94 \pm 6\%$ inhibition, average \pm S.E.M. of three assays),

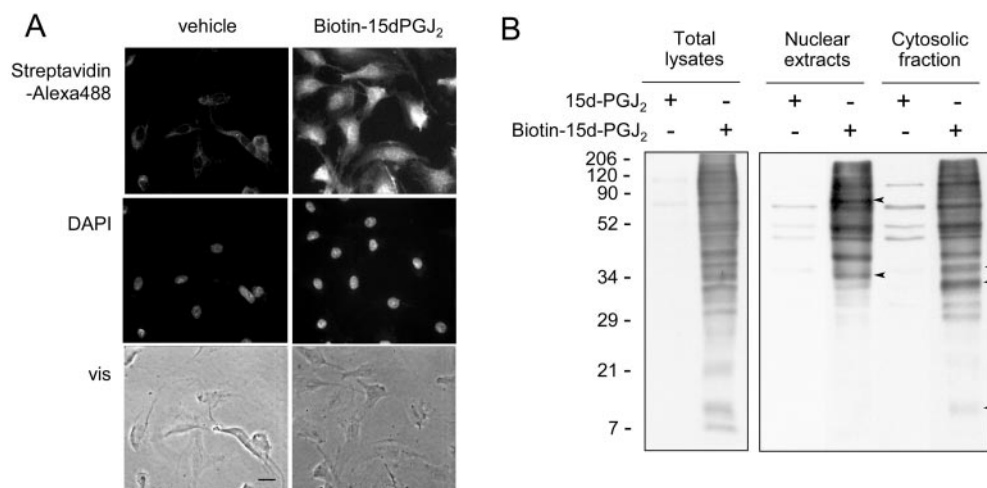


Fig. 2. Biotinylated 15d-PGJ₂ binds to nuclear and cytosolic proteins in MC. A, MC grown on glass coverslips were incubated with vehicle or 10 μ M biotinylated 15d-PGJ₂ and biotin-positive structures were visualized by incubation with Alexa488-streptavidin. The position of cell nuclei is evidenced by staining with DAPI, and cell morphology under visible light is shown at bottom (vis). Images were obtained at 40 \times magnification; scale bar, 25 μ m. B, MC were incubated in the presence of 5 μ M 15d-PGJ₂ or biotinylated 15d-PGJ₂ for 2 h, and the presence of biotinylated polypeptides in several cellular fractions was assessed by Western blot and detection with HRP-streptavidin. Arrowheads mark the position of bands that seem enriched in one of the fractions.

but it was only partially reduced by 9,10-dihydro-15d-PGJ₂ (51 ± 14% reduction) and not affected by PGE₂ (Fig. 5B). Consistent with the results shown above, the binding of biotinylated 15d-PGJ₂ to proteins in MC lysates was markedly reduced by the presence of an excess of nonbiotinylated 15d-PGJ₂, whereas 9,10-dihydro-15d-PGJ₂ and PGE₂ only moderately reduced protein labeling (Fig. 5C). The labeling in the presence of an excess of 9,10-dihydro-15d-PGJ₂, as estimated from the scanning of several bands, was reduced by 20 to 30%

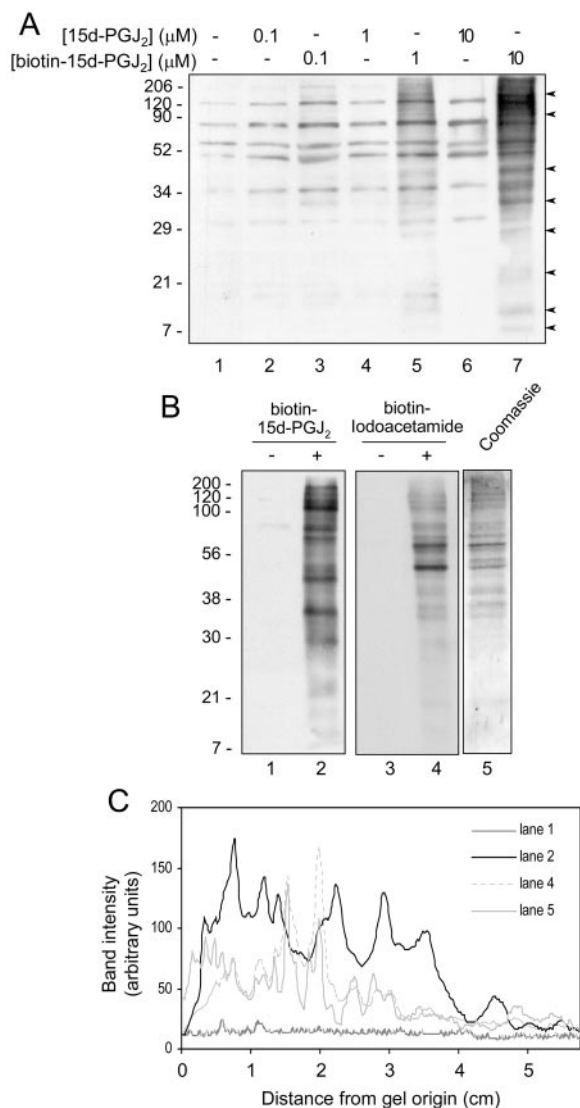


Fig. 3. Protein modification by biotinylated 15d-PGJ₂ in intact cells and in cell lysates. **A**, MC were incubated with increasing concentrations of 15d-PGJ₂ or biotinylated 15d-PGJ₂, and biotin-containing polypeptides in total cell lysates were detected by Western blot. To visualize faint bands, ECL exposures were carried out for 2 min. To show all the experimental conditions in the same exposure without saturating the signal, 2 μg of protein was loaded onto lane 7, 5 μg on lane 5, and 10 μg on the remaining lanes. **B**, MC lysates containing 6 μg of protein were incubated for 1 h at r.t. with vehicle, 10 μM biotinylated 15d-PGJ₂, or 2 mM biotinylated iodoacetamide, as indicated. Aliquots containing 6 μg (lanes 1, 2, and 5) or 0.6 μg of protein (lanes 3 and 4) were analyzed by SDS-PAGE, and biotin-containing polypeptides were detected by Western blot. Lane 5 shows the Coomassie staining of a blot run in parallel. Results shown are representative of three independent experiments. **C**, the blots shown in **B** were quantitated after image scanning, and the profiles obtained for each lane are shown. Results are expressed in arbitrary units.

compared with the labeling in the presence of PGE₂, which does not bind covalently to proteins, whereas 15d-PGJ₂ elicited an 80% inhibition. Taken together, these observations suggest that, although the 9,10-dihydro analog of 15d-PGJ₂ can form adducts with proteins to some extent, the double bond at position 9–10 in the cyclopentane ring of 15d-PGJ₂ is an important determinant for its binding to protein targets.

15d-PGJ₂ and 9,10-Dihydro-15d-PGJ₂ Activate PPAR in MC. To use 9,10-dihydro-15d-PGJ₂ as a tool to assess the relative importance of the multiple mechanisms potentially involved in the effects of 15d-PGJ₂ in MC, we compared the potency of both compounds as PPAR agonists. As shown in Fig. 6A, 9,10-dihydro-15d-PGJ₂ was able to activate PPAR to the same extent as 15d-PGJ₂, as assessed using a luciferase reporter assay. These results indicate that 15d-PGJ₂ and 9,10-dihydro-15d-PGJ₂ are equipotent as PPAR agonists in MC. The high-affinity PPAR_γ agonist rosiglitazone also activated PPRE activity, although to a lower extent than 9,10-dihydro-15d-PGJ₂, and this effect was partially blocked by the PPAR_γ antagonist T0070907 (Lee et al., 2002) (27% reduction of the rosiglitazone-elicited stimulation; Fig. 6B). In contrast, the electrophilic compounds cyclopentenone and cyclohexenone did not activate PPAR (Fig. 6B).

Role of Protein Modification in the Effects of 15d-PGJ₂ on iNOS Induction. iNOS is a key pro-inflammatory gene that can be modulated by cyPG (Ricote et al., 1998; Kwon et al., 1999). However, the mechanisms responsible for their effects have not been fully elucidated. We observed that micromolar concentrations of 15d-PGJ₂ markedly inhibited both nitrite accumulation and iNOS protein levels in MC stimulated with IL-1β plus TNF-α (Fig. 7A). In contrast, 9,10-dihydro-15d-PGJ₂ did not reduce iNOS induction. None of these compounds modulated nitrite generation or iNOS levels per se. These results indicate that the double bond at the 9 position in 15d-PGJ₂ is important for its ability to interfere with iNOS induction. The observation that both compounds activate PPAR to the same extent makes it unlikely that the inhibitory effect of 15d-PGJ₂ is related to PPAR activation. In accordance to this, the PPAR_γ antagonist GW9662 did not reduce 15d-PGJ₂ inhibition of iNOS induction (Fig. 7B). The PPAR_γ agonist rosiglitazone, which is not structurally related to 15d-PGJ₂, did not inhibit iNOS levels but increased them (Fig. 7C), although the antagonist T0070907 did not elicit appreciable changes in this assay. In addition, the compound cyclopentenone, which mimics only the cyclopentenone moiety of 15d-PGJ₂ and does not activate PPAR, at concentrations similar to those used in previous studies (Straus et al., 2000; Cipitelli et al., 2003), effectively reduced cytokine-elicited iNOS levels (Fig. 8). Moreover, another cyclic electrophile possessing an unsaturated carbonyl group, but unrelated to 15d-PGJ₂, such as cyclohexenone, blunted iNOS induction in MC (Fig. 8). Under our conditions, cyclohexenone was a more potent inhibitor than cyclopentenone and completely abrogated cytokine-elicited iNOS levels at all concentrations assayed (50 to 200 μM, not shown).

Effect of 15d-PGJ₂ on the Levels of COX-2 and ICAM-1 in MC. To assess the significance of these findings, we next explored the modulation of two additional proteins that play key roles in glomerular inflammation, COX-2 and ICAM-1. As observed with iNOS, treatment of MC with 15d-PGJ₂ before stimulation with cytokines strongly inhibited the induction of COX-2 and ICAM-1 (62 ± 10% and 79 ± 13% inhibition, aver-

age \pm S.E.M. of five and four assays, respectively, $p < 0.05$ in both cases) (Fig. 9). It is remarkable that the 9,10-dihydro analog of 15d-PGJ₂ not only did not reduce COX-2 induction but also potentiated it (4.1 ± 0.8 -fold amplification of cytokine stimulation, $n = 6$), whereas it did not affect ICAM-1 (Fig. 9A). Neither the inhibitory effect of 15d-PGJ₂ nor the amplifying effect of 9,10-dihydro-15d-PGJ₂ on COX-2 levels was reduced in the presence of the PPAR γ antagonist GW9662. Both cyclopentenone and cyclohexenone inhibited COX-2 and ICAM-1 induction by cytokines. The inhibition of COX-2 induction by cyclopentenone reached $51 \pm 10\%$, average \pm S.E.M. of three assays, $p < 0.05$ by *t* test. Cyclohexenone completely abrogated COX-2 induction (Fig. 9B). Cyclopentenone inhibited cytokine-elicited ICAM levels by $66 \pm 13\%$, average \pm S.E.M. of three assays, $p < 0.05$. Treatment with cyclohexenone before cytokine stimulation reduced ICAM protein below basal levels. These results strengthen our hypothesis that the reactivity of 15d-PGJ₂ toward cellular thiols is important for its anti-inflammatory effects.

Discussion

CyPG, and 15d-PGJ₂ in particular, have attracted considerable attention recently because of their remarkable biological effects. 15d-PGJ₂ has been reported to exert anti-inflammatory or protective effects in both cellular and animal models of inflammation or injury. These effects were first attributed to its activity as an agonist of the transcription factor PPAR γ . However, it is becoming gradually more accepted that an important determinant of 15d-PGJ₂ activity resides in its cyclopentenone structure, capable of forming covalent adducts with thiol groups by Michael addition. In this study, we have observed that 15d-PGJ₂ binds to multiple but selective protein targets in MC and that the presence of the cyclopentenone moiety is important both for protein mod-

ification and for inhibition of the levels of pro-inflammatory proteins.

In light of recent *in vitro* and *in vivo* studies, a potential for cyPG or related compounds as pharmacological tools in the treatment of inflammatory conditions has been raised. Some recent examples of the beneficial effects of cyPG include the amelioration of acute renal failure (Chatterjee et al., 2004) and the reduction of restenosis after balloon angioplasty in rats (Ianaro et al., 2003b) by 15d-PGJ₂, effects that have been attributed to its ability to inhibit NF- κ B and the expression of pro-inflammatory genes. However, the possibility that cyPG may freely react with protein thiols may constitute a drawback to their use in therapy because it could contribute to lack of specificity or multiplicity of unwanted biological effects. The experiments presented herein illustrate that covalent binding of a biotinylated analog of 15d-PGJ₂ to MC proteins is not determined by protein abundance or simply by the presence of accessible cysteine residues. This implies that the process of protein modification by these products of the arachidonic acid pathway, which could be referred to as eicosanylation or prostanylation, displays a selectivity probably related to protein or cellular context. By using two-dimensional electrophoresis, we have observed that biotinylated 15d-PGJ₂ binds to a broad but limited set of proteins in intact cells. Although the possibility that the reactivity of biotinylated 15d-PGJ₂ may not be identical to that of 15d-PGJ₂ should be taken into account, our observations provide a starting point for the identification and subsequent functional studies of the modified proteins that will help to predict the potential consequences of 15d-PGJ₂ treatment.

The cyclopentenone moiety of 15d-PGJ₂ has been proposed as an important structural feature for some of the effects of this cyPG (Straus et al., 2000; Ianaro et al., 2003a). The 9,10-dihydro analog of 15d-PGJ₂ differs only in the absence of the endocyclic double bond and is therefore not a cyclopen-

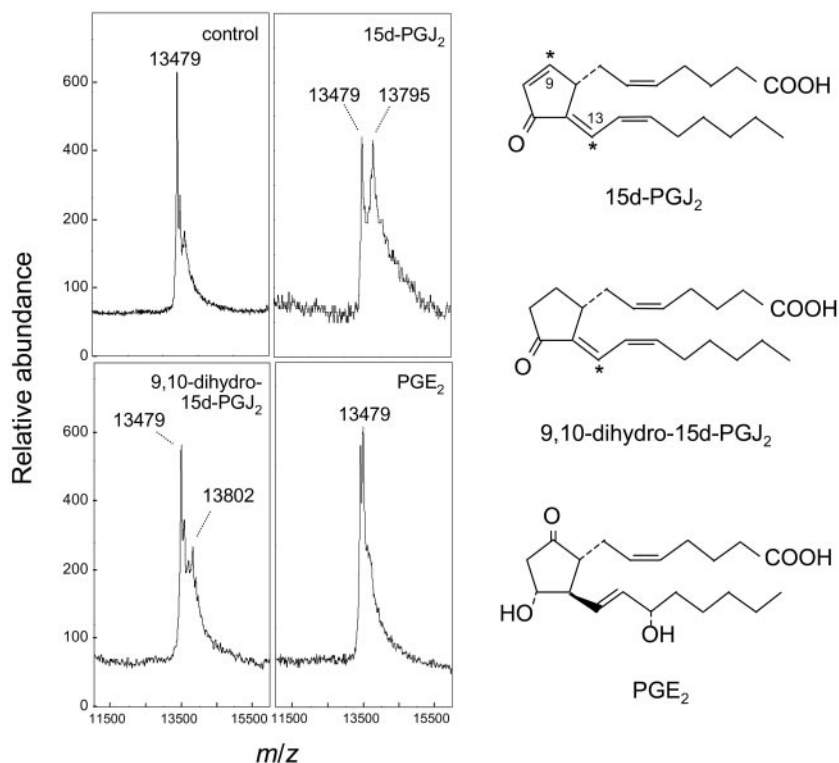


Fig. 4. MALDI-TOF mass spectrometry analysis of the interaction between c-Jun and several PG. c-Jun construct ($5 \mu\text{M}$) was incubated with $10 \mu\text{M}$ concentrations of the indicated compounds for 1 h at r.t. and analyzed by MALDI-TOF mass spectrometry. Results shown are representative of three assays. The structure of the compounds used is shown on the right. Electrophilic carbons are marked by asterisks.

tenone. This compound, designed as an analog of 15d-PGJ₂ unable to undergo conjugation with glutathione across carbon 9, has been previously proposed as a tool to explore the importance of conjugation with thiols in the effect of 15d-PGJ₂ (Cippitelli et al., 2003; Paumi et al., 2003). However, the ability of 9,10-dihydro-15d-PGJ₂ to form adducts with proteins has not been explored. Our results indicate that 9,10-dihydro-15d-PGJ₂ still retains the ability to form covalent adducts with proteins, as observed *in vitro* using a fragment of c-Jun as a model. This suggests that other electrophilic carbons present in the molecule of 15d-PGJ₂, such as carbon 13, may also participate in the formation of Michael adducts with proteins. This hypothesis is in agreement with previous reports that have identified the formation of bis-conjugates of 15d-PGJ₂ with c-Jun (Pérez-Sala et al., 2003) and of 9-deoxy- $\Delta^9, \Delta^{12}(E)$ -PGD₂, a cyPG that also possesses two electrophilic carbons, with glutathione (Atsmon et al., 1990). Nevertheless, our results suggest that 9,10-dihydro-

15d-PGJ₂ shows reduced potency as a cysteine-modifying agent, as deduced from its lesser ability to block the incorporation of biotinylated iodoacetamide or biotinylated 15d-PGJ₂ into both recombinant proteins and cellular lysates. Consistent with this, in conditions under which 9,10-dihydro-15d-PGJ₂ and 15d-PGJ₂ were equipotent at activating a PPRE reporter, 9,10-dihydro-15d-PGJ₂ did not mimic the marked inhibitory effect of 15d-PGJ₂ on the levels of iNOS, COX-2, or ICAM-1. However, a moderate inhibitory effect could be evidenced with concentrations of 9,10-dihydro-15d-PGJ₂ above 20 μ M (results not shown). Taken together, these observations suggest that the cyclopentenone structure of 15d-PGJ₂ is an important determinant both in the inhibition of the induction of pro-inflammatory genes and in the ability of 15d-PGJ₂ to modify cellular proteins. However, 15d-PGJ₂ analogs retaining electrophilic carbons cannot be considered inert compounds with respect to cysteine modification, and care should be exercised when using them as a control for the actions of cyPG.

CyPG have been shown to inhibit the induction of various pro-inflammatory genes in several experimental systems. However, the mechanisms responsible for this effect may be multiple and seem to be dependent on the system under study. An involvement of PPAR γ has been proposed in cyPG-mediated inhibition of IL-1 β -elicited iNOS induction in car-

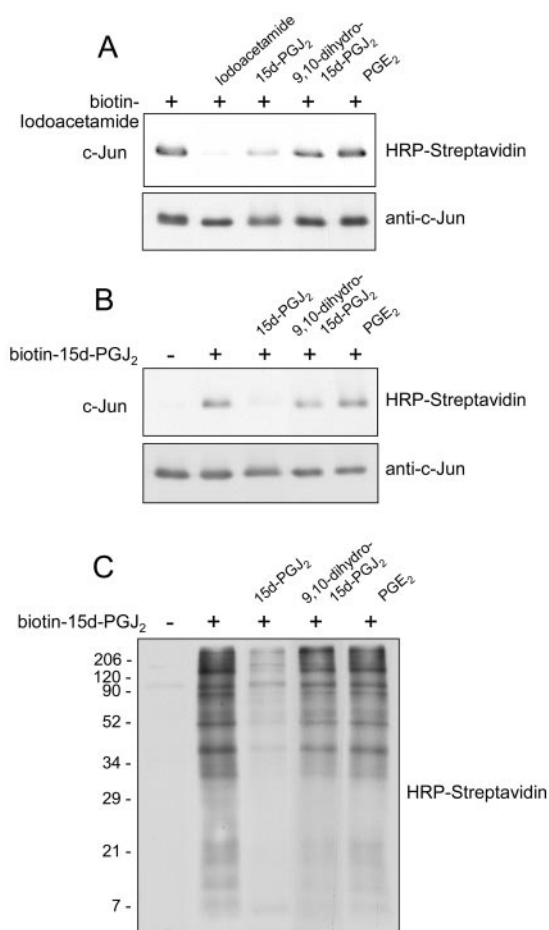


Fig. 5. Effect of several PG on the binding of biotinylated iodoacetamide or biotinylated 15d-PGJ₂ to c-Jun or cell lysates *in vitro*. A, c-Jun construct was preincubated with the indicated PG at 100 μ M final concentration or 2 mM iodoacetamide for 1 h at r.t. and subsequently incubated in the presence of 2 mM biotinylated iodoacetamide for 30 min. B, c-Jun was incubated with 1 μ M biotinylated 15d-PGJ₂ for 1 h at r.t. in the absence or presence of 100 μ M of the indicated compounds. C, aliquots from total cell lysates containing 6 μ g of protein were incubated with 10 μ M biotinylated 15d-PGJ₂ in the presence of 1 mM concentrations of the indicated PG. Incubation mixtures were subjected to SDS-PAGE and Western blot followed by detection with HRP-conjugated streptavidin or anti-c-Jun antibody, as indicated. Exposures shown are representative of at least three experiments with similar results for every assay.

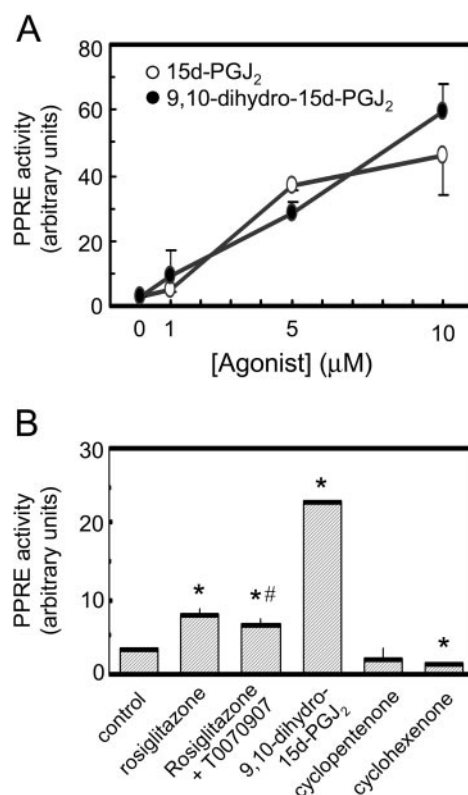


Fig. 6. Effect of 15d-PGJ₂ and 9,10-15d-PGJ₂ on the activity of a PPRE reporter. MC were transiently transfected with p4xAc-Luc plus pSG5-Renilla and treated for 16 h with the indicated concentrations of 15d-PGJ₂ or 9,10-dihydro-15d-PGJ₂ (A) or with 100 μ M cyclopentenone, 100 μ M cyclohexenone, 5 μ M rosiglitazone, 1 μ M T0070907, or 5 μ M 9,10-dihydro-15d-PGJ₂, as indicated (B). Results are expressed as the ratio between firefly and *R. reniformis* luciferase activities. Results are average values \pm S.E.M. of three independent experiments performed in duplicate. (*, $p < 0.05$ versus control by *t* test; #, $p < 0.05$ versus rosiglitazone).

diomyocytes (Mendez and LaPointe, 2003) and in human chondrocytes (Fahmi et al., 2001), as well as in the modulation of myeloperoxidase by 15d-PGJ₂ (Kumar et al., 2004) and in the protective effects of this cyPG in endotoxemia (Collin et al., 2004) and ischemia-reperfusion injury (Cuzzocrea et al., 2003). In contrast, PPAR γ -independent mechanisms have been invoked for the inhibitory actions of 15d-PGJ₂ on the expression of iNOS in pancreatic β -cells and in IFN γ -induced macrophages (Chen et al., 2003; Weber et al.,

2004), for the inhibition of NF- κ B and AP-1 in cells expressing undetectable levels of PPAR γ , such as HeLa cells (Straus et al., 2000; Pérez-Sala et al., 2003), and for some of the in vivo anti-inflammatory effects (Ianaro et al., 2003a). The effect of 15d-PGJ₂ on iNOS induction in MC and the mechanisms involved have not been previously addressed. Our results suggest that in MC the inhibition of cytokine-elicited

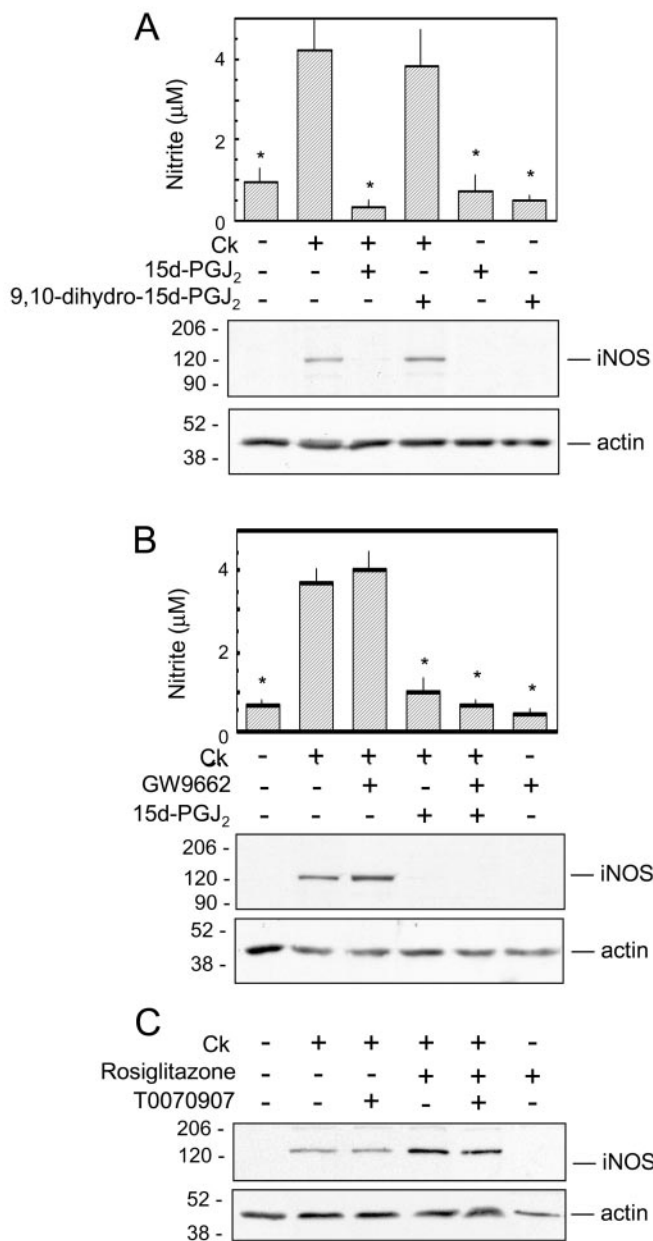


Fig. 7. Effect of 15d-PGJ₂ on iNOS induction is independent from PPAR γ activity. A, MC were preincubated for 2 h with the indicated PPAR agonists at 5 μ M before stimulation with IL-1 β plus TNF- α (Ck). B, MC were pretreated with 10 μ M GW9662 before addition of 15d-PGJ₂. C, MC were preincubated with 1 μ M T0070907 before addition of 5 μ M rosiglitazone. After 16 h of treatment with cytokines, the accumulation of nitrite in the cell supernatant was measured by the Griess method. Results are average values \pm S.E.M. of three experiments (*, $p < 0.05$ versus Ck by t test). Levels of iNOS and actin in MC extracts were assessed by Western blot. ECL exposures are representative of three experiments with similar results.

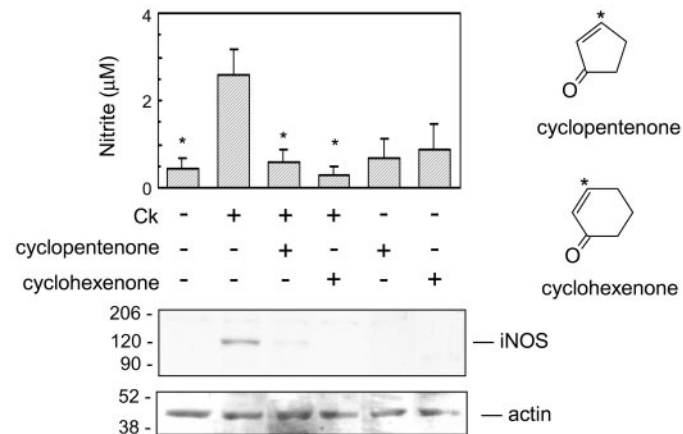


Fig. 8. Cyclopentenone and cyclohexenone inhibit iNOS induction. MC were pretreated with 100 μ M cyclopentenone or cyclohexenone for 2 h before stimulation with Ck. Levels of nitrite in the cell supernatants and of iNOS protein and actin in cell lysates were assessed as in Fig. 7. Results are average values \pm S.E.M. of three experiments (*, $p < 0.05$ versus Ck by t test). ECL exposures are representative of three experiments with similar results. The structure of the compounds used is shown on the right. Electrophilic carbons are marked by asterisks.

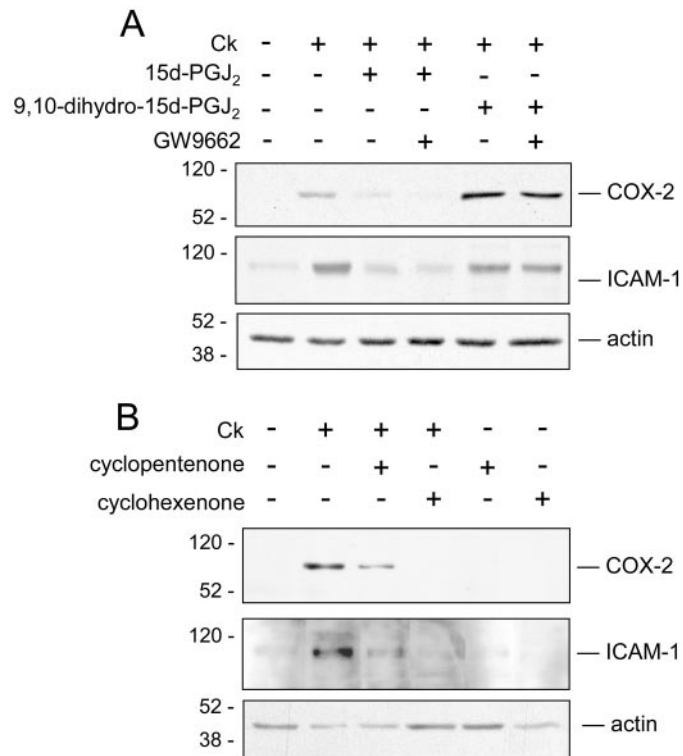


Fig. 9. Effect of several electrophilic compounds on the expression of COX-2 and ICAM-1 in MC. MC were pretreated with the indicated compounds, as specified in Fig. 7 and 8, before stimulation with Ck, and the protein levels of COX-2, ICAM-1, and actin were assessed by Western blot. ECL exposures are representative of three experiments with similar results.

iNOS induction is largely independent of PPAR γ activation. This inference is based on the fact that 15d-PGJ₂ and its 9,10-dihydro analog have different effects on iNOS induction but are equally potent as PPAR agonists, on the lack of effect of the PPAR γ antagonist GW9662 on 15d-PGJ₂-elicited inhibition, and on the inhibitory effect of other electrophiles, such as cyclopentenone and cyclohexenone. In addition, the PPAR γ ligand rosiglitazone increased iNOS levels rather than reducing them. This observation is also supported by previous results from our laboratory showing that other PPAR agonists amplify cytokine-elicited iNOS induction (Cernuda-Morollón et al., 2002). It is interesting that the inhibition of the induction of two additional pro-inflammatory genes, such as COX-2 and ICAM-1, also requires the presence of the endocyclic double bond of 15d-PGJ₂. It is remarkable that the absence of this bond, as it occurs in the 9,10-dihydro analog of 15d-PGJ₂, results in a strong potentiation of COX-2 induction. The elucidation of the mechanism of this effect, which is not blocked by GW9662, will require further investigation.

In conclusion, the observations described above point to the importance of covalent protein modification in the anti-inflammatory effects of 15d-PGJ₂. Our work illustrates the requirements, extent and selectivity of protein modification by 15d-PGJ₂ in MC. The identification of the detected targets for cyPG addition will provide a deeper insight into the mechanism of action and potential applications of these eicosanoids.

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