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Protein thiol modification by 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 addition in mesangial cells: role in the inhibition of pro-inflammatory genes*

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Running title: 15d-PGJ_2 modification of mesangial cell proteins

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Abbreviations: CyPG, cyclopentenone prostaglandin; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; ICAM-1, intercellular adhesion molecule-1; MC, mesangial cell; PPAR, peroxisome proliferator activated receptor; cyclopentenone, 2-cyclopenten-1-one; cyclohexenone, 2-cyclohexen-1-one; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence.

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Abstract

The cyclopentenone prostaglandin (cyPG) and PPARγ agonist 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) displays anti-inflammatory effects in several experimental models. Direct modification of protein thiols is arising as an important mechanism of cyPG action. However, little is known about the extent or specificity of this process. Mesangial cells (MC) play a key role in glomerulonephritis. Here we have studied the selectivity of protein modification by 15d-PGJ_2 in MC, and the correlation with the modulation of several pro-inflammatory genes. MC incubation with biotinylated 15d-PGJ_2 results in the labeling of a distinct set of proteins as evidenced by 2D-electrophoresis. 15d-PGJ_2 binds to nuclear and cytosolic targets as detected by fluorescence microscopy and subcellular fractionation. The pattern of biotinylated 15d-PGJ_2-modified polypeptides is readily distinguishable from that of total protein staining or labeling with biotinylated iodoacetamide. 15d-PGJ_2 addition requires the double bond in the cyclopentane ring. 9,10-dihydro-15d-PGJ_2, a 15d-PGJ_2 analog that shows the same potency as PPAR agonist in MC but lacks the cyclopentenone moiety, displays reduced ability to modify proteins and to block 15d-PGJ_2 binding. Micromolar concentrations of 15d-PGJ_2 inhibit cytokine-elicited levels of inducible nitric oxide synthase, cyclooxygenase-2 and intercellular adhesion molecule-1 in MC. In contrast, 9,10-dihydro-15d-PGJ_2 does not reproduce this inhibition. 15d-PGJ_2 effect is not blocked by the PPARγ antagonist 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-PGJ_2 is a selective process which plays an important role in the inhibition of MC responses to pro-inflammatory stimuli.

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Cyclopentenone prostaglandins (cyPG) are endogenous prostanoids which arise from the dehydration of their parent PG. Thus, dehydration of PGE_2 gives rise to PGA_2, while cyPG of the J series, like PGJ_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2), arise from the dehydration of PGD_2. The generation of 15d-PGJ_2 has been reported to increase under situations associated with COX-2 induction, such as inflammatory processes (Gilroy et al., 1999). 15d-PGJ_2 has been the subject of considerable study due to 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-PGJ_2 may modulate multiple cellular functions by mechanisms dependent and independent of PPAR.

15d-PGJ_2 displays anti-inflammatory and protective effects against several types of injury both in cellular systems and in animal models (Cuzzocrea et al., 2002; Ianaro et al., 2003b; Rovin et al., 2001; Zingarelli et al., 2003). 15d-PGJ_2 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) (Reilly et al., 2001; Ricote et al., 1998; Rovin et al., 2001). Although the pathophysiological importance of these findings is not clear at present, they can be of pharmacological relevance, since 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

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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 which 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-PGJ_2. Some of these proteins are involved in the modulation of inflammation, including several components of the NF-κB (Castrillo et al., 2000; Cernuda-Morollón et al., 2001; Rossi et al., 2000; Straus et al., 2000) 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 (Itoh et al., 2004; Levonen et al., 2004; Moos et al., 2003; Shibata et al., 2003). 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 since 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 which 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 constitute a relevant cellular model of inflammation. In this study we illustrate the

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covalent modification of MC proteins by 15d-PGJ_2 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.

Material and Methods

Materials

15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 was from Calbiochem-Novabiochem (San Diego, CA) or from Cayman Chemical (Ann Arbor, MI). 9,10-dihydro-15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (9,10-dihydro-15d-PGJ_2), PGE_2 and T0070907 were from Cayman Chemical. Rosiglitazone was from Alexis Biochemicals. Recombinant human IL-1β (5 x 10^7 U/mg) was from Roche Diagnostics S. L. (Barcelona, Spain). Recombinant human TNF-α was from Serotec (Oxford, U.K.). Polyclonal anti-iNOS (sc-651) and anti-c-Jun (sc-044) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-COX-2 antibody was from Oxford Biomedical Research and anti-ICAM-1 was from Jackson Laboratories (Bar Harbor, Maine). Horseradish peroxidase (HRP) conjugated anti-rabbit immunoglobulins were from Dako (Glostrup, Denmark). HRP-conjugated streptavidin and enhanced chemiluminescence (ECL) reagents were from Amersham Biosciences (Barcelona, Spain). Cell culture media and supplements were from Invitrogen Life Technologies 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 (RMC) were obtained as reported earlier (Saura et al., 1995). Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM

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glutamine, 100 U/ml penicillin and 100 µg/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-1β 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 minutes 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 previously described (Cernuda-Morollón et al., 2002). The levels of cellular actin were used as a control for inter-sample variability. None of the compounds used elicit pro-inflammatory gene expression in the absence of cytokines.

Plasmids and transient transfections

The PPAR reporter construct p4xAco-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 RMC were incubated for 3 h in transfection mixture containing 1 µg of 4xAco-Luc or empty vector (pBV-Luc) and 5 ng of pSG5-Renilla in Optimem 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 luciferases present in cell lysates were measured using a dual luciferase reporter assay system from Promega (Madison, WI). All assays were

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done in duplicate and results are expressed as the ratio between firefly and renilla luciferase activities.

Fluorescence microscopy

15d-PGJ_2 biotinylated at the carboxyl group was generously provided by Dr. F. J. Cañada. To visualize the subcellular distribution of 15d-PGJ_2 binding sites, cells were grown on glass coverslips. Subconfluent MC were incubated for 15 min in the presence of 10 μM biotinylated 15d-PGJ_2 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 μg/ml Alexa488-streptavidin (Molecular Probes Inc., Eugene, OR) for 30 min. To visualize cell nuclei, coverslips were incubated with 0.2 μg/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 CCD camera.

Incorporation of biotinylated 15d-PGJ_2 into MC proteins

MC were incubated with biotinylated 15d-PGJ_2 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 μg/ml of each of the protease inhibitors: leupeptin, pepstatin A, and aprotinin. Nuclear and cytosolic extracts were obtained as previously described (Cernuda-Morollón et al., 2001). Protein concentration was determined by the BCA protein assay from Pierce

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(Rockford, IL). Fifteen μg 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, 50mM NaCl, 1% NP-40, 0.3% sodium deoxycholate, 0.1% SDS plus protease inhibitors. Aliquots of cell lysates containing 100 μg of protein were precipitated with 10% TCA, resuspended in 130 μl of IEF sample buffer (4% Triton X-100, 2 M thiourea, 7 M urea, 100 mM DTT, 2% Bio-lyte ampholytes) and loaded on ReadyStrip IPG Strips (pH 3-7, 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-PGJ_2 into MC proteins was assessed by Western blot and detection with HRP-conjugated streptavidin and ECL, as previously described (Oliva et al., 2003; Pérez-Sala et al., 2003).

Binding of 15d-PGJ_2 to proteins in vitro

The ability of biotinylated 15d-PGJ_2 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 previously described 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-PGJ_2- or 9,10-dihydro-15d-PGJ_2-c-Jun adducts was assessed by MALDI-TOF mass spectrometry analysis as described (Pérez-Sala et al., 2003). Briefly, peptides were purified by ZipTip C18 (Millipore, Bedford, MA). The laser desorption/ionisation 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

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acid in acetonitrile: water (1:2) with 0.1 % TFA was used as the matrix. Equal volumes (0.5 µl) 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 ± SEM. Statistical analysis was performed with the use of the unpaired two-tailed Student´s *t*-test or ANOVA where applicable. Comparisons were considered statistically significant at the $p<0.05$ level.

Results

15d-PGJ$_2$ binds to multiple protein targets in MC

CyPG may exert their effects by forming covalent adducts with cellular proteins. In order to assess the extent of protein modification in MC we analyzed total lysates from cells incubated with 15d-PGJ$_2$ or biotinylated 15d-PGJ$_2$ by two-dimensional electrophoresis (Fig. 1). Western blot analysis of the 2D gels showed the presence of several spots corresponding to endogenous biotinylated proteins in the lysates from cells treated with 15d-PGJ$_2$ (Fig. 1A). In gels from cells treated with biotinylated 15d-PGJ$_2$ 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-PGJ$_2$ can bind to a defined set of targets in MC and this binding is determined by factors other than protein abundance.

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15d-PGJ$_2$ binds to proteins distributed in nuclear and cytosolic compartments in MC

To date, a limited number of proteins which can be modified by cyPG have been identified using several experimental systems. To obtain a deeper knowledge of the cellular targets for 15d-PGJ$_2$ addition we explored the distribution of the sites for covalent attachment of biotinylated 15d-PGJ$_2$ in MC by fluorescence microscopy. Incubation of MC in the presence of 10 μM biotinylated 15d-PGJ$_2$ 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-PGJ$_2$ 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-PGJ$_2$ 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-PGJ$_2$ in MC we performed subcellular fractionation. As depicted in Figure 2B, incubation of MC with the biotinylated PG resulted in the incorporation of biotin into a broad number of polypeptides which could be detected both in nuclear extracts and in the cytosolic fraction. The patterns obtained in both fractions were different, showing the presence of several polypeptides which were enriched in one of the fractions, some of which are marked by arrowheads in Figure 2B.

Binding of biotinylated 15d-PGJ$_2$ to cellular proteins is selective

We next explored the selectivity of protein modification by biotinylated 15d-PGJ$_2$. Incorporation of the biotin label into MC polypeptides after incubation of intact cells

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with biotinylated 15d-PGJ_2 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-PGJ_2, while micromolar concentrations of the biotinylated PG led to evident protein labeling. 15d-PGJ_2 can bind to purified proteins *in vitro*. We were interested in assessing the ability of 15d-PGJ_2 to modify MC proteins in cell-free extracts. After incubation of MC lysates with biotinylated 15d-PGJ_2 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-PGJ_2. The available evidence suggests that the reaction of 15d-PGJ_2 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-PGJ_2 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-PGJ_2-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-PGJ_2 and biotinylated iodoacetamide incorporation. Taken together these observations suggest that binding of biotinylated 15d-PGJ_2 occurs at a specific set of cellular proteins and does not correlate with thiol accessibility.

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Importance of the cyclopentenone moiety in the modification of protein targets by 15d-PGJ_2

We have previously reported that c-Jun is a target for 15d-PGJ_2 addition, both *in vitro* and in intact cells (Pérez-Sala et al., 2003). In order to explore the structural requirements for protein modification by 15d-PGJ_2, 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 which, that equivalent to cysteine 269 in full length human c-Jun has been shown to be the preferential site of modification by 15d-PGJ_2 (Pérez-Sala et al., 2003). The formation of adducts between 15d-PGJ_2 and the c-Jun peptide was monitored by MALDI-TOF mass spectrometry (Fig. 4). The control c-Jun peptide showed a peak of molecular mass *m/z* = 13,479, which corresponds to the calculated mass of the construct (Pérez-Sala et al., 2003). As previously reported by us, 15d-PGJ_2 (mass 316.5) readily formed an adduct with c-Jun, as indicated by the appearance of a peak of *m/z* = 13,795. We next explored the behavior of 9,10-dihydro-15d-PGJ_2, a 15d-PGJ_2 analog which lacks the cyclopentenone structure. This analog was designed to retain PPARγ agonist activity while being 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 of *m/z* 13,802 was detected in the 9,10-dihydro-15d-PGJ_2-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-PGJ_2 at forming a Michael adduct with c-Jun, as estimated from the relative intensity of the

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corresponding peaks. As expected, PGE_2, which does not possess an unsaturated carbonyl group, did not bind to the c-Jun peptide.

We next compared the performance of the various prostanoids 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. Pre-incubation of the c-Jun construct with 15d-PGJ_2 clearly reduced labeling with biotinylated iodoacetamide (49 ± 3 % inhibition, average ± S.E.M. of three assays). 9,10-dihydro-15d-PGJ_2 was much less effective than 15d-PGJ_2 (15 ± 7 % reduction) and PGE_2 was virtually ineffective. In addition, we observed that the binding of biotinylated 15d-PGJ_2 to c-Jun *in vitro* was blocked by the presence of an excess of non biotinylated 15d-PGJ_2 (94 ± 6 % inhibition, average ± S.E.M. of three assays), but it was only partially reduced by 9,10-dihydro-15d-PGJ_2 (51 ± 14 % reduction) and not affected by PGE_2 (Fig. 5B). In consistence with the results shown above, the binding of biotinylated 15d-PGJ_2 to proteins in MC lysates was markedly reduced by the presence of an excess of non biotinylated 15d-PGJ_2, while 9,10-dihydro-15d-PGJ_2 and PGE_2 only moderately reduced protein labeling (Fig. 5C). The labeling in the presence of an excess of 9,10-dihydro-15d-PGJ_2, as estimated from the scanning of several bands, was reduced by 20%-30% when compared with the labeling in the presence of PGE_2, which does not bind covalently to proteins, while 15d-PGJ_2 elicited an 80% inhibition. Taken together, these observations suggest that, although the 9,10-dihydro analog of 15d-PGJ_2 can form adducts with proteins to some extent, the double bond at position 9-10 in the cyclopentane ring of 15d-PGJ_2 is an important determinant for its binding to protein targets.

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15d-PGJ$_2$ and 9,10-dihydro-15d-PGJ$_2$ activate PPAR in MC

In order to use 9,10-dihydro-15d-PGJ$_2$ as a tool to assess the relative importance of the multiple mechanisms potentially involved in the effects of 15d-PGJ$_2$ in MC, we compared the potency of both compounds as PPAR agonists. As it is shown in Fig. 6A, 9,10-dihydro-15d-PGJ$_2$ was able to activate PPAR to the same extent as 15d-PGJ$_2$, as assessed using a luciferase reporter assay. These results indicate that 15d-PGJ$_2$ and 9,10-15d-PGJ$_2$ are equipotent as PPAR agonists in MC. The high affinity PPARγ agonist rosiglitazone also activated PPRE activity, although to a lower extent than 9,10-dihydro-15d-PGJ$_2$, and this effect was partially blocked by the PPARγ 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-PGJ$_2$ on iNOS induction

iNOS is a key pro-inflammatory gene which can be modulated by cyPG (Kwon et al., 1999; Ricote et al., 1998). However, the mechanisms responsible for their effects have not been fully elucidated. We observed that micromolar concentrations of 15d-PGJ$_2$ 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-PGJ$_2$ 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-PGJ$_2$ is important for its ability to interfere with iNOS induction. The observation that both compounds activate PPAR to the same extent, makes it unlikely the possibility that the inhibitory effect of 15d-PGJ$_2$ is related to PPAR activation. In accordance to this, the PPARγ antagonist GW9662 did not reduce 15d-PGJ$_2$ inhibition of iNOS induction (Fig.

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7B). The PPARγ agonist rosiglitazone, which is not structurally related to 15d-PGJ_2, did not inhibit, but increased iNOS levels (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-PGJ_2 and does not activate PPAR, at concentrations similar to those used in previous studies (Cippitelli et al., 2003; Straus et al., 2000), effectively reduced cytokine-elicited iNOS levels (Fig. 8). Moreover, another cyclic electrophile possessing an unsaturated carbonyl group, but unrelated to 15d-PGJ_2, 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-PGJ_2 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 which play key roles in glomerular inflammation, namely COX-2 and ICAM-1. As observed with iNOS, treatment of MC with 15d-PGJ_2 prior to stimulation with cytokines strongly inhibited the induction of COX-2 and ICAM-1 (62 ± 10 % and 79 ± 13 % inhibition, average ± S.E.M. of five and four assays, respectively, $p<0.05$ in both cases) (Fig. 9). Remarkably, the 9,10-dihydro analog of 15d-PGJ_2, not only did not reduce but potentiated COX-2 induction (4.1 ± 0.8-fold amplification of cytokine stimulation, n = 6), while it did not affect ICAM-1 (Fig. 9A). Neither the inhibitory effect of 15d-PGJ_2 nor the amplifying effect of 9,10-dihydro-15d-PGJ_2 on COX-2 levels were reduced in the presence of the PPARγ 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 ± 10 %,

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average ± 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 ± 13 % , average ± S.E.M. of three assays, $p<0.05$. Treatment with cyclohexenone prior to cytokine stimulation reduced ICAM protein below basal levels. These results strengthen our hypothesis that the reactivity of 15d-PGJ_2 towards cellular thiols is important for its anti-inflammatory effects.

Discussion

CyPG, and in particular 15d-PGJ_2, have attracted considerable attention recently due to their remarkable biological effects. 15d-PGJ_2 has been reported to exert anti-inflammatory or protective effects both in cellular and in animal models of inflammation or injury. First attributed to its activity as an agonist of the transcription factor PPARγ, it is becoming gradually more accepted that an important determinant of 15d-PGJ_2 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-PGJ_2 binds to multiple but selective protein targets in MC, and that the presence of the cyclopentenone moiety is important both for protein modification and for inhibition of the levels of pro-inflammatory proteins.

In the light of the 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-PGJ_2, effects which have been attributed to its ability to inhibit NF-κB and the expression of pro-

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inflammatory genes. However, the possibility that cyPG may freely react with protein thiols may constitute a drawback towards their use in therapy since 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-PGJ_2 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-PGJ_2 binds to a broad, but limited, set of proteins in intact cells. Although the possibility that the reactivity of biotinylated 15d-PGJ_2 may not be identical to that of 15d-PGJ_2 should be taken into account, our observations provide a starting point for the identification and subsequent functional studies of the modified proteins which will help to predict the potential consequences of 15d-PGJ_2 treatment.

The cyclopentenone moiety of 15d-PGJ_2 has been proposed as an important structural feature for some of the effects of this cyPG (Ianaro et al., 2003a; Straus et al., 2000). The 9,10-dihydro analog of 15d-PGJ_2 only differs in the absence of the endocyclic double bond, and therefore is not a cyclopentenone. This compound, designed as an analog of 15d-PGJ_2 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-PGJ_2 (Cippitelli et al., 2003; Paumi et al., 2003). However, the ability of 9,10-dihydro-15d-PGJ_2 to form adducts with proteins has not been explored. Our results indicate that 9,10-dihydro-15d-PGJ_2 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

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of 15d-PGJ_2, such as carbon 13 may also participate in the formation of Michael adducts with proteins. This hypothesis is in agreement with previous reports which have identified the formation of bis-conjugates of 15d-PGJ_2 with c-Jun (Pérez-Sala et al., 2003) and of 9-deoxy-Δ^9,Δ^{12}(*E*)-PGD_2, a cyPG possessing also two electrophilic carbons, with glutathione (Atsmon et al., 1990). Nevertheless, our results suggest that 9,10-dihydro-15d-PGJ_2 shows reduced potency as a cysteine-modifying agent, as deduced from its lesser ability to block the incorporation of biotinylated iodoacetamide or biotinylated 15d-PGJ_2 into both recombinant proteins and cellular lysates. In consistence with this, in conditions under which 9,10-dihydro-15d-PGJ_2 and 15d-PGJ_2 were equipotent at activating a PPRE reporter, 9,10-dihydro-15d-PGJ_2 did not mimic the marked inhibitory effect of 15d-PGJ_2 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-PGJ_2 above 20 μM (results not shown). Taken together these observations suggest that the cyclopentenone structure of 15d-PGJ_2 is an important determinant both in the inhibition of the induction of pro-inflammatory genes and in the ability of 15d-PGJ_2 to modify cellular proteins. However, 15d-PGJ_2 analogs retaining electrophilic carbons cannot be considered as 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 appear to be dependent on the system under study. An involvement of PPARγ has been proposed in cyPG-mediated inhibition of IL-1β-elicited iNOS induction in cardiomyocytes (Mendez and LaPointe, 2003) and in human chondrocytes (Fahmi et al., 2001), as well as in the modulation of myeloperoxidase by 15d-PGJ_2 (Kumar et al., 2004), and in the protective effects of this cyPG in endotoxemia (Collin

et al., 2004) and isquemia-reperfusion injury (Cuzzocrea et al., 2003). In contrast, PPARγ-independent mechanisms have been invoked for the inhibitory actions of 15d-PGJ_2 on the expression of iNOS in pancreatic beta-cell iNOS and in IFNγ-induced macrophages (Chen et al., 2003; Weber et al., 2004), for the inhibition of NF-κB and AP-1 in cells expressing undetectable levels of PPARγ, such as HeLa cells (Pérez-Sala et al., 2003; Straus et al., 2000) and for some of the *in vivo* anti-inflammatory effects (Ianaro et al., 2003a). The effect of 15d-PGJ_2 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 iNOS induction is largely independent from PPARγ activation. This inference is based on the different effects of 15d-PGJ_2 and its 9,10-dihydro analog on iNOS induction, while being equally potent as PPAR agonists, on the lack of effect of the PPARγ antagonist GW9662 on 15d-PGJ_2-elicited inhibition and on the inhibitory effect of other electrophiles, such as cyclopentenone and cyclohexenone. In addition, the PPARγ ligand rosiglitazone did not reduce, but increased, iNOS levels. 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). Interestingly, 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-PGJ_2. Remarkably, the absence of this bond, as it occurs in the 9,10-dihydro analog of 15d-PGJ_2, 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-PGJ_2. Our work illustrates the requirements, extent and selectivity of protein modification by 15d-PGJ_2 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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Footnotes

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Figure Legends

Fig. 1. Modification of MC proteins by biotinylated 15d-PGJ_2. MC were incubated with 5 μM 15d-PGJ_2 or biotinylated 15d-PGJ_2 for 2 h. Cell lysates containing 100 μg of protein were analyzed by 2D electrophoresis and Western blot and 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 panel C. The position of molecular weight markers is shown on the left and that of the main endogenous biotinylated proteins is marked by arrowheads.

Fig. 2. Biotinylated 15d-PGJ_2 binds to nuclear and cytosolic proteins in MC. (A) MC grown on glass coverslips were incubated with vehicle or 10 μM biotinylated 15d-PGJ_2 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 in the lower panels (vis). Images were obtained with a 40x magnification, *bar*, 25 μm. (B) MC were incubated in the presence of 5 μM 15d-PGJ_2 or biotinylated 15d-PGJ_2 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 which appear enriched in one of the fractions.

Fig. 3. Protein modification by biotinylated 15d-PGJ_2 in intact cells and in cell lysates. (A) MC were incubated with increasing concentrations of 15d-PGJ_2 or biotinylated 15d-PGJ_2 and biotin-containing polypeptides in total cell lysates were detected by Western blot. In order 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 were 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

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h at r.t. with vehicle, 10 μM biotinylated 15d-PGJ_2 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.

Fig. 4. MALDI-TOF mass spectrometry analysis of the interaction between c-Jun and several PG. c-Jun construct (5 μM) was incubated with 10 μM 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.

Fig. 5. Effect of several PG on the binding of biotinylated iodoacetamide or biotinylated 15d-PGJ_2 to c-Jun or cell lysates *in vitro*. (A) c-Jun construct was pre-incubated with the indicated PG at 100 μM 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 μM biotinylated 15d-PGJ_2 for 1 h at r.t. in the absence or presence of 100 μM of the indicated compounds. (C) Aliquots from total cell lysates containing 6 μg of protein were incubated with 10 μM biotinylated 15d-PGJ_2 in the presence of 1 mM 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-PGJ_2 and 9,10-15d-PGJ_2 on the activity of a PPRE reporter. MC were transiently transfected with p4xAco-Luc plus pSG5-Renilla and treated for 16

h with the indicated concentrations of 15d-PGJ_2 or 9,10-dihydro-15d-PGJ_2 (A) or with 100 μM cyclopentenone, 100 μM cyclohexenone, 5 μM rosiglitazone, 1 μM T0070907 or 5 μM 9,10-dihydro-15d-PGJ_2, as indicated (B). Results are expressed as the ratio between firefly and renilla luciferase activities. Results are average values ± S.E.M. of three independent experiments performed in duplicate. (*$p < 0.05$ versus control by t-test; #$p < 0.05$ versus rosiglitazone).

Fig. 7. Effect of 15d-PGJ_2 on iNOS induction is independent from PPARγ activity. (A) MC were pre-incubated for 2 h with the indicated PPAR agonists at 5 μM before stimulation with IL-1β plus TNF-α (Ck). (B) MC were pre-treated with 10 μM GW9662 before addition of 15d-PGJ_2. (C) MC were pre-incubated with 1 μM T0070907 before addition of 5 μM 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 ± SEM 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 μM 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 ± SEM 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 pre-treated 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.

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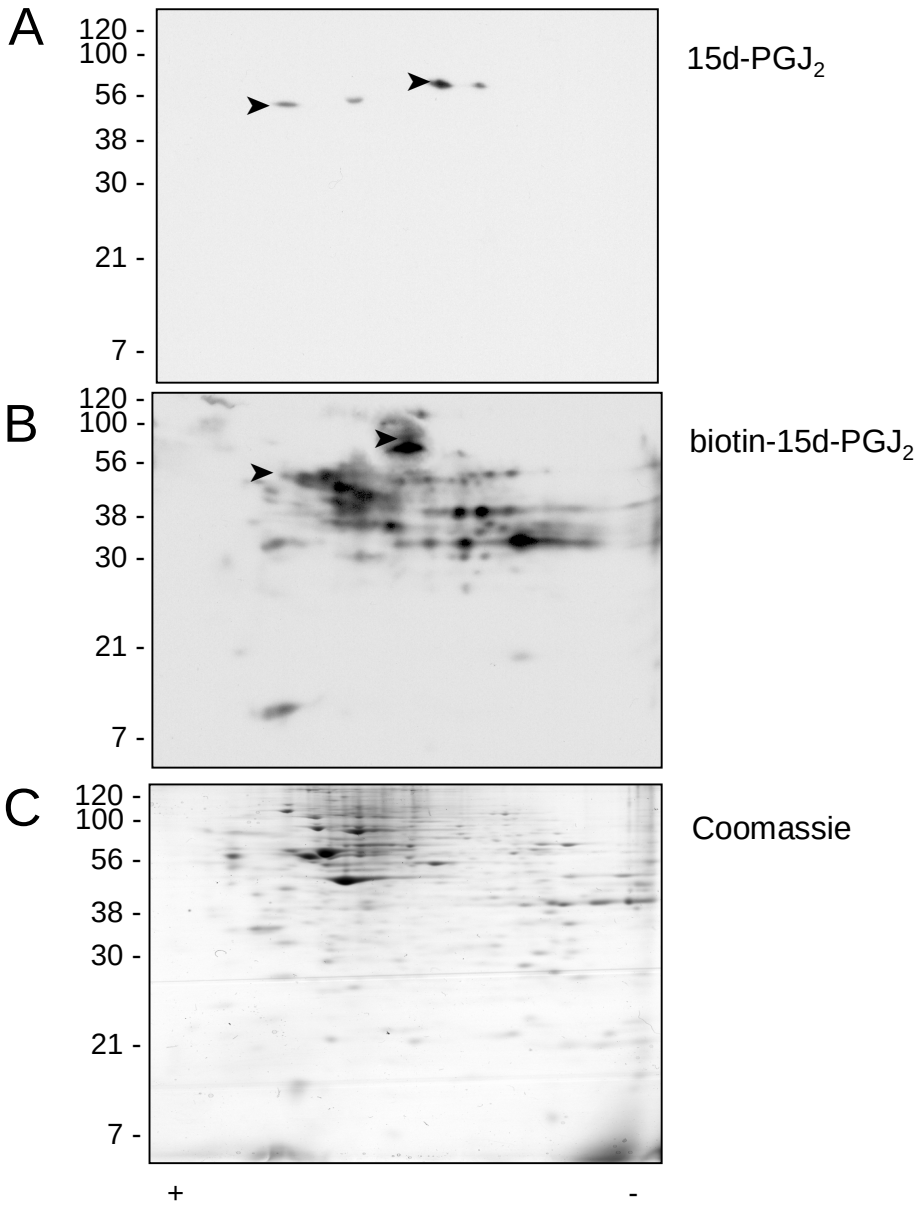

Fig. 1

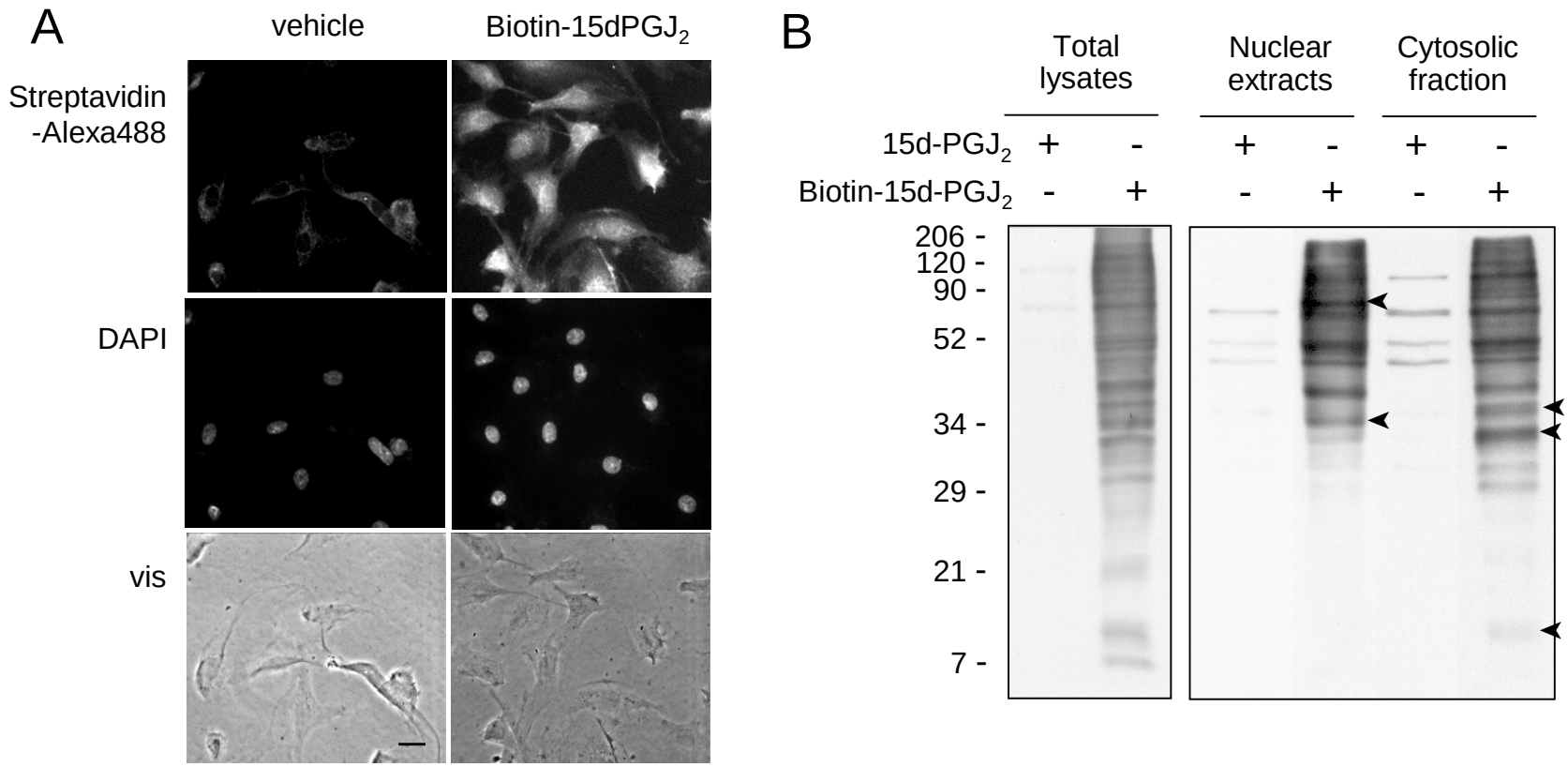

Fig. 2

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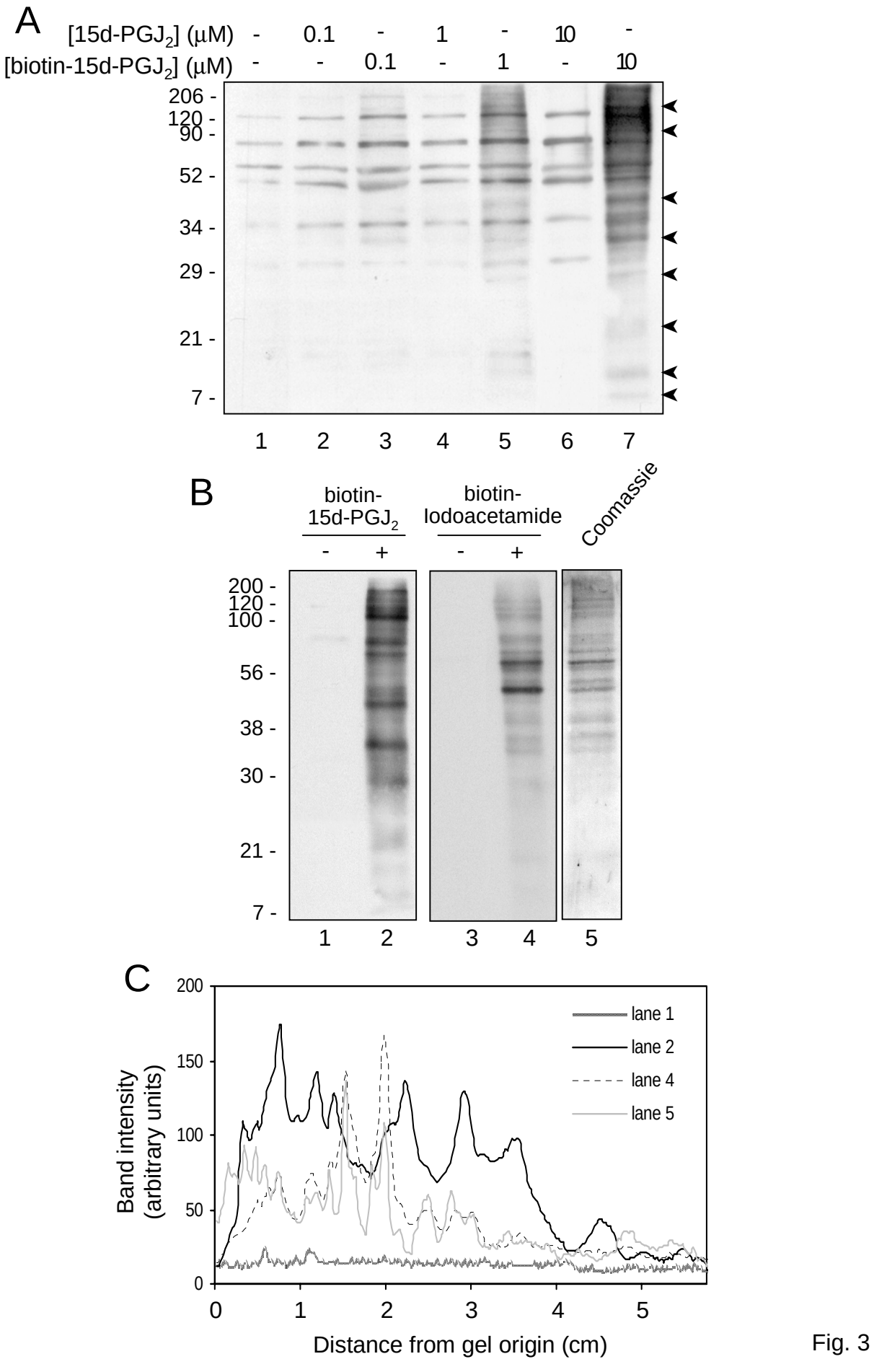

Fig. 3

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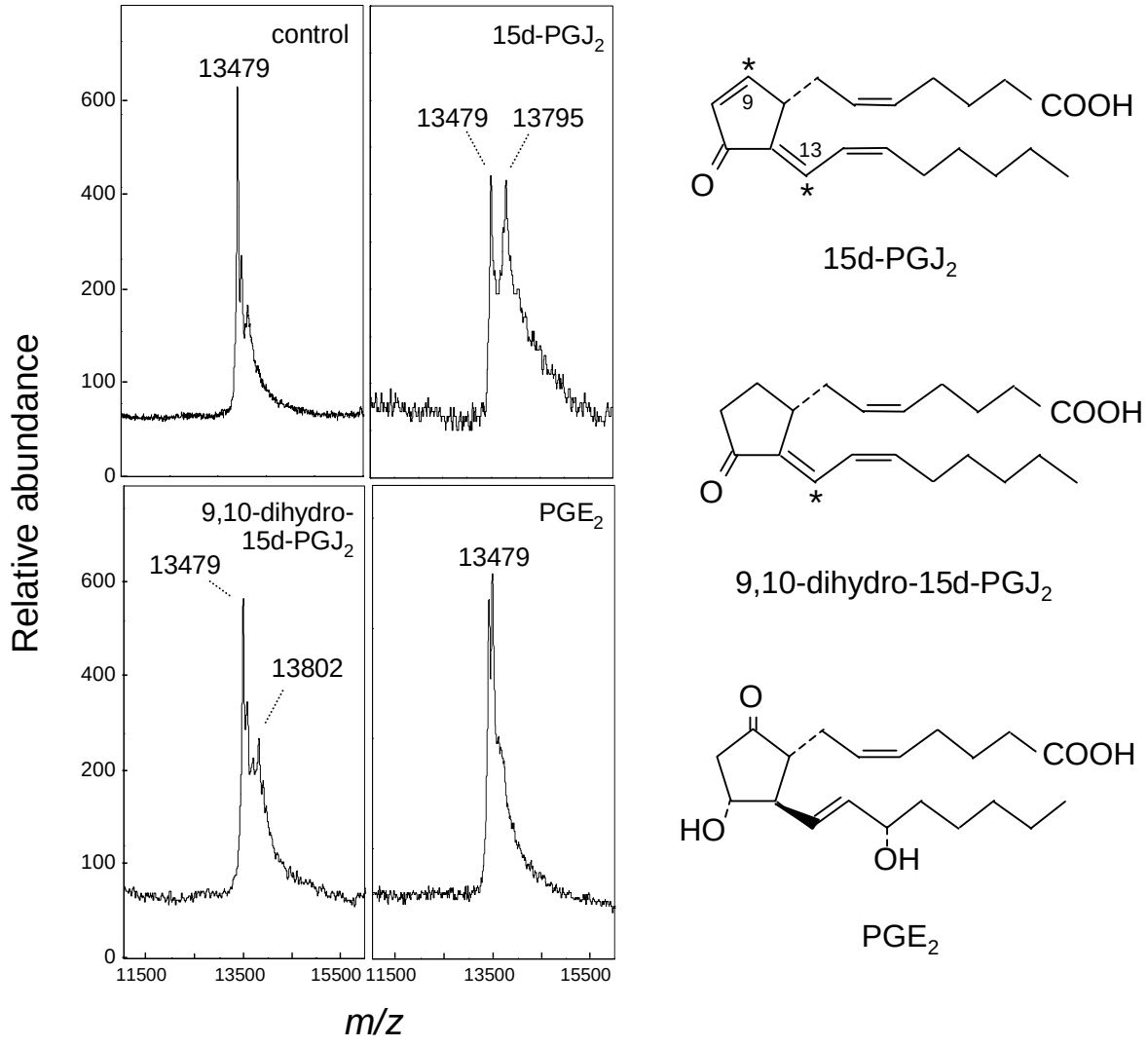

Fig. 4

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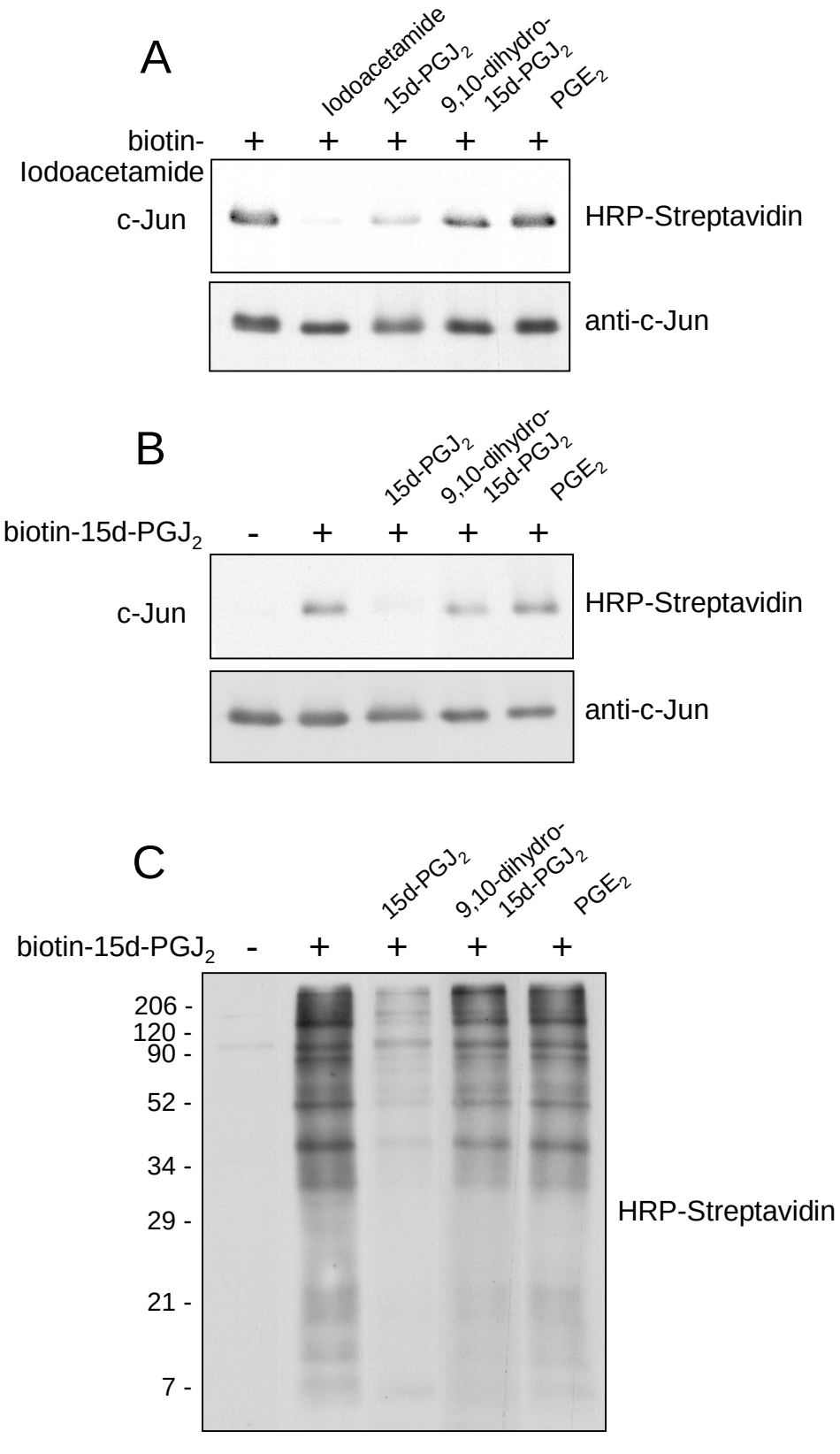

Fig. 5

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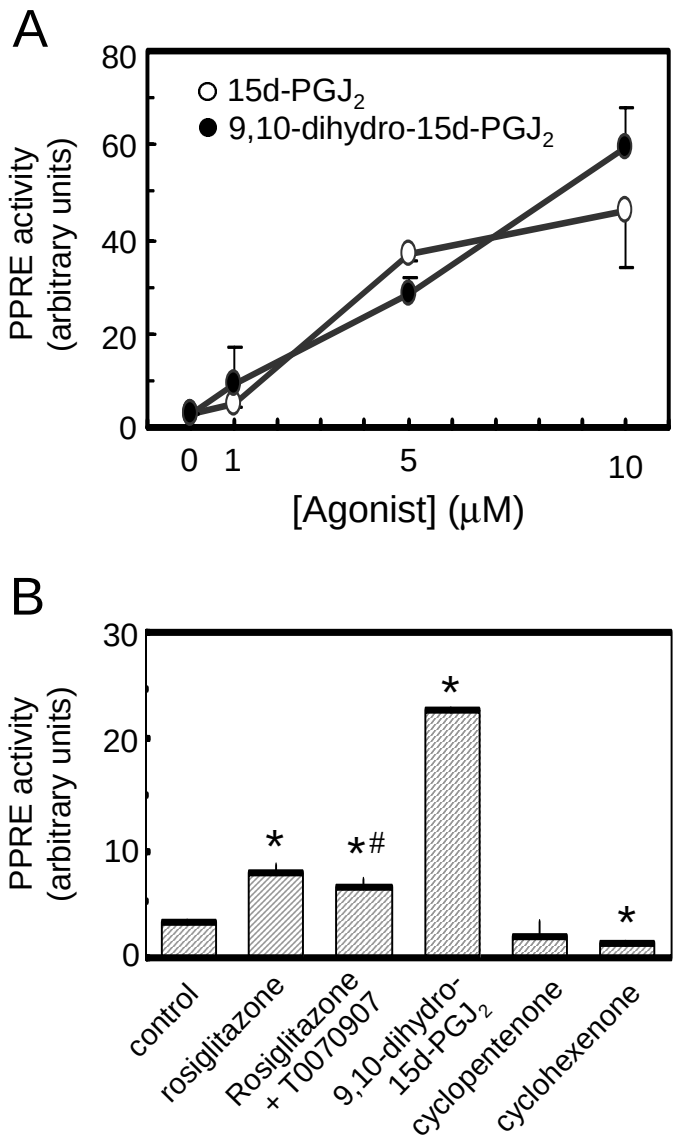

Fig. 6

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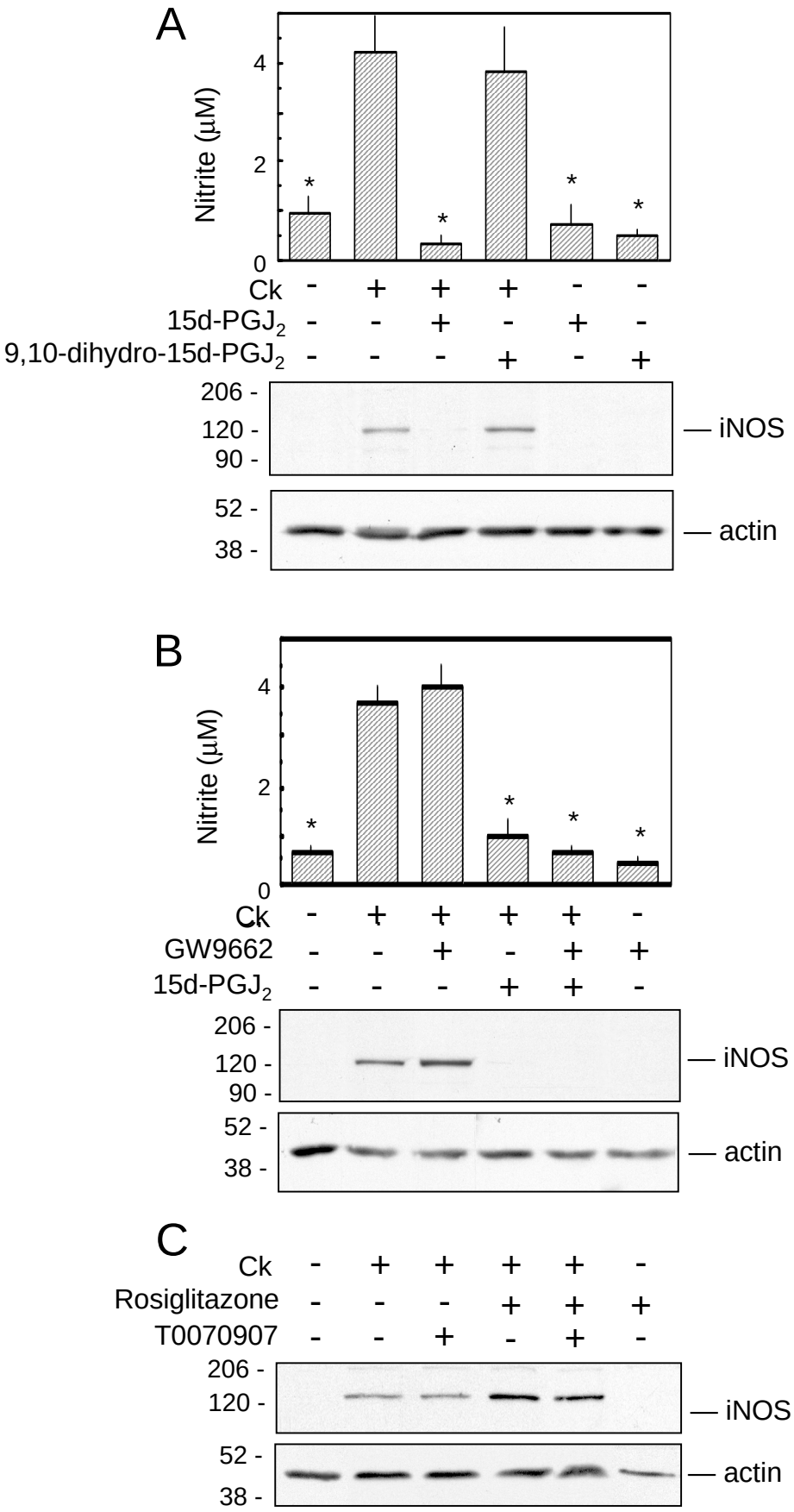

Fig. 7

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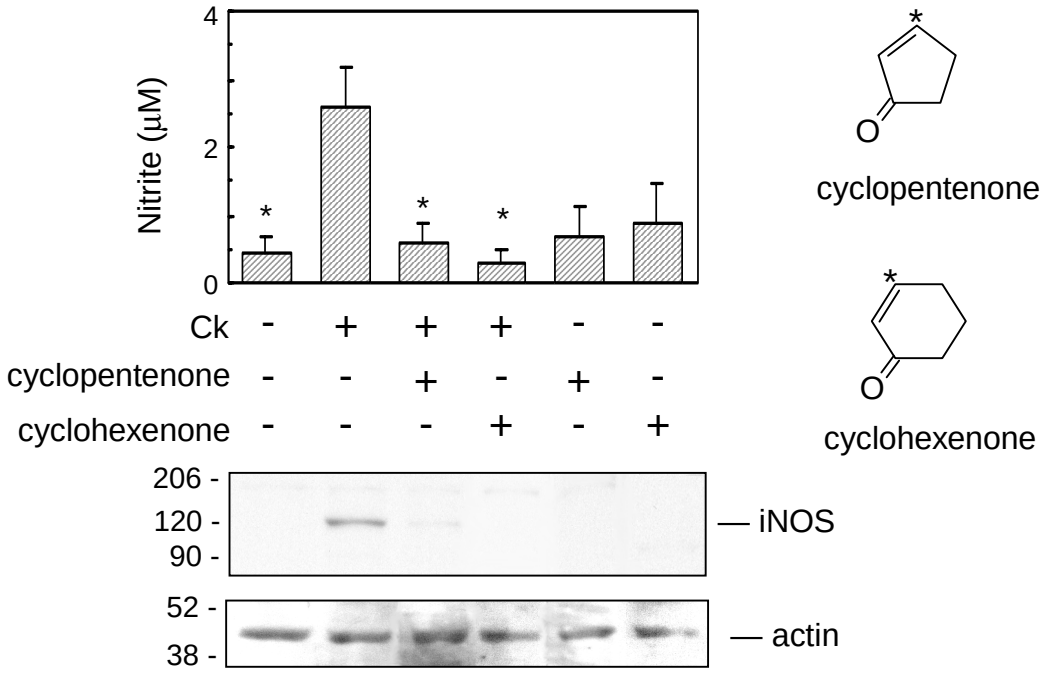

Fig. 8

Molecular Pharmacology Fast Forward. Published on August 18, 2004 as DOI: 10.1124/mol.104.002824
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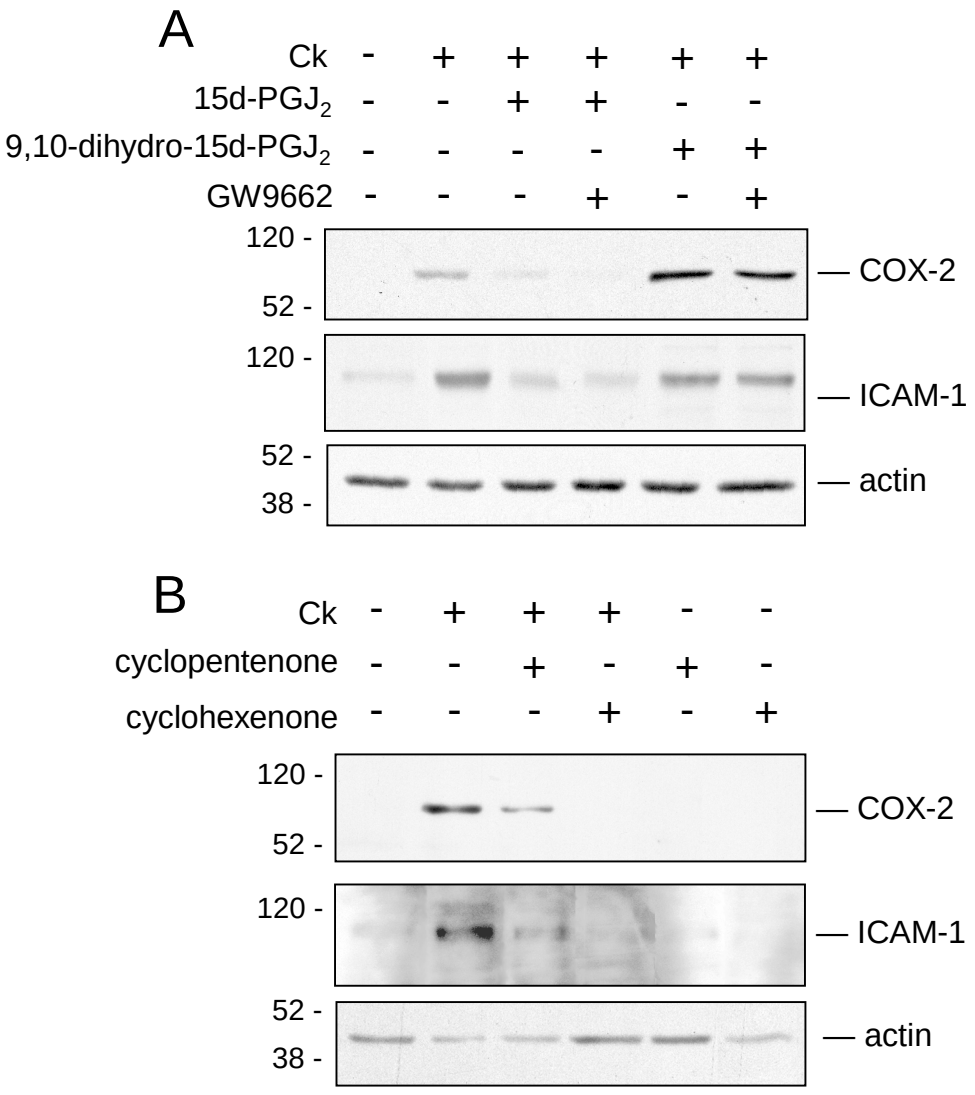

Fig. 9