15-Deoxy-\(\Delta^{12,14}\)-prostaglandin J\(_2\) Inhibition of NF-\(\kappa\)B-DNA Binding through Covalent Modification of the p50 Subunit*

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Cyclopentenone prostaglandins display anti-inflammatory activities and interfere with the signaling pathway that leads to activation of transcription factor NF-\(\kappa\)B. Here we explore the possibility that the NF-\(\kappa\)B subunit p50 may be a target for the cyclopentenone 15-deoxy-\(\Delta^{12,14}\)-prostaglandin J\(_2\) (15d-PGJ\(_2\)). This prostaglandin inhibited the DNA binding activity of recombiant p50 in a dose-dependent manner. The inhibition required the cyclopentenone moiety and could be prevented but not reverted by glutathione and dithiothreitol. Moreover, a p50 mutant with a C62S mutation was resistant to inhibition, indicating that the effect of 15d-PGJ\(_2\) was probably due to its interaction with cysteine residues by free radicals (11) or changes in the redox status of the protein. The covalent modification of p50 by 15d-PGJ\(_2\) was demonstrated by reverse-phase high pressure liquid chromatography and mass spectrometry analysis that showed an increase in retention time and in the molecular mass of 15d-PGJ\(_2\)-treated p50, respectively. The interaction between p50 and 15d-PGJ\(_2\) was relevant in intact cells. 15d-PGJ\(_2\) effectively inhibited cytokine-elicited NF-\(\kappa\)B activity in HeLa without reducing IκBα degradation or nuclear translocation of NF-\(\kappa\)B subunits. 15d-PGJ\(_2\) reduced NF-\(\kappa\)B DNA binding activity in isolated nuclear extracts, suggesting a direct effect on NF-\(\kappa\)B proteins. Finally, treatment of HeLa with biotinylated-15d-PGJ\(_2\) resulted in the formation of a 15d-PGJ\(_2\)-p50 adduct as demonstrated by neutravidin binding and immunoprecipitation. These results clearly show that p50 is a target for covalent modification by 15d-PGJ\(_2\) that results in inhibition of DNA binding.

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†‡ The abbreviations used are: NF-\(\kappa\)B, nuclear factor kappa-B; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; IκBα, inhibitory protein of NF-\(\kappa\)B; IL, interleukin; TNF, tumor necrosis factor; IKK, IκB kinase; PG, prostaglandin(s); MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; PPAR, peroxisome proliferator activated receptor.

The transcription factor NF-\(\kappa\)B\(^1\) plays a central role in inflammation, immunity, and cellular responses to stress. It is involved in the induction of numerous pro-inflammatory genes including iNOS, COX-2, adhesion molecules, and cytokines (1, 2). Its ability to rapidly elicit transcriptional responses to pro-inflammatory stimuli relies in its property of being presynthesized in the cytoplasm of cells but kept inactive by the binding of the inhibitory subunit, IκB. Pro-inflammatory stimuli such as IL-1 or TNF trigger a cascade of events that leads to the activation of the kinase complex IKK, or IκB-kinase (3, 4). Phosphorylation of IκB by IKK drives the ubiquitination and subsequent degradation of IκB by the proteasome. The active NF-\(\kappa\)B dimer, integrated by two members of the NF-\(\kappa\)B/Rel family of proteins (5), is thus free to translocate to the nucleus where it activates transcription. Additional regulation of NF-\(\kappa\)B activity may occur through the posttranslational modification of NF-\(\kappa\)B subunits, which may imply phosphorylation of p65 (6–8) or of p50 (9, 10), or modification of cysteine residues by free radicals (11) or changes in the redox status (12, 13).

Inflammatory conditions such as psoriasis, ulcerative colitis, and allergic eczema are associated with elevated levels of prostaglandins (PG). The up-regulation of COX-2 plays a key role in the overproduction of prostanooids associated with inflammatory situations (14, 15). Pro-inflammatory PG, such as PGE\(_2\) produced at the early stages of COX-2 expression, mediate vasodilatation, bone resorption, and fever (16). However, recent evidences indicate that COX-2 may play a dual role in inflammation, promoting at later stages the production of cyclopentenone PG, such as PGA\(_2\) and 15-deoxy-\(\Delta^{12,14}\)-PGJ\(_2\) (15d-PGJ\(_2\)), that may favor resolution of inflammation (17).

Cyclopentenone PG display singular biological activities, including inhibition of cell proliferation, induction of apoptosis, and inhibition of viral replication (18, 19). The COX metabolite 15d-PGJ\(_2\), is a negative modulator of cell activation by pro-inflammatory stimuli that inhibits the NF-\(\kappa\)B activation pathway acting at multiple levels (20, 21). 15d-PGJ\(_2\) acts as a ligand for the nuclear receptor PPAR\(\gamma\), which in turn may inhibit NF-\(\kappa\)B by sequestering co-activators needed for transcription (22). In addition, 15d-PGJ\(_2\) can directly inhibit NF-\(\kappa\)B activation either by blocking IKK activity (23), possibly through covalent modifications of critical cysteine residues in IKK\(\beta\) (24) or by interacting with a cysteine residue in the DNA-binding domain of the NF-\(\kappa\)B subunit p65 (25). These mechanisms may act in combination to inhibit transactivation of NF-\(\kappa\)B target genes, including COX-2 and iNOS, thus exerting a negative feedback on inflammatory processes (26, 27). These findings have lead to propose that cyclopentenone PG may be useful as a dithiothreitol; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; PPAR, peroxisome proliferator activated receptor.
anti-inflammatory agents. However, despite numerous studies on the cellular effects of NF-κB inhibition by 15d-PGJ₂, the nature of the interaction between 15d-PGJ₂ and NF-κB proteins has not been fully characterized.

The NF-κB subunit p50 possesses a critical cysteine residue in its DNA binding domain. This cysteine (Cys-62 in human p50) has been proposed to be the target for inhibition of NF-κB DNA binding activity by NO, either through S-nitrosylation (11) or NO-induced S-glutathionylation (28), and to mediate the effect of redox changes on NF-κB binding to DNA. Our results show that p50 is a target for covalent modification by 15d-PGJ₂, both in vitro and in intact cells, and that this interaction results in the inhibition of NF-κB binding to DNA.

**EXPERIMENTAL PROCEDURES**

*Materila...* 15-deoxy-Δ12,14-PGJ₂ was from Calbiochem-Novabiochem. 15-Deoxy-Δ12,14-PGJ₂ (mixture of isomers), carbaprostacyclin, PGE₂, and PGA₁ were from Cayman Chemical (Ann Arbor, MI). Horseradish peroxidase (HRP) conjugated anti-rabbit immunoglobulins were from Dako (Glostrup, Denmark). Polyonal anti-IgG (sc-731) and anti-p50, agarose conjugate (sc-371) and anti-p50, agarose conjugate (sc-1190AC), were from Santa Cruz Biotechnology (Santa Cruz, CA). The recombinant wild type and mutant DNA binding domains of human p50 (amino acids 36–385, GenBank accession number M55643) were expressed in Escherichia coli as hexahistidine fusion proteins and purified as described (28). This construct will be referred to as p50. GSH was from Sigma. Diisopropylcarbodiimide, EZ-link 5-(biotinamido)pentylamine and UltraLink-Immobilized NeutrAvidin-Plus were from Pierce. HRP-conjugated streptavidin was from Amersham Pharmacia Biotech. Cell culture media and supplements were from Life Technologies, Inc.

*Cell Culture...* HeLa cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. For experiments cells were incubated in RPMI with the indicated agents in the absence of serum. Potential toxicity of the reagents used was evaluated by trypan blue exclusion. According to these criteria, cell viability was above 90% under all experimental conditions studied.

**Preparation of Nuclear and Cytosolic Extracts**—Nuclear and cytosolic extracts were obtained as previously described in detail (29). Protein content was determined using the BCA protein assay from Pierce for cytosolic extracts and the Bradford protein assay from Bio-Rad (Munich, Germany) for nuclear extracts.

**Prostaglandin Treatment of Purified p50 and Nuclear Extracts**—Purified wild type or mutant p50 in 20 mM Tris, pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 0.7 μM 2-mercaptoethanol, 5% glycerol, 0.01% Nonidet P-40, were incubated with prostaglandins or vehicle for 2 h at 37 °C before being analyzed by EMSA or HPLC. Prostaglandinas were added in Me₂SO. Final Me₂SO concentration was 1% (v/v). This Me₂SO concentration did not interfere with EMSA. For *in vitro* treatments of nuclear extracts with 15d-PGJ₂, 3.5 μg of nuclear proteins in 20 mM Hepes, pH 7.9, 60 mM KCI, 0.25 mM EDTA, 0.125 mM EGTA, 0.2 mM DTT, 20% (v/v) glycerol plus 1 μg/ml protease inhibitors (leupeptin, pepstatin, antipain) were incubated with prostaglandins at 1 °C for 1 h at room temperature. Final Me₂SO concentration was kept below 0.6% (v/v).

**HPLC and MALDI-TOF Analysis of p50**—p50 incubated in the absence or presence of 15d-PGJ₂ was diluted (2:1) with 8 N guanidinium chloride and 0.1% trifluoroacetic acid, injected into a reverse-phase HPLC column (Vydac 218TP5415) equilibrated with solvent A (0.1% trifluoroacetic acid), and eluted with a gradient of 0 to 100% eluant B (90% acetonitrile in solvent A) fractions containing p50 were pooled and concentrated by evaporation. 0.5 μl of the fractions to be analyzed were added onto target and dried out along with 0.5 μl of saturated α-ciano-4-hydroxycinnamic acid matrix in water:acetonitrile (1:1) containing 0.1% trifluoroacetic acid. Mass spectrometry analysis by MALDI-TOF was performed using a Reflex II instrument (Bruker, Bremen, Germany), operating in reflectron mode. Calibration was performed externally using bovine serum albumin and control p50 as standards.

**EMSA and Western Blot Analysis**—Sense and antisense oligonucleotides corresponding to the upstream NF-κB site from the human COX-2 promoter (‘-GGGAGAGGGATCCCTGCGG-‘), located at –452 to –433 bases from the transcriptional start site, were annealed and labeled by incubation with [α-32P]dCTP (PerkinElmer Life Sciences) as previously described (29). Two ng of the labeled double stranded oligonucleotide (~20,000 cpm) were used per binding reaction. For EMSA of purified p50, 15 nM wild type or C62S mutant p50 treated with vehicle or cyclopentenone PG were incubated for 30 min at room temperature in the presence of labeled oligonucleotide, 0.2 mg/ml bovine serum albumin, and 1 μg of poly(dI-dC) carrier in binding buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 5% glycerol, 0.01% Nonidet P-40). For EMSA of nuclear proteins, binding reactions containing 3.5 μg of nuclear extract were carried out for 20 min on ice. Unless otherwise stated, DTT final concentration was 0.1 mM. Protein-DNA complexes were separated by electrophoresis on a non-denaturing 6% polyacrylamide gel and visualized by autoradiography. For competition experiments, a 200-fold excess of unlabeled NF-κB oligonucleo...
tide or of an unrelated oligonucleotide of sequence 5′-GGG CTT GAT GAG TCA GCC GGA CC-3′ were added to the binding reaction to ensure the specificity of the NF-κB-DNA complexes. The intensity of the complexes formed was quantitated by densitometry of the autoradiographic exposures of gels. Levels of p65 and p50 proteins in nuclear extracts and of IκB present in the cytosolic fraction of HeLa were assessed by SDS-polyacrylamide gel electrophoresis and immunoblot as previously described (29).

Preparation of Biotinylated 15d-PGJ2—The carboxyl group of 15d-PGJ2 was modified by amidation with EZ-link 5-(biotinamido)pentylamine by a modification of a previously described procedure (30). Briefly, 1 mg of 15d-PGJ2 (isomer mixture containing 90–95% of the trans, cis-12, 14 isomer) was made to react with 1 mg of (5-biotinamido)pentylamine in the presence of the condensing agent diisopropylcarbodiimide. The reaction was carried out overnight at room temperature in acetonitrile. Biotinylated 15d-PGJ2 was then extracted with ethyl acetate and purified through a silica gel column eluted with ethyl acetate:methanol (9:1). The recovery of biotinylated PG was estimated as 50% according to the instructions of the manufacturer for the determination of biotin. The modified PG was then dried under argon and dissolved in Me2SO for further use.

Biotinylated 15d-PGJ2 Labeling of p50 in HeLa Cells—HeLa cells at 50% confluence were incubated with 10 μM biotinylated 15d-PGJ2 for 2 h. Cells were washed twice with phosphate-buffered saline, harvested and lysed in 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 0.5% SDS, 0.1 mM 2-mercaptoethanol, plus protease inhibitors. Cell lysates containing 200 μg of protein were incubated batch-wise with 50 μl of NeutrAvidin-Plus beads for 1 h at 4 °C with constant shaking. Beads were washed five times with lysis buffer by centrifugation at 6000 rpm for 1 min. Proteins were eluted by incubating the beads in 0.05N NaOH for 1 min at room temperature to hydrolyze the protein-PG linkage (31, 32) and analyzed by SDS-polyacrylamide gel electrophoresis followed by immunodetection with anti-p50 antibody. In addition, cell lysates were immunoprecipitated with agarose-conjugated anti-p50 antibody according to the manufacturer’s instructions, and biotinylated proteins were subjected to Western blot and detection with HRP-conjugated streptavidin and ECL (Amersham Pharmacia Biotech).

RESULTS

15d-PGJ2 Inhibits p50 DNA Binding—To test the hypothesis that p50 may be a target for the inhibitory effect of 15d-PGJ2 on NF-κB activity we first studied the interaction between 15d-PGJ2 and p50 in vitro. Treatment of purified p50 with 15d-PGJ2 strongly inhibited its ability to bind DNA as determined by EMSA (Fig. 1). This effect was dose-dependent. Concentrations of the prostaglandin as low as 1 μM were sufficient to cause a significant inhibition (p < 0.05, n = 4). The potency of

![Fig. 3. Effect of GSH and DTT on the inhibitory effect of 15d-PGJ2. A, reaction mixtures containing 3 mM GSH and/or 5 μM 15d-PGJ2 were incubated for 15 min at 37 °C before the addition of p50 as depicted in the scheme. After an additional 2 h incubation DNA binding activity was estimated by EMSA in the absence or presence of 3 mM GSH, as indicated. B, p50 was incubated with 5 μM 15d-PGJ2 in the presence of the indicated concentrations of DTT for 2 h before being subjected to EMSA in the presence of 0.1 mM DTT for lanes 1–4 or 1.1 mM DTT for lanes 5 and 6. Results are average values ± S.E. of six (A) and four experiments (B) (*p < 0.05 by t test).

![Fig. 4. Effect of 15d-PGJ2 on the DNA binding activity of wild type or C62S mutant p50. Wild type (WT) or mutant p50 was incubated with the indicated concentrations of 15d-PGJ2 for 2 h at 37 °C before being analyzed by EMSA. Representative autoradiographic exposures are shown. Results are average values ± S.E. from four experiments and are expressed as percentage of the values obtained in the absence of 15d-PGJ2 (*p < 0.05 with respect to vehicle by t test).]
the inhibition was also proportional to the time of pre-incubation of p50 with 15d-PGJ2.

**Effect of Various Prostanoids on p50 DNA Binding**—The structural requirements for the inhibition by 15d-PGJ2 were explored by studying the effect of several PG on p50 DNA binding (Fig. 2). Of the compounds tested, 15d-PGJ2 caused the most potent inhibitory effect. PGA1 moderately interfered with p50 DNA binding while PGE2 and carbaprostacyclin (cPGI2) were virtually ineffective.

**Effect of GSH on the Inhibitory Effect of 15d-PGJ2**—The detoxification of PG in cells may involve conjugation with GSH (33). We therefore studied the ability of GSH to prevent the inhibitory effect of 15d-PGJ2 in vitro by adding GSH before or after treatment of p50 with 15d-PGJ2, as depicted in Fig. 3A. Treatment of p50 with 15d-PGJ2 in the absence of GSH induced a 50% inhibition of DNA binding (Fig. 3A, lanes 1 and 2). However, incubation of 15d-PGJ2 with 3 mM GSH prior to addition of p50 strongly diminished the inhibitory effect (8% inhibition, Fig. 3A, compare lanes 3 and 4). In contrast, addition of GSH after incubation of p50 with 15d-PGJ2 was not able to fully restore DNA binding activity (39% inhibition persisted in this case; Fig. 3A, compare lanes 5 and 6). As expected based on previous observations (34), in all incubations containing GSH, the overall DNA binding activity of p50 was improved. These results suggest that GSH may prevent but not revert the inhibitory effect of 15d-PGJ2 on p50 DNA binding.

We next explored whether DTT could exert a protective effect similar to that of GSH. Incubation of p50 with 15d-PGJ2 in the
results show that 15d-PGJ2 covalently modifies p50. In extracts and in intact cells. Both 15d-PGJ2 and biotinylated log and studied its interaction with p50 both in isolated nuclear in response to cytokine treatment (Fig. 6).

15d-PGJ2 Forms a Covalent Adduct with p50—To gain insight into the interaction between 15d-PGJ2 and p50 we first subjected the protein to reverse-phase HPLC analysis (Fig. 5A). Control p50 eluted as a single peak with a retention time of 43.7 min. 15d-PGJ2-treated p50 eluted in a major peak with a retention time of 44.5 min and several minor peaks. In contrast, the HPLC elution profile of the C62S p50 mutant did not vary significantly after treatment with 15d-PGJ2 (Fig. 5A, right panel). The fractions corresponding to the major peaks from control and 15d-PGJ2-treated wild type p50 were subsequently analyzed by mass spectrometry. The MALDI-TOF spectrum of control p50 showed a peak of \( m/z = 40,600 \), which corresponds to the calculated molecular mass of the p50 construct used, together with peaks of \( m/z = 20,300 \) (doubly charged) and 81,230 (Fig. 5B, lower panel). The spectrum of 15d-PGJ2-treated p50 showed peaks of \( m/z = 41,241 \), \( m/z = 20,618 \) (doubly charged), which are compatible with the formation of a covalent adduct between one molecule of p50 and two molecules of 15d-PGJ2 (expected \( m/z = 41,232 \) and 20,616 respectively), and a peak of \( m/z = 82,526 \) (Fig. 5B, upper panel). These results show that 15d-PGJ2 covalently modifies p50.

Effect of 15d-PGJ2 on NF-kB Activity in HeLa Cells—We were then interested in assessing the relevance of cyclopentenone PG modification of p50 in cells. For this purpose we first explored the effect of 15d-PGJ2 on cytokine-elicited NF-kB activation in intact cells. Treatment of HeLa with 15d-PGJ2 before stimulation with IL-1β plus TNF-α clearly reduced NF-kB activity in nuclear extracts as determined by EMSA (Fig. 6A). However, the inhibition of NF-kB DNA binding activity did not correlate with a reduction in the nuclear levels of p65 and p50 subunits, suggesting that NF-kB activation was not impaired in 15d-PGJ2-treated cells. This was confirmed by the observation that 15d-PGJ2 did not interfere with the phosphorylation of IkBα as estimated from the appearance of a slower migrating form or with the degradation of IkBα protein in response to cytokine treatment (Fig. 6B). These observations indicate that in intact HeLa cells, the direct inhibition of the DNA binding ability of nuclear NF-kB may be the main mechanism by which 15d-PGJ2 interferes with NF-kB activity.

Modification of p50 by a Biotinylated 15d-PGJ2 Derivative in Intact Cells—To assess if inhibition of NF-kB DNA binding elicited by 15d-PGJ2 in HeLa could be due to the covalent modification of p50 we prepared a biotinylated 15d-PGJ2 analog and studied its interaction with p50 both in isolated nuclear extracts and in intact cells. Both 15d-PGJ2 and biotinylated 15d-PGJ2 Inhibition of NF-κB by p50 Modification

**DISCUSSION**

Cyclopentenone PG display varied biological activities. They have been reported to modulate adipocyte differentiation (35), presence of DTT reduced the potency of the inhibition in a dose-dependent manner (Fig. 3B). The extent of inhibition of p50-DNA complex formation was reduced from 81% when p50 had been pre-incubated with 15d-PGJ2 in the absence of DTT (lanes 1 and 2) to 63% when 0.1 mM DTT had been included in the pre-incubation mixture (lanes 3 and 4). In both cases EMSA was performed in the presence of 0.1 mM DTT, which was not sufficient to revert the inhibition. When 1 mM DTT was used throughout the experiment, the inhibitory effect of 15d-PGJ2 was completely prevented (Fig. 3B, lanes 5 and 6). These results suggest that the inhibitory effect of 15d-PGJ2 may arise from its interaction with cysteine residues in p50.

**Inhibition of p50 DNA Binding by 15d-PGJ2 Requires Cys-62**—The p50 DNA binding domain contains a cysteine residue that has been proposed to be a target for redox regulation (12). We therefore explored whether this cysteine was important for the effect of 15d-PGJ2. As it can be observed in Fig. 4, the DNA binding ability of a p50 mutant in which Cys-62 was substituted by serine was virtually unaffected by 15d-PGJ2 treatment. These results suggest that 15d-PGJ2 may inhibit p50 DNA binding by interacting with Cys-62.

**15d-PGJ2 Inhibition of NF-κB**

**A**

![Image](http://www.jbc.org/)

**Fig. 7. Modification of p50 by biotinylated 15d-PGJ2. A** nuclear extracts obtained from HeLa stimulated for 30 min with IL-1β/TNF-α were incubated with 15d-PGJ2 or biotinylated 15d-PGJ2, at the indicated concentrations for 1 h at room temperature before being analyzed by EMSA. Results are average values ± S.E. of four experiments. B, exponentially growing HeLa cells were treated with biotinylated 15d-PGJ2 for 2 h. Cell lysates were incubated with Immobilized NeutrAvidin or with anti-p50-agarose, as indicated. The presence of p50 in the indicated fractions from the neutravidin assay was detected by Western blot, and the incorporation of biotinylated 15d-PGJ2 into p50 immunoprecipitates was detected with HRP-streptavidin and ECL. ECL exposures were carried out for 20 s for lanes 1 and 2, and for 2 min for lanes 3 and 4.

15d-PGJ2 directly inhibited the DNA binding of the native NF-κB dimers present in nuclear extracts isolated from IL-1β/TNF-α-stimulated HeLa, although the biotinylated PG was slightly less effective (Fig. 7A).

To demonstrate the modification of p50 by cyclopentenone PG in intact cells, HeLa were treated with biotinylated 15d-PGJ2, and cell lysates were incubated batch-wise with NeutrAvidin beads. After extensive washing, proteins bound to the resin through biotinylated PG were eluted by hydrolysis of the protein-PG linkage with 0.05 M NaOH (31), and p50 present in the eluate was detected by immunoblot (Fig. 7B). Alternatively, cell lysates were subjected to immunoprecipitation with an anti-p50 antibody and the presence of biotinylated 15d-PGJ2-modified proteins was assessed by Western blot and detection with HRP-conjugated streptavidin. (Fig. 7B). These results clearly show that p50 is a target for covalent modification by cyclopentenone PG in intact cells.
cell proliferation, and apoptosis (19), heat shock response, and viral replication (36). The mechanisms that mediate these effects may be multiple. Certain cyclopentenone PG, in particular 15d-PGJ₂, has been shown to act as an endogenous ligand for the transcription factor PPARγ, causing the activation of this factor that plays key roles in adipocyte differentiation and modulation of inflammation (37). In addition, recent evidence points to the occurrence of direct effects of cyclopentenone PG, which are independent from PPARγ. Cyclopentenone PG are reactive compounds that possess an α, β-unsaturated carbonyl group in the cyclopentenone ring. This group may react with sulphydryl groups of cysteine residues of proteins or of the tripeptide glutathione (38). 15d-PGJ₂ also has a double bond at position 12 that may participate in these interactions. This PG abolishes the inhibitory effect, thus supporting the irreversible modification of p50 C62S mutant indicates that the cysteine(s) modified by 15d-PGJ₂, although the lack of inhibitory effect of 15d-PGJ₂ on the p50 C62S mutant indicates that the modification of this cysteine accounts for the inhibition of DNA binding. Moreover, treatment of this mutant with 15d-PGJ₂ did not alter its elution profile in reverse phase HPLC analysis. Nevertheless, given the high reactivity of 15d-PGJ₂, the modification of p50 at other cysteine residues under our incubation conditions cannot be excluded. Mass spectrometry of a fraction from the HPLC analysis of 15d-PGJ₂-treated p50 eluting at 47 min gave a molecular mass of 41,877, which could be compatible with the formation of an adduct between one molecule of p50 and four PG molecules.

The formation of adducts between cyclopentenone PG and glutathione has been shown to take place by Michael’s addition (38). This process may be involved in the detoxification of cyclopentenone PG by cells and it can occur both non-enzymatically and/or, at a higher rate, in a process catalyzed by GST (38, 39). Here we show that pre-incubation of 15d-PGJ₂ with glutathione effectively reduces the inhibitory effect of the former on p50 DNA binding. This could be due to the sequestration of the PG in the form of GSH-15d-PGJ₂ adducts. Interestingly, addition of GSH after treatment of p50 with 15d-PGJ₂ did not abolish the inhibitory effect, thus supporting the irreversible nature of the 15d-PGJ₂–protein interaction under physiological conditions (32). Our results also add biochemical support to evidences pointing out the importance of cellular GSH levels and/or GST activity in determining the susceptibility to the inhibitory effects of 15d-PGJ₂ (25, 32).

The modification of p50 by 15d-PGJ₂ can be evidenced in intact cells. Several approaches have been previously used to follow the fate of cyclopentenone PG in cells. By using radioactively labeled 15d-PGJ₂, it was shown that this PG was taken up by cells and accumulated into nuclei where it was bound to the protein fraction (31). The labeling of several cellular proteins in the 40- to 60-kDa range was observed by incubating K562 cells with biotinylated PG₉₅ (30). We have used biotinylated 15d-PGJ₂ to explore the incorporation of this PG into p50. Our results show that biotinylated 15d-PGJ₂ displays a similar potency to that of 15d-PGJ₂ as inhibitor of p50 DNA binding under in vitro conditions, and that it modifies p50 when added to intact cells. These results raise the possibility that p50 is a target for the anti-inflammatory effects of 15d-PGJ₂, at least at pharmacological doses employed in most biochemical studies. The relevance of the modification of p50 and the inhibition of DNA binding by endogenously generated 15d-PGJ₂ remains to be established. In a model of pleurisy in rats, cyclopentenone PG have been shown to reach concentrations between 2.5 and 5 nM in the cell-free inflammatory exudate (17), although no information was available about the intracellular or intranuclear levels. In addition, the formation of reactive cyclopentenone compounds has been documented in vivo, and they have been detected esterified to lipids in the liver of rats in amounts that increased markedly after oxidant injury (up to 600 ng/g) (39). Because cyclopentenone PG tend to accumulate in the nuclei of cells (31), it is possible to speculate that the concentrations of endogenous 15d-PGJ₂ reached in cell nuclei under certain pathophysiological conditions might be compatible with the formation of the p50 modification.

In conclusion, our results identify the p50 subunit of the NF-κB transcription factor as a target for modification by 15d-PGJ₂, providing a biochemical basis for some of the anti-inflammatory actions of this PG. In addition, the use of biotinylated 15d-PGJ₂ might prove a valuable approach for the identification of other potential targets for the actions of cyclopentenone PG in cells.

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15d-PGJ₂ Inhibition of NF-κB by p50 Modification
