Cyclopentenone prostaglandins may interfere with cellular functions by multiple mechanisms. The cyclopentenone 15-deoxy-Δ^{12,14}-prostaglandin J₂ (15d-PGJ₂) has been reported to inhibit the activity of the transcription factor AP-1 in several experimental settings. We have explored the possibility of a direct interaction of 15d-PGJ₂ with AP-1 proteins. Here we show that 15d-PGJ₂ covalently modifies c-Jun and directly inhibits the DNA binding activity of AP-1. The modification of c-Jun occurs both in vitro and in intact cells as detected by labeling with biotinylated 15d-PGJ₂ and mass spectrometric analysis. Attachment of the cyclopentenone prostaglandin occurs at cysteine 269, which is located in the c-Jun DNA binding domain. In addition, 15d-PGJ₂ can promote the oligomerization of a fraction of c-Jun through the formation of intermolecular disulfide bonds or 15d-PGJ₂-bonded dimers. Our results identify a novel site of interaction of 15d-PGJ₂ with the AP-1 activation pathway that may contribute to the complex effects of cyclopentenone prostaglandins on the cellular response to pro-inflammatory agents. They also show the first evidence for the induction of protein cross-linking by 15d-PGJ₂.

Cyclopentenone prostaglandins (cyPG)⁷ are products of the cyclooxygenase pathway, which are generated by the spontaneous dehydration of certain PG. Dehydration of PGD₂ gives rise to PGJ₂ and 15-deoxy-Δ^{12,14}-prostaglandin J₂ (15d-PGJ₂), whereas PGD₂ is converted into PGA₂ (1). cyPG have been detected in situations associated with an increase in cyclooxygenase-2 expression and PG production, such as acute and chronic inflammatory conditions (2–4). In addition, generation of cyPG has been reported in several experimental models, such as activated macrophages and mesangial cells (3, 5). cyPG have received considerable attention during the past decade due to their action as antiproliferative agents and inducers of apoptosis in a variety of normal and cancer cell types (6, 7). cyPG also display anti-inflammatory effects both in cellular and in animal models (2, 8, 9). This has led to the proposal that cyPG, and in particular 15d-PGJ₂, may contribute to the resolution of inflammation through various mechanisms that include the inhibition of the expression of pro-inflammatory genes, the induction of apoptosis in activated macrophages, and the activation of the transcription factor peroxisome proliferator activated receptor γ (PPARγ) (10, 11). Thus, cyPG have been envisaged as potential therapeutic agents against inflammatory and proliferative diseases (1).

cyPG have been found to reduce the activity of transcription factors key for the inflammatory response, like NF-κB and AP-1, by acting at multiple levels. 15d-PGJ₂ has been shown to interfere with the signaling pathway leading to NF-κB activation through the direct inhibition of the IκB kinase (12, 13), the PPARγ-dependent sequestration of coactivators (14), and the impairment of the ability of NF-κB to bind DNA (15, 16). Inhibition of DNA binding is the consequence of the formation of an adduct between 15d-PGJ₂ and NF-κB proteins, which has been demonstrated both in vitro and in intact cells (16). The transcription factor AP-1 is formed by homo- or heterodimers of proteins of the Fra family. 15d-PGJ₂ has been shown to reduce AP-1 activation in several experimental systems (11, 17) by mechanisms that may involve activation of PPARγ (11, 18) and/or inhibition of c-Jun NH₂-terminal kinase (JNK) (19). However, the possibility of a direct interaction of cyPG with AP-1 proteins has not been explored.

cyPG are reactive compounds that possess an α,β-unsaturated carbonyl group in the cyclopentenone ring. This group may react with sulfhydrol groups of cysteine residues of proteins by Michael’s addition (20), resulting in an alteration of protein function (16). The DNA binding domain of human c-Jun possesses two functionally important cysteine residues. Cys²⁶⁹ is located in close contact with DNA, and it is involved in the reoxidation-dependent regulation of DNA binding by means of specifically targeted S-glutathionylation (21–23). Cys³⁵² is located in the leucine zipper region and participates in the homodimerization of proteins of the Fos family through the formation of an intermolecular disulfide bridge (24). Here we show that 15d-PGJ₂ can form a covalent adduct with c-Jun and directly inhibit DNA binding. These observations unveil a novel PPARγ-independent mechanism that may contribute to the overall effect of cyPG on the AP-1 signaling pathway.

**EXPERIMENTAL PROCEDURES**

Materials—Human recombinant c-Jun (full-length protein) was from Promega (Madison, WI). The hexahistidine fusion construct of the re-
combinant human c-Jun fragment, amino acids 223–327 of the translated sequence was from the GenBank™ accession number AF241111 (sequence of the construct, MBGISHHHHHHHSQALKERQFTQVMPPGE-TPPLSIDPESQERIIARKRNRAIAKCRKRLERKVLRTLQNSLMAANLREQVALQKVMVNVNSCQMLLTTQQ), and the C2695s mutant were the generous gifts of Dr. Pineda-Molina and Dr. S. Lamars and have been described previously (22, 23). Anti-c-Jun antibodies sc-044 and sc-045, anti-c-Fos sc-413, and anti-PPARγ sc-7196 were from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Dako (Glostrup, Denmark). 15-Deoxy-Δ9,11,14-prostaglandin J2 was from Calbiochem-Novabiochem and from Cayman Chemical (Ann Arbor, MI). Biotinylated 15d-PGJ2 was prepared as described previously (16).

**Cell Culture**—Rat mesangial cells (RMC) (25) and HeLa cells were grown in RPMI 1640 medium (Invitrogen) containing 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. NIH-3T3 fibroblasts and RAW264.7 macrophages were grown in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum and antibiotics. Treatment with 15d-PGJ2 was carried out in serum- and phenol red-free medium. Levels of 15d-PGJ2 in the cell culture supernatant of RAW264.7 macrophages, incubated in the absence or presence of 10 μM bacterial lipopolysaccharide for 24 h, were assessed with an enzyme immunoassay from Assay Designs, Inc. (Ann Arbor, MI) following the instructions of the manufacturer.

**Preparation of Total Cell Lysates and Nuclear Extracts**—Cells were lysed in 50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 0.1 mM β-mercaptoethanol, 1% Nonidet P-40, or RIPA buffer containing protease inhibitors for 30 min on ice. DNA was disrupted by several forced passes through a narrow needle, and cellular debris was removed by centrifugation for 5 min at 10,000 × g at 4 °C. Nuclear extracts were obtained as described previously in detail (26). Protein content was determined using the BCA protein assay from Pierce for the cell lysates and the Bradford protein assay from Bio-Rad for the nuclear extracts.

**Western Blot and Detection of Incorporation**—Cell lysates and nuclear extracts were analyzed in 10% or 15% polyacrylamide gels. Gels were stained with Coomassie Blue R-250 and analyzed by densitometry of the ECL exposures.

**MALDI-TOF Analysis**—For MALDI, peptides were analyzed by MALDI-TOF mass spectrometry in the reflectron mode. A saturated solution of c-cyano-4-hydroxy-cinnamic acid in acetonitrile/water (1:2) with 0.1% trifluoroacetic acid was used as the matrix. External calibration was performed, using the monoisotopic peaks of angiotensin (m/z 1046.5), adrenocorticotropin hormone (m/z 2,465.2), and the matrix (c-cyano-4-hydroxycinnamic acid, m/z 379) recorded in a single spectrum. Typically, 50–100 laser shots were summed into a single mass spectrum for analysis.

**Analysis of c-Jun Dimerization**—Dimerization assays were performed essentially as described (22). c-Jun (223–327) was incubated under the conditions described above, except that DTT final concentration was 0.5 mM, in the absence or presence of 10 μM 15d-PGJ2, or 1 mM H2O2 for 30 min at 37 °C. Reactions were stopped by the addition of iodoacetamide at a final concentration of 50 mM and a further 30-min incubation at r.t. The reversibility of dimer formation was assessed by addition of 10 mM DTT and incubation at r.t. for 30 min before iodoacetamide addition. Aliquots of the incubation mixtures containing 1.7 μg of protein were analyzed by non-reducing SDS-PAGE on 15% polyacrylamide gels. Gels were stained with Coomassie Blue R-250 and analyzed by image scanning.

**RESULTS**

**15d-PGJ2 Inhibits AP-1 DNA Binding Activity Both in Intact Cells and in Vitro**—15d-PGJ2 has been reported to inhibit the transcriptional activity of AP-1 and to reduce its DNA binding activity in several cell types (11, 19, 27), through PPARγ-dependent or -independent mechanisms. We were interested in studying whether the direct modification of AP-1 constituents by 15d-PGJ2 may be involved in the inhibitory effect. Treatment of HeLa cells with 10 μM 15d-PGJ2 reduced the DNA binding activity of AP-1, as evaluated by EMSA, by ~30% (Fig. 1A). A similar inhibitory effect was evidenced in RMC (Fig. 1B).

**Figure 1** C-Jun in Intact Cells—HeLa or NIH-3T3 cells were incubated in the presence of 10 μM biotinylated 15d-PGJ2 for 2 h. Cells were lysed in RIPA, and biotinylated proteins were purified by adsorption onto Soft-Link avidin (Pierce, Madison, WI) following the manufacturer’s instructions. The presence of c-Jun, or other proteins of interest, in the eluate was assessed by Western blot. In addition, cell lysates were immunoprecipitated with anti-c-Jun antibodies, and the incorporation of biotinylated 15d-PGJ2 into the immunoprecipitated protein was monitored by Western blot detection with HRP-conjugated streptavidin.

**Analysis of the Interaction between 15d-PGJ2 and Recombinant c-Jun in Vitro**—Full-length recombinant human c-Jun or purified c-Jun fragments were incubated at a final concentration of 5 μM in 20 mM Tris-HCl, pH 7.0, 45 mM NaCl, 5 mM MgCl2, 0.14 mM β-mercaptoethanol, 0.1 mM DTT, 0.01% Nonidet P-40, 1% glycerol in the presence of 15d-PGJ2 (150 μM) or vehicle (MeSO4) for 2 h at 4 °C. Several incubation mixtures were analyzed either by MALDI-TOF mass spectrometry or by SDS-PAGE on 15% polyacrylamide gels followed by staining with Coomassie Blue or Western blot and detection of incorporated biotin with HRP-conjugated streptavidin and ECL (Amersham Biosciences). By using the c-Jun-(223–327) construct as an acceptor, the interaction between biotinylated 15d-PGJ2 (2 to 20 μM) was dose-dependent and proportional to the concentration of protein and PG, as estimated from the densitometry of the ECL exposures.

**MALDI-TOF Analysis**—For analysis of control and 15d-PGJ2-treated c-Jun, trifluoroacetic acid was added to the incubation mixtures at a final concentration of 0.1%, and peptides were purified on ZipTip C18 (Millipore, Bedford, MA). The laser desorption/ionization data 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 μl) of the sample solution and the matrix were spotted on the target and analyzed. External calibration was performed, using the calibration standard II (Bruker Daltonics, Bremen, Germany), and samples were analyzed in the linear mode. For analysis of c-Jun monomer and dimer, 15d-PGJ2-treated c-Jun was subjected to SDS-PAGE, and gels were stained with Coomassie Blue. Bands corresponding to the monomer and dimer species were excised and digested in-gel with trypsin. The resulting tryptic peptides were analyzed by MALDI-TOF mass spectrometry in the reflectron mode. A saturated solution of c-cyano-4-hydroxy-cinnamic acid in acetonitrile/water (1:2) with 0.1% trifluoroacetic acid was used as the matrix. External calibration was performed, using the monoisotopic peaks of angiotensin (m/z 1046.5), adrenocorticotropic hormone (m/z 2,465.2), and the matrix (c-cyano-4-hydroxycinnamic acid, m/z 379) recorded in a single spectrum. Typically, 50–100 laser shots were summed into a single mass spectrum for analysis.

**Binding of Biotinylated 15d-PGJ2 to c-Jun in Intact Cells—**HeLa or NIH-3T3 cells were incubated in the presence of 10 μM biotinylated 15d-PGJ2 for 2 h. Cells were lysed in RIPA, and biotinylated proteins were purified by adsorption onto Soft-Link avidin (Promega, Madison, WI) and washed three times with RIPA buffer containing 0.1% Nonidet P-40, or RIPA buffer containing protease inhibitors for 30 min on ice. The resulting peptides were analyzed by MALDI-TOF mass spectrometry or by SDS-PAGE on 15% polyacrylamide gels. Gels were stained with Coomassie Blue R-250 and analyzed by image scanning.
for 16 h were pretreated with 10 μM 15d-PGJ2 for 10 min before stimulation with a combination of interleukin-1β (2 ng/ml) plus tumor necrosis factor-α (25 ng/ml) (Ck) for 15 min. Nuclear extracts were obtained and analyzed by EMSA. When indicated EMSA was performed in the presence of an excess of unlabeled AP-1 (comp) or an unrelated oligonucleotide. C, the components of the AP-1-DNA complex in nuclear extracts from RMC were explored by performing EMSA in the presence of the indicated antibodies against several members of the Fra family. D, the levels of c-Jun and c-Fos in the nuclear extracts from RMC, under the same experimental conditions as in B, were assessed by Western blot. Results shown are representative of three experiments with similar results.

Fig. 1. 15d-PGJ2 inhibits AP-1 DNA binding in intact cells. HeLa cells (A) or rat mesangial cells (B) serum-starved for 16 h were pretreated with 10 μM 15d-PGJ2 for 10 min before stimulation with a concentration of interleukin-1β (2 ng/ml) plus tumor necrosis factor-α (25 ng/ml) (Ck) for 15 min. Nuclear extracts were obtained and analyzed by EMSA. When indicated EMSA was performed in the presence of an excess of unlabeled AP-1 (comp) or an unrelated oligonucleotide. C, the components of the AP-1-DNA complex in nuclear extracts from RMC were explored by performing EMSA in the presence of the indicated antibodies against several members of the Fra family. D, the levels of c-Jun and c-Fos in the nuclear extracts from RMC, under the same experimental conditions as in B, were assessed by Western blot. Results shown are representative of three experiments with similar results.

under denaturing conditions, as evidenced by Western blot and detection with HRP-conjugated streptavidin (Fig. 3A). Labeling of c-Jun was completely prevented by excess 15d-PGJ2, suggesting that this interaction is specific. The incorporation of 15d-PGJ2 was dose-dependent and could be detected with nanomolar concentrations of this cyPG (Fig. 3B). We have shown above that the interaction of 15d-PGJ2 with c-Jun precludes DNA binding. We then explored whether DNA binding by c-Jun would prevent incorporation of 15d-PGJ2. As shown in Fig. 3C, preincubation of c-Jun with an oligonucleotide containing the consensus site for binding of AP-1 markedly reduced incorporation of biotinylated 15d-PGJ2. In contrast, a mutated AP-1 oligonucleotide or an oligonucleotide with a consensus sequence for binding of NF-kB did not display this protective effect. These results suggest that the interaction of 15d-PGJ2 with c-Jun involves a region closely implicated in DNA binding. The region of c-Jun that is in close contact with DNA contains a cysteine residue (cysteine 269), which has been shown to be specifically targeted by S-glutathionylation under oxidative and nitrosative stress conditions. Treatment of c-Jun with the thiol reagent iodoacetamide blocked incorporation of biotinylated 15d-PGJ2 (Fig. 3D). This confirms the requirement of free cysteine residues for attachment of this cyPG. Moreover, preincubation of c-Jun with oxidized glutathione or nitrosoglutathione under conditions previously found to specifically induce the glutathionylation of cysteine 269 also precludes binding of biotinylated 15d-PGJ2 (Fig. 3D). These results point to the involvement of cysteine 269 of c-Jun in the interaction with 15d-PGJ2.

The interaction between 15d-PGJ2 and c-Jun was next studied by mass spectrometry. Untreated c-Jun (223–327) d did not display this protective effect. These results suggest that the interaction of 15d-PGJ2 with c-Jun involves a region closely implicated in DNA binding. The region of c-Jun that is in close contact with DNA contains a cysteine residue (cysteine 269), which has been shown to be specifically targeted by S-glutathionylation under oxidative and nitrosative stress conditions. Treatment of c-Jun with the thiol reagent iodoacetamide blocked incorporation of biotinylated 15d-PGJ2 (Fig. 3D). This confirms the requirement of free cysteine residues for attachment of this cyPG. Moreover, preincubation of c-Jun with oxidized glutathione or nitrosoglutathione under conditions previously found to specifically induce the glutathionylation of cysteine 269 also precludes binding of biotinylated 15d-PGJ2 (Fig. 3D). These results point to the involvement of cysteine 269 of c-Jun in the interaction with 15d-PGJ2.
Interaction of 15d-PGJ2 with c-Jun

(Fig. 5A). The electrophoretic mobility of the 15d-PGJ2-induced c-Jun dimer was different from that induced by H2O2 treatment. The 15d-PGJ2-induced dimer appeared as a diffuse doublet, whereas H2O2 treatment elicited predominantly the appearance of the lower component of this doublet. The proportion of dimer formation was 13 and 17% for 15d-PGJ2 and H2O2 treatment, respectively (estimates from three independent experiments). H2O2-induced c-Jun dimer formation could be reversed by treatment with excess DTT. In contrast, 15d-PGJ2-elicited dimerization was only partially abrogated by DTT. These observations suggest that 15d-PGJ2 may induce both reversible and non-reversible dimerization of c-Jun.

cyPG may form Michael adducts with cysteine residues of glutathione or proteins. However, the possibility that 15d-PGJ2 may induce protein cross-linking has not been raised. To address this question we treated c-Jun with 15d-PGJ2 and assessed the appearance of higher molecular weight species by SDS-PAGE under reducing conditions (Fig. 5B). 15d-PGJ2 treatment induced the appearance of c-Jun dimer in a dose-dependent fashion (Fig. 5B, right panel). Concentrations as low as 2 μM 15d-PGJ2 induced significant dimer formation, and a maximal effect was attained with concentrations between 10 and 100 μM. c-Jun dimerization could be detected as early as 5 min after addition of 15d-PGJ2, and it was maximal after 2 h of incubation (not shown). The ability of 15d-PGJ2 to induce c-Jun dimerization may be due to the presence of several electrophilic carbons in the molecule, two of which can simultaneously form Michael adducts with thiols. In accordance with this, PGA1, which is also a cyclopentenone but possesses only one electrophilic carbon, did not induce c-Jun dimerization (Fig. 5C).

15d-PGJ2 Binds to Cysteine 269 of c-Jun—To identify the site of modification of c-Jun by 15d-PGJ2, c-Jun was treated with 15d-PGJ2 as in Fig. 5B. After SDS-PAGE, the protein bands corresponding to the monomer and dimer were excised, subjected to in-gel digestion with trypsin, and analyzed by MALDI-TOF mass spectrometry (Fig. 6A and Table I). Analysis of the monomer band showed the presence of a peak of m/z = 595, which is compatible with the incorporation of one 15d-PGJ2 molecule into the Cys269→Arg270 dipeptide (expected m/z = 594.62). These results indicate that 15d-PGJ2 binds to the cysteine residue located in the basic DNA binding domain of c-Jun (cysteine 269). Analysis of the dimer band showed the presence of a peak of m/z = 873, which is compatible with a dimer composed of two dipeptides containing Cys269 and Arg270, cross-linked through the cysteine residues by one 15d-PGJ2 molecule (expected m/z = 872.74), as depicted in the inset of Fig. 6A. This peak was absent from the monomer sample. The presence of 15d-PGJ2 in the c-Jun dimer was also confirmed by incubation of c-Jun with biotinylated 15d-PGJ2 and analysis of the resulting species by SDS-PAGE under reducing conditions. Incorporation of biotinylated 15d-PGJ2 could be observed both in the monomer and in the c-Jun dimer, as evidenced by Western blot and detection with HRP-conjugated streptavidin (Fig. 6B). We then assessed the role of 15d-PGJ2 addition to cysteine 269 in the inhibition of c-Jun DNA binding. As shown in Fig. 6C, the inhibition by 15d-PGJ2 was strongly reduced when a C269S mutant of the c-Jun construct was used. This mutant showed improved DNA binding activity over the wild type construct, as reported previously (21), and was more resistant to the inhibition observed under strong oxidizing conditions achieved by treatment with H2O2. This indicates that modification of cysteine 269 is relevant for 15d-PGJ2-elicited inhibition, whereas oxidative modifications of other parts of the molecule do not account for the inhibitory effect.

Biotinylated 15d-PGJ2 Binds to c-Jun in Intact Cells—We next studied the interaction between 15d-PGJ2 and c-Jun in

c-Jun preparation of 315 and 620 Da, compatible with the incorporation of one and two 15d-PGJ2 molecules, respectively, into a putative c-Jun dimer.

15d-PGJ2 Induces c-Jun Dimerization—Dimerization of c-Jun molecules through the formation of intermolecular disulfide bonds has been shown to occur under oxidative conditions, and it has been proposed as one of the potential mechanisms involved in redox regulation of AP-1 DNA binding (30). To assess the effect of 15d-PGJ2 on c-Jun dimer formation, we separated c-Jun monomers from covalently linked dimers by non-reducing SDS-PAGE, by a method established previously (22, 23). Incubation of c-Jun constructs with 15d-PGJ2 induced the appearance of higher molecular weight species compatible with dimer and, in a smaller proportion, multimer formation
intact cells. Incubation of cells in the presence of biotinylated 15d-PGJ2 led to the incorporation of the biotinylated PG into several polypeptides, the most intensely labeled of which displayed apparent molecular masses of 90, 45–55, and 34 kDa. The labeled polypeptides were clearly distinguishable from endogenous biotinylated proteins, which showed molecular masses of ~120 and 80 kDa (Fig. 7A). Immunoprecipitation of c-Jun from biotinylated 15d-PGJ2-treated cells showed the incorporation of biotin into the c-Jun band (Fig. 7B). This signal was absent in the immunoprecipitates from cells treated with non-biotinylated 15d-PGJ2. In addition, c-Jun from biotinylated 15d-PGJ2-treated cells, but not from cells incubated with 15d-PGJ2, was retained on avidin beads, as shown by Western blot after avidin pull-down. Similar results were obtained using NIH-3T3 fibroblasts (Fig. 7B) and HeLa cells (not shown). These results indicate that 15d-PGJ2 modifies c-Jun in intact cells. To assess the selectivity of this modification, we explored the presence of other transcription factors with cysteines important for function in the avidin-binding fraction. As shown in Fig. 7C, c-Fos, which is highly homologous to c-Jun and possesses a cysteine residue in its DNA binding domain, was detected in the avidin pull-down, whereas PPARγ was not. In order to explore the potential significance of this finding, we wondered whether c-Jun modification occurred under conditions close to those encountered in a cellular model of inflammation. For this purpose we first estimated the levels of 15d-PGJ2 in the culture medium of RAW264.7 macrophages. Under our experimental conditions 15d-PGJ2 levels increased from 1 ± 0.01 nM in basal conditions to 524 ± 84 nM (average of four determinations ± S.E.) after stimulation with bacterial lipopolysaccharide. We then incubated cells in the presence of 500 nM biotinylated 15d-PGJ2. This concentration was sufficient to elicit the modification of c-Jun in intact cells (Fig. 7D).

DISCUSSION

cyPG have been proposed to act as regulators of inflammatory and proliferative responses. The results herein show that the cyPG 15d-PGJ2 can directly interact with the component of the transcription factor AP-1, c-Jun, leading to an inhibition of its ability to bind DNA. In addition, 15d-PGJ2 participates in the formation of intermolecular cross-links that result in the dimerization of c-Jun under in vitro conditions.

15d-PGJ2 has been shown to inhibit AP-1 DNA binding in various experimental systems, but the mechanisms involved have not been fully elucidated. We have observed that 15d-PGJ2 reduces AP-1 DNA binding activity in several cell types, an effect that is not associated with a reduction in the nuclear levels of the AP-1 constituents c-Jun and c-Fos. Our results also show that 15d-PGJ2 reduces the formation of AP-1-DNA complexes in identical aliquots of isolated nuclear extracts, therefore acting in a manner independent from the amount or nature of the AP-1 proteins present in the assay. Moreover, 15d-PGJ2 also inhibits recombinant c-Jun DNA binding, thus suggesting the involvement of a direct interaction between the PG and the protein in the inhibitory effect. These observations may help to explain the results of previous reports (31) in which
15d-PGJ₂ induced a decrease in AP-1-DNA binding in the presence of unaffected or even increased nuclear levels of c-Jun protein. We have observed that 15d-PGJ₂ directly binds to c-Jun at a cysteine residue located in the DNA binding domain, cysteine 269. This interaction occurs by means of Michael's addition, a covalent linkage between the cysteine sulfhydryl group and the electrophilic carbon in the cyclopentenone ring of the PG. Modifications of this cysteine residue have been shown to result in loss of the DNA binding ability (21–23). Other members of the Jun and Fos families possess equivalent cysteine residues in their DNA binding domains. Moreover, the DNA binding domain of other transcription factors, including NF-κB, p53, CREB, or c-Myb, possess critical redox-sensitive cysteine residues that may be potential targets for modification by cyPG. Our results suggest that c-Fos is also modified by 15d-PGJ₂. However, although both p65 and p50 NF-κB subunits have been shown to be targets for cyPG (15, 16), neither p53 (32) nor PPARγ (this work) react to an appreciable extent with biotinylated cyPG in intact cells. Taken together, the available studies indicate that protein modification by cyPG is not a random process but displays some specificity determined by factors potentially related to protein or cell context.

One important issue regarding many of the reported effects of cyPG is whether they can be considered either of biological or pharmacological significance. The levels of 15d-PGJ₂, measured in cell-free exudates or extracellular media in several experimental models of inflammation are in the nanomolar range (2, 3). However, most of the biological effects of cyPG have been observed using micromolar concentrations (13, 32–34). Several considerations have been made to reconcile these discrepancies. First, it is known that cyPG accumulate inside cells, particularly in cell nuclei (35); therefore, concentrations measured in the culture medium would be expected to be lower than those present intracellularly. Second, cyPG readily form adducts with glutathione or proteins. This has been shown to result in the underestimation of the generation of cyPG when only the free forms are measured (36). Third, as discussed previously (32), although individual eicosanoids with a,β-unsaturated ketone substituents may not occur in micromolar concentrations at the site of inflammation, inflammatory exudates contain a variety a,β-unsaturated aldehydes and ketones with reactivity similar to that of 15d-PGJ₂, which can reach concentrations of 10 μM in tissues under conditions of oxidative stress. Our results indicate that covalent protein modification can occur in the presence of concentrations of 15d-PGJ₂ in the range of those measured in the culture medium of activated macrophages, thus suggesting the relevance of this mechanism in the effects of cyPG. Further studies will be needed to ascertain whether concentrations of cyPG sufficient to elicit the
anti-inflammatory or antiproliferative effects described in most studies may occur locally under pathophysiological conditions. The results described here unveil a novel aspect of the post-translational modification of proteins by cyPG as is the ability of 15d-PGJ2 to induce c-Jun cross-links. This effect is apparently due to the formation of covalent bonds between two re-

TABLE I

Peptides of 15d-PGJ2-treated c-Jun-(223-327) identified by MALDI-TOF mass spectrometry

| Experimental m/z | Theoretical m/z | Position     | Sequence   |
|------------------|----------------|--------------|------------|
| 561.7            | 562.32         | 269-272      | CRKR       |
| 587.7            | 588.4          | 284-288      | VKTLK      |
| 595.0            | 594.62         | 289-270      | CR+ 15dPGJ2|
| 658.3            | 659.29         | 255-259      | AERKR      |
| 733.0            | 732.4          | 259-263      | RMRNR      |
| 749.0            | 748.41         | 264-270      | IAASKCR    |
| 815.2            | 815.46         | 303-309      | EQVAQLK    |
| 829.1            | 829.54         | 271-276      | KRRKLER    |
| 859.0            | 858.50         | 277-283      | IARLEEK    |
| 861.0            | 860.5          | 258-263      | KRMNR      |
| 873.1            | 872.74         | 269-270      | CR+ 15dPGJ2+CR|
| 877.1            | 876.51         | 264-271      | IAASKCRK   |
| 901.0            | 900.57         | 253-259      | IKAERKR    |
| 947.0            | 946.53         | 255-261      | AERKRMK    |
| 992.3            | 992.63         | 255-261      | AERKRMK (MSO)|
| 1202.9           | 1202.69        | 259-268      | RMRNIAASK  |
| 1218.7           | 1218.68        | 259-268      | RMRNIAASK (MSO)|
| 1428.0           | 1427.89        | 277-288      | IARLEEKVKTLK|
| 1484.2           | 1483.89        | 274-285      | LERIARLEEKVK|
| 1570.3           | 1569.75        | 6xHis-226    | GSHHHHHHGSQALK|
| 1687.1           | 1686.96        | 255-268      | AERKRMNIAASK|
| 1787.9           | 1786.09        | 272-285      | RKLERIARLEEKVK|
| 1803.1           | 1802.83        | 312-328      | VMNHVNSGCQMLTQQ|
| 2923.2           | 2923.33        | 227-252      | EEPQTVMPGPETPLSPIDMESQER|
| 2939.3           | 2939.33        | 227-252      | EEPQTVMPGPETPLSPIDMESQER (MSO)|
| 2955.2           | 2955.32        | 227-252      | EEPQTVMPGPETPLSPIDMESQER (2MSO)|

Interaction of 15d-PGJ2 with c-Jun.

A. c-Jun was incubated in the presence of 15d-PGJ2 as described above and subjected to SDS-PAGE. The protein bands corresponding to the monomer (upper panel) and dimer (lower panel) species were isolated and digested in-gel with trypsin, and the resulting peptides were analyzed by MALDI-TOF mass spectrometry. The monoisotopic mass of some of the detected peptides is given. The amino acid sequence is shown in Table I. Asterisks mark the position of the peaks corresponding to putatively 15d-PGJ2-modified c-Jun fragments, the structure of which is shown in insets. B, c-Jun was incubated in the absence or presence of biotinylated 15d-PGJ2, and incorporation of 15d-PGJ2 into the monomer and dimer forms was assessed as described in the legend of Fig. 3. Results shown are representative of four assays. C, c-Jun wild type and C269S constructs were incubated in the presence of 15d-PGJ2 or H2O2 as described in Fig. 5, after which DNA binding activity was analyzed by EMSA. Results are representative of three assays.
FIG. 7. 15d-PGJ₂ modifies c-Jun in intact cells. A, NIH-3T3 cells were incubated with 10 μM 15d-PGJ₂ or biotinylated 15d-PGJ₂ for 2 h. Aliquots from cell lysates containing 20 μg of protein were analyzed by SDS-PAGE followed by Western blot and detection of biotinylated proteins with HRP-conjugated streptavidin. The presence of biotin-labeled proteins in the c-Jun immunoprecipitates was assessed by Western blot, and detection was with HRP-conjugated streptavidin and ECL. The total amount of c-Jun in lysates was shown. B, lysates from cells treated as in A were subjected to immunoprecipitation with anti-c-Jun antibodies (upper panels) or to a pull-down assay with avidin-agarose (lower panels). The presence of c-Jun from biotinylated 15d-PGJ₂-treated cells on avidin beads was confirmed by Western blot with anti-c-Jun antibody. The total amount of c-Jun in lysates is shown. C, the presence of c-Fos and PPARγ in the avidin-binding fraction of 15d-PGJ₂ or biotinylated 15d-PGJ₂-treated cells and the total amount present in lysates was assessed by Western blot. D, cells were incubated with 500 nM biotinylated 15d-PGJ₂, and labeling of c-Jun was explored by avidin pull-down as in B.

SCHEME 1. Potential sites of interaction of 15d-PGJ₂ with the AP-1 activation pathway. The activation of AP-1 can be regulated both at transcriptional and post-transcriptional levels. 15d-PGJ₂ can modulate AP-1 by interacting at multiple levels along the AP-1 activation pathway including modulation of the transcription of Jun and Fos proteins (A); alteration of the cellular redox state, interaction with redox regulated proteins, and/or direct interaction with AP-1 proteins (B); modulation of the phosphorylation state of c-Jun by activation or inhibition of JNK (C); and activation of PPARγ and sequestration of coactivators (D) (see text for details). ERK, extracellular signal-regulated kinase; JNK, c-Jun NH₂-terminal kinase; JAK, Janus-activated kinase; CBP, CREB-binding protein; RXR, retinoid X receptor; SRC-1, steroid receptor coactivator-1; TR, thioredoxin reductase; Trx, thioredoxin; GSH, reduced glutathione.
active carbons in the PG molecule and cysteine residues of two c-Jun monomers. Interestingly, other cyPG, which also possess two electrophilic carbons, like 9-deoxy-3,11E-PGJ2, have been described to form a conjugate with two molecules of glutathione (37). Thus, it would be interesting to explore the implication of 15d-PGJ2 in the pathophysiology of inflammatory and neurodegenerative diseases. These inflammatory and neurodegenerative settings are characterized by increased transactivation activity and association with coactivators such as CBP/p300 (46, 47). Moreover, cyPG can directly modify and inhibit both thioredoxin and thioredoxin reductase (32). Finally, cyPG can directly modify and inhibit both thioredoxin and thioredoxin reductase (32). Consequently, this modification will be addressed in future studies.

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