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15-Deoxy-$\Delta^{12,14}$-prostaglandin J2 (15d-PGJ2) mediates repression of TNF-α by decreasing levels of acetylated histone H3 and H4 at its promoter

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Abstract

Prostaglandin metabolite 15-Deoxy-$\Delta^{12,14}$-prostaglandin J2 (15d-PGJ2) is known to inhibit a number of pro-inflammatory cytokines as well as being a ligand for nuclear receptor PPARγ. We investigated the ability of 15d-PGJ2 to inhibit TNF-α gene expression through mechanisms that involve histone modification. Pretreatment with 15d-PGJ2 (10 μM) inhibited LPS-stimulated TNF-α mRNA in THP-1 monocytes or PMA-differentiated cells to nearly basal levels. A specific PPARγ ligand, GW1929, failed to inhibit LPS-induced TNF-α mRNA expression nor did a PPARγ antagonist, GW9662, alter the repression of TNF-α mRNA in LPS-stimulated cells pretreated with 15d-PGJ2 suggesting a PPARγ-independent inhibition of TNF-α mRNA in THP-1 cells. Transfection studies with a reporter construct and subsequent treatment with 15d-PGJ2 demonstrated a dose-dependent inhibition of the TNF-α promoter. Additional studies demonstrated that inhibition of histone deacetylases with trichostatin A (TSA) or overexpression of histone acetyltransferase CBP could overcome 15d-PGJ2-mediated repression of the TNF-α promoter, suggesting that an important mechanism whereby 15d-PGJ2 suppresses a cytokine is through factors that regulate histone modifications. To examine the endogenous TNF-α promoter, chromatin immunoprecipitations (ChIP) were performed. ChIP assays demonstrated that LPS stimulation induced an increase in histone H3 and H4 acetylation at the TNF-α promoter, which was reduced in cells pretreated with 15d-PGJ2. These results highlight the ability of acetylation and deacetylation factors to affect the TNF-α promoter and demonstrate that an additional important mechanism whereby 15d-PGJ2 mediates TNF-α transcriptional repression by altering levels of acetylated histone H3 and H4 at its promoter.
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Prostaglandin metabolite 15-Deoxy-$\Delta^{12,14}$-PGJ2 (15d-PGJ2) has received considerable attention for its anti-inflammatory effects both in vivo and in vitro. Though 15d-PGJ2 is a ligand of the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ), it mediates its effects in PPARγ-dependent and independent pathways [1–7] and is cell and gene specific [8,9]. The interpretation of many studies involving 15d-PGJ2 and other PPARγ ligands remains difficult due to PPARγ-dependent and independent actions as well as its cell dependent response. Various mechanisms have been implicated by which 15d-PGJ2 suppresses pro-inflammatory cytokines in monocytes and macrophages [1,2,11–14]. The ability of 15d-PGJ2 to repress transcription at a gene promoter has been shown for TNF-α and IL-2 [1,2]. Additionally, PPARγ ligand rosiglitazone has been shown to inhibit transcription of iNOS in mouse macrophages [14]. However, the means by which 15d-PGJ2 and other PPARγ

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ligands may block cytokine transcription at a gene promoter is still unclear.

There is increasing evidence that transcriptional regulation within a gene promoter involves histone modifications. Numerous studies have revealed the importance of transcriptional activation and its association with the levels of histone acetylation [17–21], and increased acetylation levels of histone H3 and H4 has been correlated with expression of TNF-α in THP-1 cells [15,16]. The effect of 15d-PGJ2 on histone modifications at a gene promoter to date are largely unknown. A study has shown that 15d-PGJ2 can block IL-1β-induced histone H3 acetylation at the COX-2 promoter in human synovial fibroblasts [22], though no study to date has examined the ability of a PPARγ ligand to modify histone acetylation in monocytes or macrophages.

This study investigated the ability of 15d-PGJ2 to affect cytokine TNF-α expression through transcriptional repression involving mechanisms of histone modifications. We found that in THP-1 cells 15d-PGJ2 greatly inhibits LPS-induced TNF-α mRNA likely through a PPARγ-independent process. A synthetic human PPARγ agonist was unable to significantly inhibit TNF-α mRNA. Additionally, blocking PPARγ with an antagonist did not affect the ability of 15d-PGJ2 to inhibit TNF-α mRNA. In studies examining the human TNF-α promoter, we demonstrate the ability of 15d-PGJ2 to repress TNF-α transcription and reversal of this repression with histone deacetylase inhibitor Trichostatin A (TSA) or overexpression of histone acetyltransferase CBP. Additionally, chromatin immunoprecipitation (ChIP) assays examining the endogenous TNF-α promoter show that 15d-PGJ2 pretreatment inhibits LPS-induced levels of histone H3 and H4 acetylation.

Materials and methods

Materials. THP-1 cells were from ATCC (Manassas, VA), and RPMI 1640 was from Mediatech, Inc. (Herdon, VA). 15-Deoxy-$\Delta^{12,14}$-prostaglandin J2 (15d-PGJ2) and GW9662 were from Biomol (Plymouth Meeting, PA). Phorbol 12-myristate 13-acetate (PMA), Trichostatin A (TSA), and LPS were purchased from Sigma (St. Louis, MO). SYBR green was from Applied Biosystems (Foster City, CA). Salmon sperm DNA/protein A agarose, acetylated H3, H4, and non-specific IgG antibodies were from Upstate Biotechnology/Millipore (Chicago, IL).

Cell culture and treatment. THP-1 cells were grown in suspension at 37 °C in 5% CO_2 in RPMI 1640 supplemented with 5 mM L-glutamine, 100 U/ml penicillin and streptomycin, 10% FBS. For experiments, cells were cultured in media containing 1% FBS. For differentiation, cells were cultured with 100 nM PMA for 24 h, then washed with media and subsequently incubated for additional 24 h prior to stimulation. Conditions with 15d-PGJ2 or GW19129 involved a 10 μM pretreatment for 2 h prior to LPS (1 μg/ml) stimulation for 4 h. GW9662 at 10 μM pretreatment was done for 1 h prior to addition of 15d-PGJ2 at 10 μM for 2 h and then LPS stimulation for 4 h.

RNA preparation, reverse transcription, and PCR. Total RNA was isolated from THP-1 cells (1×10^6/sample) using RNeasy Mini Kit (Qiagen, Valencia, CA). RNA (0.5 μg) was reverse transcribed with Superscript II kit (Invitrogen, Carlsbad, CA) using the manufactures' suggested guidelines. Real-time PCR was performed with Applied Biosystems (ABI) Prism 7500 Sequence Detection System and software. PCR conditions were the standard protocol of ABI Prism 7500 Sequence Detection System with SYBR green dye. Primers for real-time PCR of cDNA for TNF-α were forward (5′-CTGCCCCAATCCCTTTATT-3′) and reverse (5′-CCCAATTCTCTT TTTGAGCC-3′). TNF-α data were normalized for expression with the housekeeping gene GAPDH. Primers for GAPDH were forward (5′-GA AGGTGAAGGTCGGAGTCAAC-3′) and reverse (5′-CAGAGTTAAA AGCAGCCCTGGT-3′). Real-time PCR cycling parameters were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min each. Results were expressed as fold stimulation over cells after normalizing with paired GAPDH levels. A critical threshold cycle (C_t) value was determined for each reaction using ABI software. To calculate relative transcript levels the $x = 2^{-\Delta\Delta C_t}$ formula was used, in which $\Delta\Delta C_t = \Delta E - \Delta C$ and $\Delta E = C_{t_{(\text{treatment})}} - C_{t_{(\text{reference})}}$, $\Delta C = C_{t_{(\text{LPS-Ctrl})}} - C_{t_{(\text{reference})}}$.

Plasmid construction of TNF-α reporter construct and pCBP expression vector. The wild-type human TNF-α promoter (−991 to +1) was generated by PCR with primers containing flanking HindIII sites 5′-GCGCA GCTCCTGGGAGATATGGCCAC-3′ and 5′-GCGCGGGTGTGCCA ACAACTGCCTTT-3′ and subsequently cloned into pGL3-Basic luciferase vector (Promega, Madison, WI) to generate luciferase reporter pTNF-α luc. Plasmid pCBP was obtained from Ref. [25].

Transient transfection and luciferase assays. The RAW264.7 cell line (ATCC) was maintained at 37 °C in 5% CO_2 in DMEM supplemented with 100 U/ml penicillin and streptomycin and 10% FBS. Transfections were carried out in six-well plates with $0.5–1 \times 10^6$ cells per well. Cells were transfected with Fugene (Roche Indianapolis, IN) using 200 ng of luciferase reporter pTNF-α luc per well for 16 h, after which the cells underwent different treatment conditions. Histone acetyltransferase CBP expression vector (pCBP) was cotransfected with pTNF-α luc and empty vector was used to ensure equal DNA loaded per well. The following day after transfection, the media were changed to DMEM containing 1% FBS prior to addition of 15d-PGJ2 and or LPS. Cells were incubated with 15d-PGJ2 (1 or 5 μM) for 2 h prior to LPS (1 μg/ml) stimulation for 16 h. TSA was added 45 min prior to the addition of 15d-PGJ2. Luciferase assays were carried out using Dual-Luciferase Reporter Assay System using control plasmid *Renilla* pRL-TK vector (Promega, Madison, WI) according to manufactures' guidelines. For dose-dependent 15-PGJ2 treatment or pCBP experiments, luciferase values were normalized to *Renilla* control expression. TSA treated sample values were normalized via protein assay. Relative fold luciferase values were determined by fold induction of treated cells containing pTNF-α luc over untreated samples.

Chromatin immunoprecipitation (ChIP) assays. ChIP assays were performed following the protocol from Upstate Biotechnology/Millipore with some minor modifications. Briefly, cells were cross-linked with 1% formaldehyde for 10 min, washed twice with cold phosphate-buffered saline, resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.1, 1× protease inhibitor mixture (Roche Applied Science)), and sonicated on wet ice five to six times for 10 s each at 40% maximum setting of the sonicator (Branson Sonifier, model 250) followed by centrifugation for 10 min at 4 °C at 12,000 rpm. Supernatants were diluted in buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris–HCl, pH 8.1, 1× protease inhibitor mixture) followed by immunoclearing with 45 μl of protein-A–Sepharose/sheared salmon sperm DNA (Upstate Biotechnology/Millipore) for 1 h at 4 °C. One-tenth of the total lysate was used for total genomic DNA as input. Immunoprecipitation was performed for 16 h at 4 °C with 1–2 μg each of specific antibodies. Rabbit IgG antibody (Upstate Biotechnology/Millipore) was used as a non-specific antibody control. Precipitates were washed at 4 °C sequentially two times for 5 min each in the following solutions: Buffer I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris–HCl, pH 8.1), Buffer II (buffer I with 500 mM NaCl), and Buffer III (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris–HCl, pH 8.1). Following the washes, precipitates were then washed twice with TE buffer (10 mM Tris–HCl, pH 7.5, 0.1 mM EDTA) and extracted twice for 15 min each with 1% SDS containing 0.1 M $NaHCO_3$. Eluates were heated at 65 °C for at least 6 h to reverse the formaldehyde cross-linking. DNA fragments were purified by phenol:chloroform extraction. For PCR, 2 μl from a 20 μl DNA extraction was used. PCR primers corresponding to sequences within the promoter regions as follows: TNF-α, forward (5′-CCCTCCA GTTCTAGTTCTATC-3′) and reverse (5′-GGGGAAAGAATCATTC

AACCAG-3′). PCR was performed using ABI Prism 7500 Sequence Detection System. Cycling parameters were 50 °C for 2 min, 95 °C for 10 min, followed by 30 cycles of 95 °C for 15 s and 60 °C for 1 min each. Products were shown to be in the log phase by real-time PCR analysis. Products were subsequently run on a 2% agarose gel with ethidium bromide.

Statistics. Values are means ± SEM of triplicate values with $n = 4$ experiments for the mRNA studies and $n = 3$–4 for the transfection studies. Significant difference of LPS treatment group to that of 15d-PGJ2 treatment conditions was determined using the Students t test. Significance was determined by a $P < 0.05$.

Results

15d-PGJ2 inhibits TNF-α mRNA in THP-1 monocytes or PMA-differentiated cells

Treatment with LPS (1 μg/ml) caused an increase in TNF-α mRNA expression which was greater for the PMA-differentiated THP-1 cells than undifferentiated monocytes. LPS induced a 10- to 15-fold expression in TNF-α mRNA in differentiated THP-1 and a 5- to 6-fold expression in undifferentiated monocytes. In differentiated cells or monocytes, pretreatment with 15d-PGJ2 at 10 μM inhibited LPS-induced TNF-α mRNA expression to nearly basal levels (Fig. 1). To examine if this inhibition of LPS-induced TNF-α mRNA was dose related, THP-1 monocytes or differentiated cells were pretreated with 1, 5 or 10 μM of 15d-PGJ2 followed by stimulation with LPS. 15d-PGJ2 at concentrations of 5 and 10 μM significantly inhibited TNF-α mRNA whereas a concentration of 1 μM only slightly altered mRNA levels (Fig. 1B).

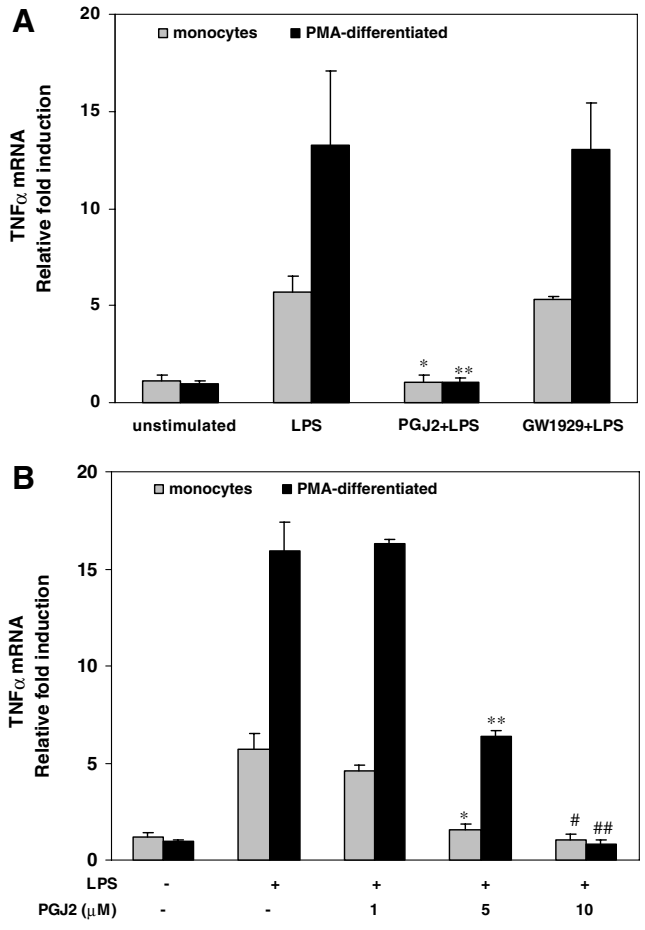

Fig. 1. Effect of 15d-PGJ2 or GW1929, a specific PPARγ agonist, on inhibition of TNF-α mRNA levels in THP-1 cells. Cells were pretreated with 10 μM 15d-PGJ2 or GW1929 for 2 h in RPMI-1640 medium containing 1% FBS then stimulated with LPS (1 μg/ml) for 4 h. 15d-PGJ2 pretreatment at concentrations of 1, 5, or 10 μM (B). For differentiation, cells were cultured with 100 nM PMA for 24 h, washed, then pretreated and stimulated as above. GW1929, a specific PPARγ agonist, does not significantly repress TNF-α mRNA production in THP-1 cells. Values are means ± SEM of $n = 4$ experiments. (A) $^{*}P < 0.001$ and $^{**}P < 0.005$, and (B) $^{*}P < 0.004$ and $^{**}P < 0.002$ and $^{\#}P < 0.01$ and $^{\#\#}P < 0.005$.

PPARγ-independent inhibition of TNF-α mRNA by 15d-PGJ2

The use of a specific human PPARγ agonist and antagonist addressed PPARγ-dependent versus independent effects of 15d-PGJ2. As shown in Fig. 1A, a specific human PPARγ agonist GW1929 failed to significantly affect LPS-induced TNF-α mRNA in THP-1 cells. The idea of a PPARγ-independent effect of 15d-PGJ2 was further supported by the use of a specific PPARγ antagonist as shown in Fig. 2. Given the possibility that 15d-PGJ2 might require PPARγ-dependent and independent mechanisms together to inhibit LPS-induced TNF-α mRNA in THP-1 cells, we blocked PPARγ receptor activation by using a specific human PPARγ antagonist (GW9662) and examined the ability of 15d-PGJ2 at 10 μM to inhibit LPS-induced TNF-α mRNA. PPARγ antagonist GW9662 did not alter the inhibitory effects of 15d-PGJ2 on LPS-induced TNF-α mRNA. Also, GW9662 did not significantly alter LPS-induced expression of TNF-α mRNA under these conditions.

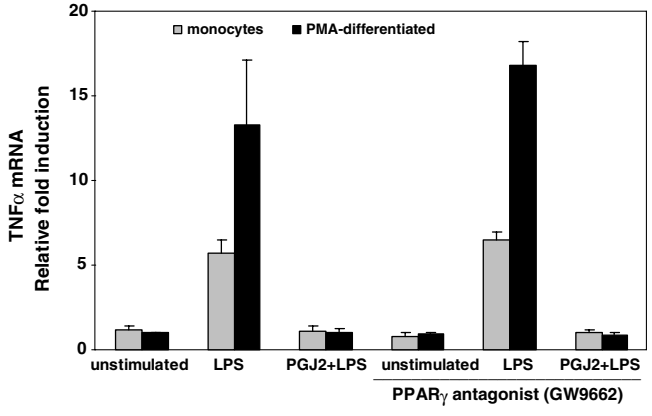

Fig. 2. A specific PPARγ antagonist, GW9662, fails to block inhibition of TNF-α in 15d-PGJ2-treated cells. THP-1 cells were pretreated with specific PPARγ antagonist GW9662 at 10 μM for 1 h followed by a 15d-PGJ2 treatment with for 2 h in RPMI-1640 medium containing 1% FBS, and then stimulated with LPS (1 μg/ml) for 4 h. GW9662 was not capable of altering the ability of 15d-PGJ2 to inhibit stimulated-TNF-α mRNA levels. Values are means ± SEM of $n = 4$ experiments.

15d-PGJ2 treatment inhibits human TNF-α promoter gene expression

To confirm that the reduction of TNF-α mRNA was due to inhibition of promoter activity, we transfected RAW264.7 cells with a luciferase reporter construct containing the human TNF-α promoter. As demonstrated in Fig. 3A, 15d-PGJ2 exhibited a dose–response inhibition of the TNF-α promoter. At concentrations of 1 and 5 μM 15d-PGJ2 pretreatment demonstrated significant TNF-α promoter inhibition. In the transfected cells under these conditions, concentration of 15d-PGJ2 of 10 μM demonstrated toxicity and were not used in the analysis.

Reversal of 15d-PGJ2-mediated repression of human TNF-α promoter by TSA treatment or histone acetyltransferase (CBP) overexpression

We sought to determine the effect of known histone acetylation modifiers on 15d-PGJ2-mediated repression of TNF-α gene expression. A prior study has demonstrated that overexpression of histone modifier p300 was capable reversing 15d-PG2 repression of IL-1β-induced COX-2 in human synovial fibroblasts [22]. We investigated if similar mechanisms might play a role in LPS-induced TNF-α in macrophages. The effect of histone deacetylase inhibitor TSA was examined on the human TNF-α promoter. As shown in Fig. 3B, TSA treatment alone has a modest effect

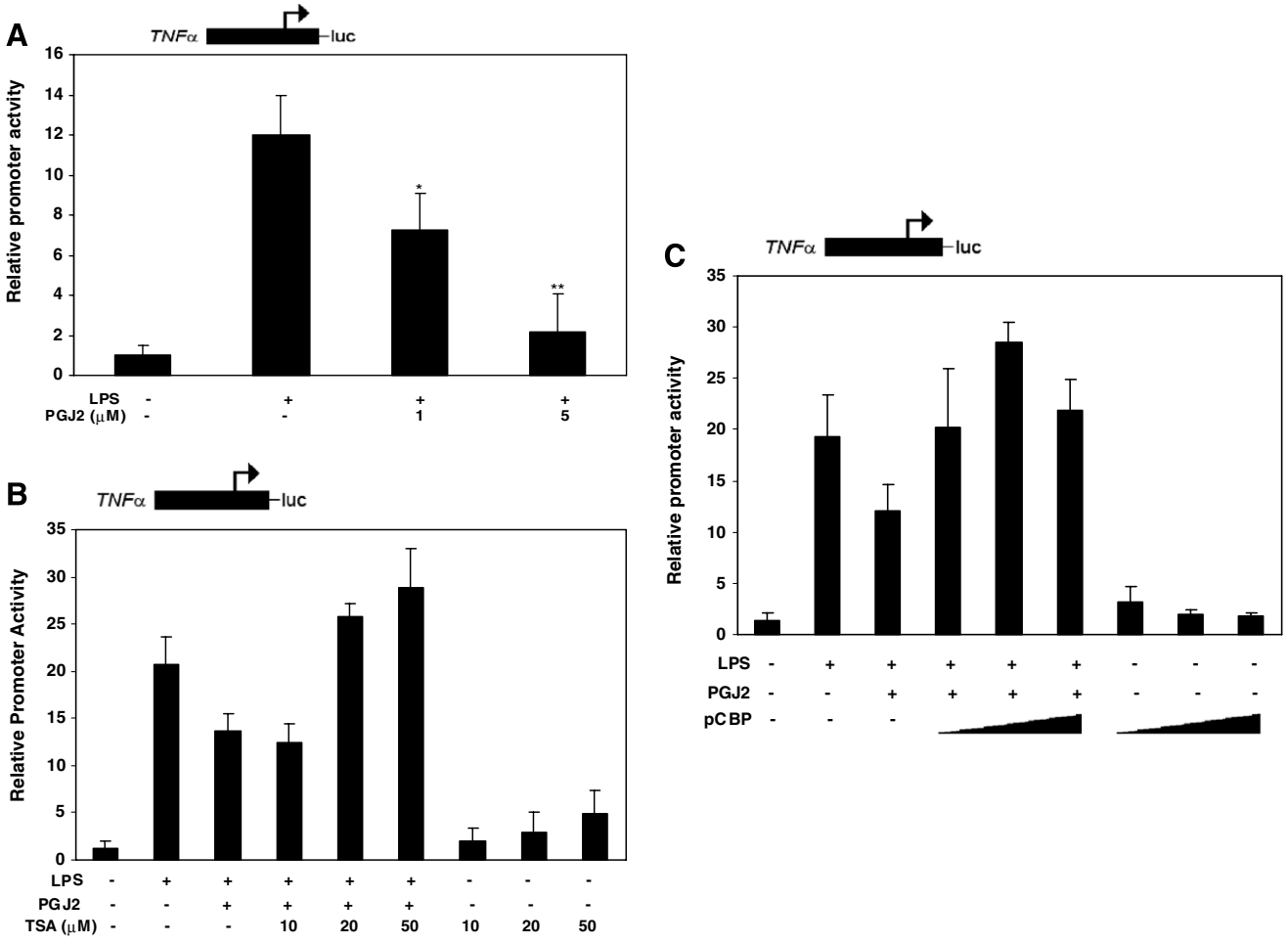

Fig. 3. Histone deacetylase inhibitor trichostatin A (TSA) or overexpression of histone acetyltransferase CBP reversal of 15d-PGJ2 inhibition of the TNF-α promoter. (A) RAW264.7 cells were transfected with 200 ng per well of luciferase reporter containing the human TNF-α promoter (pTNF-α luc) for 16 h, after which the cells underwent pretreatment with 1 or 5 μM of 15d-PGJ2 for 2 h prior to LPS (1 μg/ml) stimulation for 16 h. (B) In separate experiments, cells were transfected with luciferase reporter vector and treated with 15d-PGJ2 or co-treated with TSA at concentrations indicated. (C) Cells were transfected with 200 ng per well of pTNF-α luc with the addition of 100, 200, or 500 ng of pCBP, after which the cells underwent pretreatment with 15d-PGJ2 prior to LPS stimulation. Relative fold luciferase values were determined by fold induction of treated cells containing pTNF-α luc over untreated samples. For (A) values are means ± SEM of $n = 3$ experiments, $^{*}P < 0.05$, $^{**}P < 0.01$ (B) and (C) values are means ± SEM of $n = 4$ experiments.

on the TNF-α promoter with an induction of promoter activity from 2- to 5-fold. However, in the presence of LPS, TSA was capable of completely reversing 15d-PGJ2-mediated suppression of TNF-α. We next examined the effect of overexpression of a histone acetyltransferase (CBP) in this process. Overexpression of CBP alone had minimal effect of the TNF-α promoter. However, in the presence of LPS, addition of CBP, like the addition of TSA, was capable of reversing 15d-PGJ2 suppression of TNF-α.

15d-PGJ2 treatment decreases levels of histone H3 and H4 acetylation at the TNF-α promoter in LPS-stimulated cells

We examined the effect of 15d-PGJ2 on acetylation of histones H3 and H4 in THP-1 monocytes or PMA-differentiated cells at the endogenous TNF-α promoter. As shown by chromatin immunoprecipitation assay (ChIP) in Fig. 4, cells alone have a basal level of histone H3 and H4 acetylation at the TNF-α promoter which following LPS treatment is increased. This is in accordance with prior studies correlating increased acetylation levels of histone H3 and H4 with TNF-α expression in monocytes [15,16]. This result was more subtle for monocytes than differentiated cells, however, clearly detectable in either circumstance. Pretreatment with 15d-PGJ2 decreased LPS-induced levels H3 and H4 acetylation at the TNF-α promoter region similar to that of basal acetylation. The reduction of acetylated H3 and H4 was shown to be true for monocytes or differentiated THP-1 cells implying similarity of the action of 15d-PGJ2 at the TNF-α promoter region. Input samples confirmed equal starting material. Samples immunoprecipitated with a non-specific antibody (normal rabbit IgG) showed no PCR product indicating the specificity of the ChIP assay.

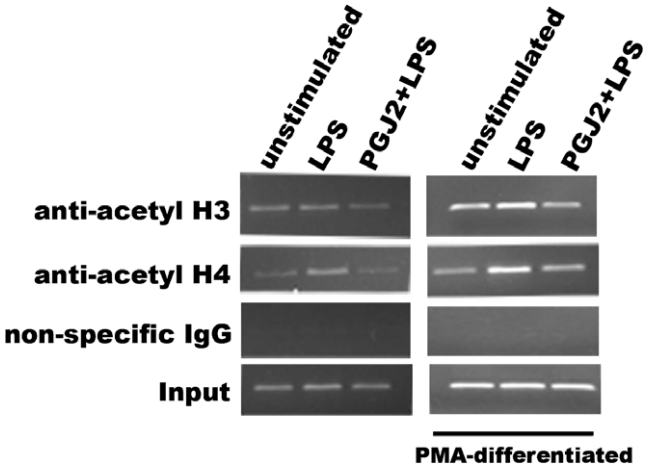

Fig. 4. 15d-PGJ2 decreases levels of LPS-induced histone H3 and H4 acetylation at the TNF-α promoter. Chromatin immunoprecipitation (ChIP) assays in THP-1 cells pretreated with 10 μM of 15d-PGJ2 for 2 h in RPMI-1640 medium containing 1% FBS, and then stimulated with LPS (1 μg/ml) for 2 h. Cells were differentiated as described in Fig. 1. Samples were immunoprecipitated using an antibody recognizing acetylated histone H3 or H4 or a non-specific antibody, normal rabbit immunoglobulin. The products were analyzed with primers for a region within the TNF-α promoter and after PCR of 30 cycles, products were run on a 2% agarose gel with ethidium bromide. The non-specific antibody control showed no PCR product. Gels are representative results of three independent experiments with similar findings.

Discussion

Prostaglandin metabolite 15d-PGJ2, a ligand for nuclear receptor PPARγ, has been shown to inhibit LPS-induction of many cytokines including IL-1β, TNF-α, IL-6 [23], as well cyclooxygenase-2, matrix metalloproteinases, and iNOS [10]. The ability of PPARγ ligands to influence cytokine gene expression has been shown to affect numerous pathways that are cell, stimulus, and gene specific [8,9]. In this study, we demonstrate the ability of 15d-PGJ2 to inhibit LPS-induced TNF-α mRNA expression in a human monocytic cell line. In THP-1 monocytes or PMA-differentiated cells, 15d-PGJ2 inhibited TNF-α mRNA to nearly basal levels at the most commonly used 15d-PGJ2 concentration of 10 μM. It is likely that 15d-PGJ2 inhibits TNF-α in THP-1 cells independent from PPARγ since a specific PPARγ antagonist (GW9662) failed to interfere with this inhibition. Furthermore, a specific human PPARγ agonist (GW1929) did not inhibit LPS-induced TNF-α mRNA expression.

Our data demonstrating the ability of 15d-PGJ2 to inhibit inflammatory gene promoter activation is supported by additional studies [1,2,10]. A common model of transcriptional regulation at an endogenous gene promoter involves histone protein modifications through a process of histone deacetylases (HDACs) and histone acetyltransferases (HATs) allowing for the assembly of transcription factor complexes. Work in THP-1 cells has demonstrated that cells in high glucose-containing media mimicking diabetic conditions have increased levels of TNF-α and increased levels of histone H3, H4 acetylation and CBP binding to the TNF-α promoter [15]. Additionally, HDAC inhibitor TSA is capable of inducing TNF-α expression [16]. Supporting the role of HDACs in this process, HDAC3 has been shown to repress LPS-induced TNF gene expression in promonocytic cells [24].

We show that 15d-PGJ2-mediated suppression of TNF-α expression can be overcome by inhibition of HDACs or overexpression of CBP. This result strongly suggests that acetylation modifiers play an important role in the ability of 15d-PGJ2 to suppress TNF-α. As further mechanisms in this process we demonstrate that LPS increases histone H3 and H4 acetylation at the TNF-α promoter, which is inhibited by pretreatment with 15d-PGJ2 in THP-1 cells, suggesting that 15d-PGJ2 represses endogenous pro-inflammatory gene expression by chromatin modification. Our work supports the importance of histone H3 and H4 acetylation in pro-inflammatory TNF-α gene expression in monocytes as demonstrated in the literature. This study demonstrates a novel mechanism whereby 15d-PGJ2 inhibits its LPS-induced gene expression of TNF-α by decreasing levels of acetylated histone H3 and H4 in monocytes.

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