Online Supplement for

15-deoxy-Δ^{12,14}-PGJ$_2$ promotes inflammation and apoptosis in cardiomyocytes via the DP2/MAPK/TNFα axis

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2. MATERIALS AND METHODS

2.1 Cell culture

HL-1 cells (a murine atrial cardiomyocyte cell line) were cultured in fibronectin (0.5%, w/v)/gelatin (0.02%, w/v) (Sigma-Aldrich, MO, USA)-coated flasks as described [27]. Cells were supplied with Claycomb medium (Sigma-Aldrich) containing 10% (v/v) FBS (Sigma-Aldrich), 0.1 mM norepinephrine (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 100 IU/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Lofer, Austria) and maintained at 37°C under 5% CO₂. For experiments cells were seeded in six-well plates at a density of 1-2x10⁴/well.

2.2 Isolation of primary murine ventricular cardiomyocytes

Male C57BL/6 mice (8–12 weeks, 20-30 g) were obtained from the Institute of Biomedical Research (Medical University of Vienna, Austria) and were kept on a 12 h light/dark cycle with free access to food and water. Ventricular cardiomyocytes were isolated as previously described [28]. Mice were anesthetized with isoflurane (Abbott, Wiesbaden, Germany) and sacrificed by cervical dislocation. Hearts were quickly removed and perfused on a Langendorff setup for 4 min with Ca²⁺-free Tyrode's solution (135 mM NaCl, 4.7 mM KCl, 0.6 mM KH₂PO₄, 0.6 mM Na₂HPO₄, 1.2 mM MgSO₄, 10 mM HEPES, 30 mM taurine) to which Liberase TM (75 µg/ml, Roche Diagnostics, Germany) was then added and perfusion was continued for another 4 min. Following mechanical dispersion of the cells, extracellular [Ca²⁺] concentrations were increased stepwise to 1 mM. Primary cardiomyocytes were then transferred to M199
cell culture medium (Sigma-Aldrich) containing penicillin 50 IU/ml, streptomycin 50 µg/ml (both Sigma-Aldrich). Six-well plates were coated with 20 µg/ml poly-ornithin (Sigma-Aldrich) and 5 µg/ml laminin (Sigma-Aldrich) in cold sterile water and kept at 37°C for 2 h. Coating solutions were removed and plates were washed with PBS (pH 7.4). Afterwards, primary cardiomyocytes were kept in coated six-well plates at 37°C under 5% CO₂ for 2 h. Supernatants containing nonadherent cells were removed and fresh M199 medium was added. The percentage of attached viable cells was > 90% in all cell preparations.

2.3 Incubation protocol

HL-1 cells were grown to 70% confluence followed by serum starvation overnight (12 h). Cells were incubated with 15d-PGJ₂ (Cayman, MI, USA) at indicated concentrations and time periods. Alternatively, cells were preincubated for 30 min with the p42/44 MAPK kinase inhibitor (25 µM PD98059, Merck Biosciences, Darmstadt, Germany), the p38 MAPK inhibitors (10 µM SB203580 or 25 µM PD169316, Merck Biosciences), N-acetyl-L-cysteine (5 mM NAC, Sigma-Aldrich), pyrrolidine dithiocarbamate (1 mM PDTC, Sigma-Aldrich), Tempol (1 mM, Tocris, MA, USA), PPARγ inhibitor (20 µM T0070907, Cayman), DP1 antagonist (100 nM MK0524, Cayman), DP2 antagonist (1 µM CAY10471, Cayman), TNFα receptor I/II antagonist (30 µM R-7050, Santa Cruz, CA, USA) or ethyleneglycol-bis(β-aminoethyl)-N,N,N′,N′-tetraacetoxymethyl ester (50 µM EGTA-AM, BioMol, Hamburg, Germany) followed by 15d-PGJ₂ treatment. Alternatively, cells were incubated with 13,14-dihydro-15-keto PGD₂ (1 µM, Cayman) or 9,10-dihydro-15d-PGJ₂ (15 µM, Cayman) for indicated time
periods. All reagents were dissolved in DMSO and DMSO (0.1%, [v/v]) was used as a vehicle control.

Primary murine cardiomyocytes were incubated with 15d-PGJ₂ (Cayman) at indicated concentrations and time periods. Alternatively, cells were preincubated for 30 min with DP2 antagonist (1 µM CAY10471, Cayman) followed by 15d-PGJ₂ treatments. DMSO (0.1%, [v/v]) was used as a vehicle control.

2.4 Western blot analysis

Protein expression was determined by Western blot technique as described [22, 29]. Briefly, HL-1 cells or primary cardiomyocytes were lysed in 100 µl of ice-cold lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 10 mM Na₄P₂O₇, 2 mM Na₃VO₄, 10 mM NaF, 1% [v/v] Triton X-100, 10% [v/v] glycerol, pH 7.4) containing a protease inhibitor cocktail tablet (Roche Diagnostics, Vienna, Austria) for 10 min on ice. Cells were scraped and centrifuged at 10,000 rpm (4°C, 10 min) to pellet debris. Protein estimations of cell lysates were performed using BCA protein assay kit (Thermo Scientific, IL, USA). Fifty µg of total protein was added to 10 µl of 4x NuPAGE LDS sample buffer (Invitrogen) containing 2 µl sample reducing agent (Invitrogen) and heated (70°C, 10 min). Proteins were separated by electrophoresis on NuPAGE 4-12% Bis-Tris gel and transferred to nitrocellulose membranes (Invitrogen). Membranes were blocked with 5% (w/v) non-fat milk in TBST (Tris-buffered saline containing Tween 20) or with 3% [w/v] BSA-TBST solution (in the case of MAPK) at 25°C (2 h) and incubated with primary antibodies (overnight at 4°C). The following primary antibodies were used (diluted in 5% [w/v] BSA-TBST): anti-phospho-p38 MAPK (1:1000, Cell Signalling-
anti-phospho-p44/42 MAPK (1:1000, Cell Signalling-9106), anti-bax (1:1000, Cell Signalling-2772), anti-caspase-3 (1:1000, Cell Signalling-9662), anti-caspase-9 (1:1000, Cell Signalling-9504), anti-Poly (ADP-ribose) polymerase (PARP) (1:1000, Biomol-SA250) or anti-phospho-histone H2AX (termed γH2AX) antibody (1:1000, Cell Signaling-2577), anti-PPARγ (1:500, Santa Cruz-sc7273), anti-NF-κB (p65) (1:500, Santa Cruz-sc372), anti-phospho-p53 (Ser\textsuperscript{46} or Ser\textsuperscript{15}) (1:1000 each, Cell Signalling-2521 and -9284). After being washed membranes were incubated with HRP-conjugated goat anti-mouse IgG (1:100,000 Biomol-8101102) or goat anti-rabbit IgG (1:200,000 Biomol-6293) (25°C, 2 h). Immunoreactive bands were visualized using Super Signal West Pico Chemiluminescent substrate (Thermo Scientific) and developed by Bio-Rad ChemiDoc MP Imaging System. For normalization, membranes were stripped with stripping buffer (58.4 g/L NaCl, 7.5 g/L glycine, pH 2.15) and incubated with anti-p38 (1:2000 Sigma-Aldrich-M0800), anti-p42/44 MAPK (1:1000 Santa Cruz-SC94) or anti-β-actin (1:1000 Santa Cruz-SC47778) as primary antibodies.

2.5 RNA isolation and real time RT-PCR (qPCR)

Gene quantification was done using real time RT-PCR as described [30]. Total RNA was isolated from HL-1 cells or primary cardiomyocytes using QIAshredder and RNeasy Mini Kit (Qiagen, UK) and 1 μg was subjected to reverse transcription. Six ng cDNA per template was used for gene quantification using SYBR Green PCR Kit (Qiagen) and gene specific primers. The qPCR protocol was performed using LightCycler 480 system (Roche Diagnostics). Gene specific primers were used for GAPDH (Mm_Gapdh_3_SG), DP1 (Mm_Ptgdr_1_SG), DP2/CRT3H2.
Sequences for cytokine specific primers are given in **Supplement Table I**. Relative gene expression levels compared to GAPDH were calculated using $\Delta\Delta CT$ method.

### 2.6 TNFα RNA interference

TNFα silencing was performed as described [22]. Briefly, HL-1 cells were transfected with four siRNAs specific for TNFα (40 nM in total, SI00207676/83/90/97, Qiagen) or with a scrambled negative control siRNA (40 nM si-scr; Allstars negative control siRNA, Qiagen). The siRNA transfection was performed with Lipofectamine (Invitrogen) according to the manufacturer’s suggestions. Briefly, HL-1 cells were grown to 50% confluence and transfected with 1 ml medium (without penicillin/streptomycin) containing 4 µl of Lipofectamine and the respective siRNA (40 nM final concentrations) for 6 h at 37°C. The transfection medium was aspirated and replaced with medium containing penicillin/streptomycin. Cells were grown for another 24 h, RNA was isolated and TNFα mRNA expression was quantitated using qPCR (see above). In parallel, transfected HL-1 cells were treated with 15d-PGJ2 at indicated concentrations and time periods to analyze expression of proteins of interest by Western blotting (see above) or cell viability using the MTT assay (see below).

### 2.7 ROS measurement

Intracellular ROS levels were assessed using 5-(and -6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H$_2$DCFDA, Invitrogen) as previously described [31]. The cell-permeable dye carboxy-H$_2$DCFDA becomes fluorescent upon
oxidation by ROS. HL-1 cells were incubated with 10 µM DCFDA in PBS for 30 min at 37°C, washed twice with ice-cold PBS and lysed with 300 µl of 3% (v/v) Triton X-100 in PBS (30 min) followed by addition of 50 µl absolute ethanol (15 min) on a rotary shaker (1350 rpm; Heidolph Instruments, Schwabach, Germany) at 4°C (in the dark). The cell lysates were transferred to microfuge tubes and cell debris was removed by centrifugation (13,000 rpm, 4°C, 10 min). One hundred µl of the supernatant was transferred into 96-well microtiter plates and DCF (2',7'-dichlorofluorescein) fluorescence was measured on a Victor Multilabel Counter (Perkin-Elmer, Waltham, MA, USA) with excitation/emission wavelengths at 485/540 nm, respectively. All steps concerning carboxy-H$_2$DCFDA were performed under light-protected conditions. Protein contents of the cell lysates were determined using the BCA$^{\text{TM}}$ Protein Assay Kit according to the manufacturer's instructions. Each treatment was done in triplicate and values are expressed as percentage of controls.

2.8 Cell viability assay

Cell viability was assessed as described [31]. HL-1 cells (50% confluent) were treated with 15d-PGJ$_2$ at indicated concentrations and time periods followed by incubation with MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (0.5 mg/ml; dissolved in serum-free medium) for 30 min at 37°C. The converted insoluble dark purple coloured dye (formazan) was solubilized with 300 µl acidic isopropanol (0.04 M HCl in absolute isopropanol). Reduction of MTT fluorescence was assessed by measuring excitation/emission at 570/630 nm using a microplate reader (Victor
Multilabel Counter). Each treatment was done in triplicate and values are expressed as percentages of controls.

2.9 TNFα ELISA

To determine TNFα levels in the cell culture medium, HL-1 cells (70% confluent) or primary cardiomyocytes were treated with 15d-PGJ₂ at indicated concentrations and time periods. The cell culture medium was collected and ELISA (Enzo Life Sciences, NY, USA) specific for mouse TNFα was performed according to manufacturer’s instructions. Briefly, ELISA plate wells were incubated with TNFα standard dilutions at indicated concentrations (15.63-2000 ng/ml) or 50 µl cell culture medium. After washing with buffer, anti-TNFα antibody (50 µl) was added to all wells followed by addition of conjugate (50 µl). Wells were washed and incubated with substrate (50 µl) followed by stop solution (50 µl). Optical densities were measured according to the manufacturer’s instructions at 450 nm and 590 nm, respectively, using a microtiter plate reader (Victor Multilabel Counter). All standard and sample values were subtracted with blank control values and TNFα concentrations were calculated by comparison with an external calibration curve.

2.10 Statistics

All values are represented as mean±SEM and n represents the number of experiments. Statistical significances were tested by Student’s t-test or one-way ANOVA with adequate post hoc tests (Tukey, Dunett), using IBM SPSS software; p values ≤ 0.05 were considered statistically significant. All tests were 2-sided.
References

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# Supplement Table I

List of primers including gene accession numbers, sequences and amplicon size.

| Gene (accession no.) | Primers | Amplicon size |
|----------------------|---------|---------------|
| **TNFα (NM_013693)** | F: 5’-CTTCCAGAAGCTCAGGCCTG-3’<br>R: 5’-GGCTACAGGCTTGTCACTCG-3’ | 211 |
| **IL-1α (NM_010554)** | F: 5’-GAAGACTACAGTTCTGCCATT-3’<br>R: 5’-GACGTTTCAGAGGTTCCTGTA-3’ | 126 |
| **IL-2 (NM_008366)** | F: 5’-GTGCTCTTTGCAACAGCG-3’<br>R: 5’-GGGGAGTTTCAGGTTCCTGTA-3’ | 171 |
| **IL-6 (NM_031168)** | F: 5’-TAGTCCTTCTACCTCACATTCC-3’<br>R: 5’-TTGGTCTTTAGCCACTCCTTC-3’ | 76 |
| **IL-8 (NM_011339)** | F: 5’-CAAGGCTGGCTTCCATGCTCC-3’<br>R: 5’-TGCTATCACTTCTTTCTGTTGC-3’ | 183 |
| **IL-10 (NM_010548)** | F: 5’-GCTTTAATGAGCTGGCATGAG-3’<br>R: 5’-CGCAGCTCTAGGAGCATGTG-3’ | 105 |
Supplement Figure I

The chemical structure of PGD\textsubscript{2} and its metabolites. * indicates electrophilic carbon atom.
Supplement Figure II

Densitometric evaluation of immunoreactive (A) pp38 MAPK/p38 MAPK, (B) pp42/44 MAPK/p42/44 MAPK, (C) p38 MAPK/β-actin and (D) p42/44 MAPK/β-actin bands in response to 15d-PGJ₂ treatment (1 h) in a concentration-dependent manner (see Fig. 1A). Values are expressed as mean ± SEM (n=3); *p≤0.05 vs. untreated. The ratio of p38 MAPK/β-actin (C) and p42/44 MAPK/β-actin (D) at indicated 15d-PGJ₂ concentrations was 1.047 ± 0.034 and 1.039 ± 0.032, respectively.
Supplement Figure III

Densitometric evaluation of immunoreactive (A) pp38 MAPK/p38 MAPK and (B) pp42/44 MAPK/p42/44 MAPK bands in response to 15d-PGJ₂ treatment (15 µM) in a time-dependent manner (see Fig. 1B/C). Densitometric evaluation of immunoreactive (C) pp38 MAPK/p38 MAPK and (D) pp42/44 MAPK/p42/44 MAPK bands in response to 15d-PGJ₂ treatments (15 µM, 1 h) in the absence or presence of ROS scavengers (see Fig. 1E). Values are expressed as mean±SEM (n=3); *p≤0.05 vs. untreated and #p≤0.05 vs. 15d-PGJ₂ treatment.
**Supplement Figure IV**

Densitometric evaluation of immunoreactive (A) pp38 MAPK/p38 MAPK and (B) pp42/44 MAPK/p42/44 MAPK bands in response to 15d-PGJ2 treatment (15 µM, 1 h) in the absence or presence of indicated inhibitors (see Fig. 2B). Values are expressed as mean±SEM (n=3); *p≤0.05 vs. untreated and #p≤0.05 vs. 15d-PGJ2 treatment.
HL-1 cells were incubated with p38 MAPK inhibitor (10 µM SB203580, 25 µM PD169316) or p42/44 MAPK kinase inhibitor (25 µM PD98059) for 30 min prior to 15d-PGJ$_2$ treatment (15 µM, 1 h). Immunoreactive bands for pp38 and pp42/44 MAPK expressions were followed by Western blot. For Western blot analysis protein lysates were subjected to SDS-PAGE, proteins were transferred to nitrocellulose membranes and immunoreactive bands were visualized using specific primary and secondary antibodies. For normalization, membranes were stripped and probed with primary antibodies against total p38 and p42/44 MAPK.
Supplement Figure VI

Densitometric evaluation of immunoreactive (A) Bax, caspase 8, 9 and 3 compared to β-actin and (B) cleaved PARP (cPARP) and γH2AX compared to β-actin bands in response to 15d-PGJ2 treatment (15 μM) in a time-dependent manner (see Fig. 5B). Densitometric evaluation of immunoreactive (C) Bax, caspase 8, 9 and 3 compared to β-actin and (D) cPARP, γH2AX compared to β-actin bands in response to 15d-PGJ2 treatment (15 μM, 4 h) in scrambled negative control siRNA (si-scr, 40 nM) or siRNA against TNFα (si-TNFα, 40 nM) transfected cells (see Fig. 5D). Values are expressed as mean±SEM (n=3); *p≤0.05 vs. untreated and **p≤0.05 vs. si-scr treatment, ##p≤0.05 vs. si-scr+15d-PGJ2 treatment.
Supplement Figure VII

HL-1 cells were transfected with scrambled negative control siRNA (si-scr, 40 nM) or siRNA against TNFα (si-TNFα, 40 nM) for 6 h and grown for 24 h to follow TNFα mRNA expression using qPCR. Cells were lysed, RNA was isolated and qPCR for TNFα was performed using specific primers. Values are expressed as mean±SEM (n=6); *p≤0.05 vs. untransfected.
Supplement Figure VIII

Densitometric evaluation of immunoreactive caspase 8, 9 and 3 compared to β-actin bands in response to 15d-PGJ$_2$ treatments (15 µM, 4 h) in the absence or presence of R-7050 (30 µM) (see Fig. 5F). Values are expressed as mean±SEM (n=3); *p≤0.05 vs. untreated and #p≤0.05 vs. 15d-PGJ$_2$ treatment.
Supplement Figure IX

Densitometric evaluation of immunoreactive (A) pp38 MAPK/p38 MAPK and (B) pp42/44 MAPK/p42/44 MAPK bands in response to 15d-PGJ$_2$ (15 µM), dh-15d-PGJ$_2$ (15 µM) or dk-PGD$_2$ (1 µM) treatments for 1 h (see Fig. 6C, lower panel). Values are expressed as mean±SEM (n=3); *p≤0.05 vs. untreated.
Supplement Figure X

HL-1 cells were incubated with 15d-PGJ$_2$ (15 µM) for indicated time periods. Immunoreactive bands for pp53 (Ser$^{46}$ and Ser$^{15}$) expressions were followed by Western blot. For Western blot analysis protein lysates were subjected to SDS-PAGE, proteins were transferred to nitrocellulose membranes and immunoreactive bands were visualized using specific primary and secondary antibodies. For normalization, membranes were stripped and probed with primary antibodies against β-actin.