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Inflammation pro-resolving potential of 3,4-dihydroxyacetophenone through 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 in murine macrophages

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Abstract

3,4-dihydroxyacetophenone (DHAP), an active component isolated from leaves of Tumaodongqing (*Ilex Pubescens Hook. Et Arn. Var glaber Chang*), is initially used to treat cardiovascular diseases. Previously, we found it had anti-inflammatory effect on macrophages by reducing the production of TNF-alpha *in vitro*. To further determine whether DHAP could influence inflammatory resolution, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15dPGJ$_2$), an arachidonic acid metabolite and also crucial pro-resolving mediator in inflammation, was chosen as the research target. It showed that 10^{-5} M DHAP resulted in obvious increase of 15dPGJ$_2$ in LPS-activated macrophages. Further, inflammation related cytokines and cell apoptosis were also studied. We found DHAP could markedly inhibit LPS-stimulated production of TNF-alpha. However, it could not change the level of IL-10 obviously. At the same time, LPS-triggered apoptosis of macrophage was enhanced by DHAP significantly. After different kinds of cyclooxygenase (COX) inhibitors were administrated, it showed that the effects of DHAP on TNF-alpha and apoptosis were COX-2 dependent. While, inhibition of both COX-1 and COX-2 with indomethacin and administration of 15dPGJ$_2$ simultaneous reserved the effect of DHAP to inhibit TNF-alpha and enhance apoptosis in LPS-activated macrophages at least partly. The level of COX-2 mRNA and protein were also detected. It was showed that DHAP could increase the expression of COX-2 at both mRNA and protein levels in LPS-activated macrophages. Our results suggest that DHAP could accelerate resolution phase of acute inflammation though enhance the production of 15dPGJ$_2$, which was also proved to mediate the function of DHAP to inhibit TNF-alpha and enhance apoptosis *in vitro*. These results are potentially valuable for future use of DHAP.

Keywords: Traditional Chinese herbs; 3,4-dihydroxyacetophenone; Inflammatory resolution; Apoptosis; 15dPGJ$_2$

1. Introduction

3,4-dihydroxyacetophenone (DHAP) is an active component isolated from leaves of Tumaodongqing (*Ilex Pubescens Hook. Et Arn. Var glaber Chang*) that has been used as traditional Chinese herbal medicine [1]. DHAP was initially used to promote blood circulation and remove blood stasis [2]. And it has gradually shown good pharmacologic effect on the patients with cardiovascular diseases such as pregnancy-induced hypertension and coronary heart disease [2–5]. This might be partly due to the effect that DHAP could reduce the ratios

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of thromboxane A_2 (TXA_2) and prostacyclin (PGI_2), and then improve the haemorheology [6].

Since most metabolites of arachidonic acid (AA), like TXA_2 and PGI_2, are also important inflammatory mediators [7], we further investigated whether DHAP could influence inflammation response. As a result, it was interesting to find that DHAP had anti-inflammatory effect on macrophages by reducing the production of TNF-alpha *in vitro* [8].

As being emphasized in the past few years, the initiation of inflammation response is under the control of "go signals" which drive cells trafficking, edema formation and pro-inflammatory cytokines production, while, the resolution phase is equally coordinated by "switching-off signals", such as the endogenous pro-resolving factors that mediate cell clearance in a non-phlogistic manner [9,10]. Till now, the mechanism by which inflammation begins has been extensively characterized but the approach of interrupting the single pro-inflammatory mediator or pathway has provided the mainstay of conventional anti-inflammatory therapies [11]. These therapies are proved to be not so satisfactory or even still having some shortcomings. For example, anti-TNF-alpha therapy can depress the hosts' ability to deal with infection [12], at the same time, specific cyclooxygenase-2 (COX-2) inhibitor refecoxib causes an increased risk of stroke and myocardial infarction [13,14]. Thus, it is important to search for new drugs which could facilitate the inflammation resolution.

Since it is newly found that 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 ($15dPGJ_2$), also AA metabolite, is critical one among these "switch off" signals [15], we are quite interested in whether DHAP could influence the generation of $15dPGJ_2$ in inflammatory cells and finally affect inflammatory progress. Therefore, the aim of this research was to investigate the potential influence of DHAP on $15dPGJ_2$ and its possible downstream effect. The murine macrophage cell line RAW264.7 was used for this purpose. Since the bacterial lipopolysaccharide (LPS) was the major inducer of macrophages inflammation process, it was served as a stimulus for establishing inflammatory model *in vitro*.

2. Materials and methods

2.1. Regents

DHAP (molecular weight 152) was purchased from the Beijing Pharmaceutical Research Institute, with the purity over 98%. RPMI-1640 cell culture medium and fetal calf serum were obtained from Gibco BLR Life Technologies Inc (New York). Propidium Iodide (PI), Hoechst dye 33258 stain kit were the products of KPL. TNF-alpha and IL-10 ELISA kit were obtained from Jingmei Biotech Co., China. SC-560 and NS-398 were purchased from Cayman Chemicals. TRIzol Reagent was from Invitrogen (Carlsbad, CA, USA). SYBR green PCR Master Mix was the product of ABI (Applied Biosystems, Foster City, CA). COX-2 and β-actin polyclonal antibody were purchased from Santa Cruz (Santa Cruz, CA, USA) and peroxidase-conjugated IgG was purchased from Pierce (Rockford, IL, USA). $15dPGJ_2$, LPS from *Escherichia coli* 055:B5 purified by phenol extraction and all other materials were purchased from Sigma Chemical Co.

2.2. Cell culture and stimulation

RAW 264.7 cells were purchased from Chinese Type Culture Collection (Shanghai). Cells were cultured at 37 °C in RPMI-1640 containing 10% heat-inactivated fetal calf serum, 2 g/L $NaHCO_3$ (pH=7.4), penicillin (100 units/mL) and streptomycin sulfate (100 μg/mL) in a humidified atmosphere of 5% CO_2. Cells were allowed to grow to confluence over a period of 24 h before different stimuli were administrated into the culture medium.

2.3. Analysis of $15dPGJ_2$ in cell culture medium by high performance liquid chromatography (HPLC)

In order to determine the amount of $15dPGJ_2$ secreted by macrophages into cell culture medium , VARIAN Prostar HPLC system was adopted, including a Prostar 230 pump, a Prostar 410 automatic injector, a variable wavelength detector and a Prostar 363 fluorescence detector.

The cell culture medium (0.5 mL) was collected and centrifuged (300 g, 10 min, 4 °C). After acidification with 1 M HCl, the samples were extracted by ethyl acetate, dried by nitrogen and then redissolved in acetonitrile. Since the concentration of $15dPGJ_2$ in culture medium was hardly to be determined directly, the precolumn derivatization reaction with 4-bromomethyl-7-methoxycoumarin (BrMMC) and dicyclohexyl-18-crown-6 as catalyst were adopted.

After derivatization, 30 μL of solution was tested on a Kromasil C-18 reverse-phase column (4.6×150 mm, 5 μm) , using the mixture of acetonitrile–water(60:40, V/V) as the mobile phase, with the flow rate of 0.8 mL/min and UV detection wavelength of 315 nm and 370 nm(λex=315 nm, λem=370 nm). For peak assignment, the standard compounds of $15dPGJ_2$ were applied to HPLC and eluted under the same chromatographic conditions as those in experimental groups. The quantity of $15dPGJ_2$ was determined by external standard peak area.

2.4. Cytokines assay by enzyme-linked immunosorbent assay (ELISA) method

The quantities of TNF-alpha and IL-10 in cell culture medium were measured by a standard sandwich cytokine ELISA procedure to assess the pharmacological effect of DHAP on cytokines production. Standards (recombinant TNF-alpha or IL-10 at 0, 31.25, 62.5, 125, 250, 500, 1000, 2000 pg/mL

concentrations) and samples were added at a volume of 100 μL per well. Absorbance results were assessed using an ELISA microplate reader (Sunrise, Tecan Co, Austria) of which wavelengths were set and corrected according to the manufacturer's instruction. TNF-alpha and IL-10 quantities in the samples were calculated from standard curves of recombinant cytokines using a regression linear method.

2.5. Flow cytometry analysis of apoptotic cells

According to the protocol, cells were detached using 0.25% trypsin. Cell suspension was washed in phosphate-buffered saline and centrifuged (1000 r/min) for 5 min twice. After fixing with 80% iced ethanol, the cell pellets were incubated in PBS buffer containing 0.33 mg/L PI and 100 mg/L RNase at room temperature in the dark for 30 min. After that, cells were run in FACScan cytometer with excitation wavelength of 488 nm and emission wavelength of 625 nm. Apoptotic cells were determined on a PI histogram as a hypodiploid. For each sample, 20,000 cells were analyzed.

2.6. Morphological analysis of apoptotic cells

Cells were stained with Hoechst 33258, a classical dye for identifying apoptosis, to observe nuclear morphology. Cells were washed with PBS and stained with 2.5 g/L Hoechst dye 33258 fluorochrome for 5 min at room temperature, observed on a fluorescence microscope (Olympus BX50) with an excitation wavelength of 355 to 366 nm and an emission wavelength of 465 to 480 nm.

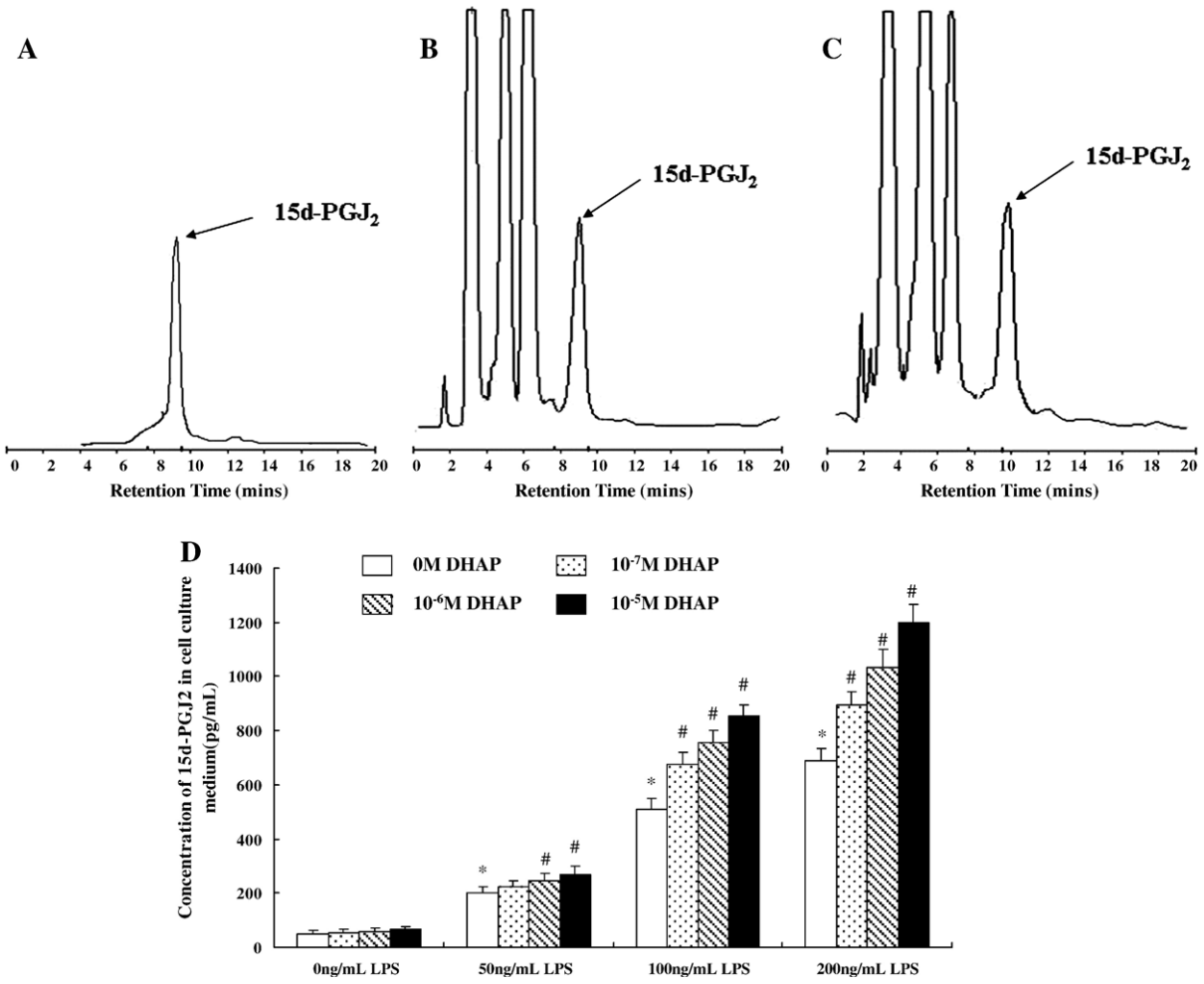

Fig. 1. DHAP increased 15dPGJ$_2$ production in LPS-activated RAW264.7 cells. A, HPLC chromatogram of standard 15dPGJ$_2$. B, HPLC chromatogram of cell culture medium added with standard 15dPGJ$_2$. C, HPLC chromatogram of cell culture medium added with LPS or DHAP. D, Columns represent the content of 15dPGJ$_2$ in cell culture medium. Cells were treated for 18 h under indicated condition. Cell culture medium was collected and analyzed by HPLC. *P<0.01 vs. control group. #P<0.01 vs. groups treated with same dose of LPS but without DHAP. Each experiment was performed 4 times and represented as the mean value±SD.

2.7. Analysis of COX-2 RNA by quantitative real-time PCR

Total RNA extraction and reverse transcription was performed as previously reported with minor modifications [16]. Briefly, Total RNA was extracted with TRIzol Reagent. The concentrations of total RNA were measured using a UV spectrophotometer (UV-1201, Shimadzu corp., Kyoto, Japan). Four micrograms of total RNA was used in a reverse transcription reaction using 200 units of M-MLV Reverse Transcriptase in a 25 μL reaction mixture. First strand cDNAs were amplified using a real-time PCR thermal cycler. Quantitative PCR was performed with SYBR green PCR Master Mix according to the protocols. The following primers were used to amplify COX-2 cDNA: sense, 5′-GCATTCTTTGCCCAGCACTT-3′, and antisense, 5′-AGACCAGGCACCGACCAAAGA-3′. GAPDH cDNA: sense, 5′-CCTGCACCACCAACTGCTTA-3′, and antisense, 5′-TCATGAGCCCTTCCACAATG-3′. Optimal reaction conditions were 40 cycles of a two stage PCR (denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min) after an initial denaturation step (95 °C for 10 min). The generation of specific PCR products was confirmed by melting-curve analysis. To ensure the comparability between 2 PCR assays, the efficiency of each individual assay was determined by measuring serial dilutions of cDNA in triplicate. Only Ct values < 40 were used for calculation of the PCR efficiency from the given slope generated with the SDS 2.2 software according to the equation: PCR efficiency = $(10^{[-1/\text{slope}]}-1)\times 100$. Relative COX-2 cDNA expression was analyzed by the $2^{-\Delta Ct}$ method. The PCR cycle number at the threshold was represented as Ct, and the difference between Ct for the target and Ct for the internal control, ΔCt ($\Delta Ct = C_{t\ COX\text{-}2} - C_{t\ GAPDH}$), was calculated. The $2^{-\Delta Ct}$m values were used to represent the amount of COX-2 mRNA in comparison to that of GAPDH.

2.8. Analysis of COX-2 protein by western blotting

Cell pellets(10^7) were resuspended in 50 μL of buffer A (0.4% Nonidet P-40, 10 mmol/L EDTA, 10 mmol/L EGTA, 10 mmol/L KCl, 10 mmol/L HEPES to that of 1 mmol/L DTT, 0.5 mmol/L PMSF, 10 g/L leupeptin, 1 g/L pepstatin, 1 g/L trypsin inhibitor, and 1 g/L antipain which were freshly added) and incubated on ice for 5 min. Cell pellets were sedimented by centrifuging the lysates at 2500 g for 5 min. The cell pellets were resuspended in 50 μL of buffer B [1 mmol/L EDTA, 1 mmol/L EGTA, 0.4 mol/L NaCl, 20 mmol/L HEPES (pH7.9), 5 mmol/L $MgCl_2$, 25% glycerol, with fresh additions as above] and incubated for 10 min on ice. The suspensions were clarified by centrifuging at 12,000 g for 15 min. The supernatants were recovered as cell extracts and stored at −80 °C. Cell extracts (60 μg) were separated by 10% SDS-PAGE gel and blotted onto nitrocellulose membranes. The blots were incubated with rabbit polyclonal antibodies against COX-2, and reacted with anti-rabbit horseradish peroxidase-coupled secondary antibody using an ECL system. β-actin protein was served as loading control.

2.9. Statistical analyses

The results were expressed as the means ± SD of the indicated number of experiments. The statistical significance was determined using a Student's *t*-test for unpaired observations. A P value of <0.05 was considered to be significant.

3. Results

3.1. Effects of DHAP on 15dPGJ$_2$ production in macrophages

In order to determine the effects of DHAP on the production of 15dPGJ$_2$ in LPS-activated macrophages, cells treated with

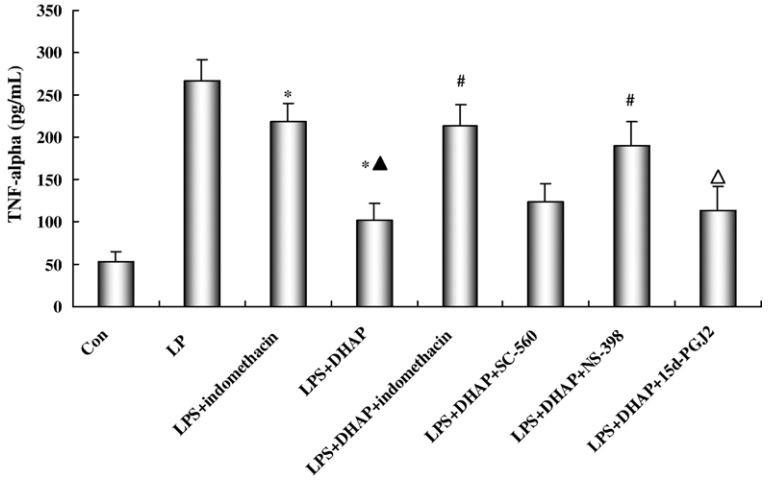

Fig. 2. TNF-alpha levels in cell culture medium assessed by ELISA in RAW264.7 cells. Cells were treated with indicated conditions (100 ng/mL LPS, 10^{-5} M DHAP, 10 mM indomethacin, 100 nM SC-560, 100 μM NS-398) for 18 h and content of TNF-alpha in cell culture medium was measured by ELISA kits. *$P<0.01$ in relation to LPS group. #$P<0.01$ in relation to LPS + DHAP group. △$P<0.01$ in relation to LPS + DHAP + indomethacin group. ▲$P<0.05$ in relation to LPS + indomethacin group.

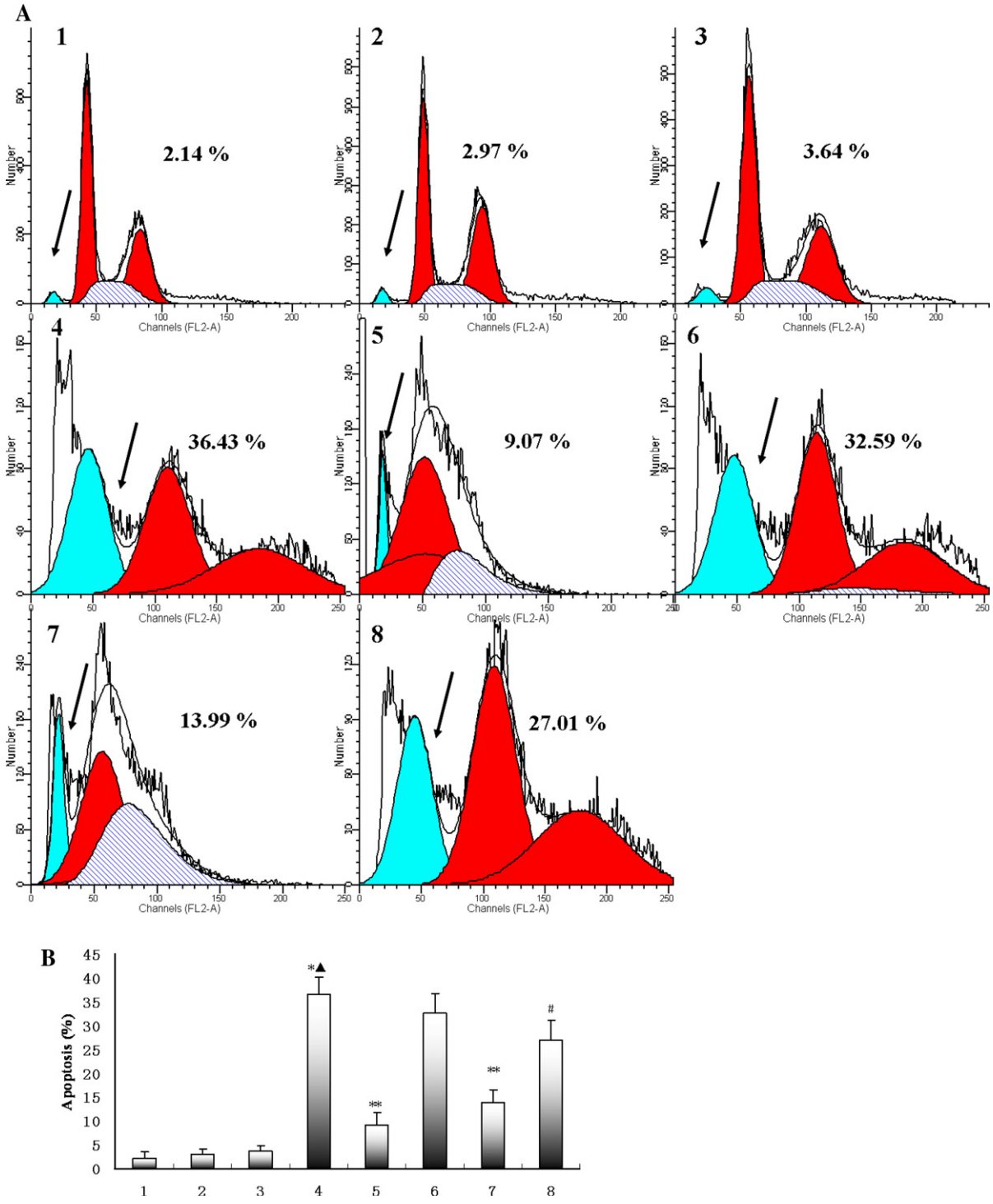

Fig. 3. Apoptotic rate of RAW 264.7 cells assessed by FACS. Cells were treated with indicated conditions for 24 h, stained with 0.33 mg/L PI and then analyzed by flow cytometry. (A) 1, Control group; 2, 100 ng/mL LPS group; 3, 100 ng/mL LPS + 10 mM indomethacin group; 4, 100 ng/mL LPS + 10^{-5} M DHAP group; 5, 100 ng/mL LPS + 10^{-5} M DHAP + 10 mM indomethacin group; 6, 100 ng/mL LPS + 10^{-5} M DHAP + 100 nM SC-560 group; 7, 100 ng/mL LPS + 10^{-5} M DHAP + 100 μM NS-398 group; 8, 100 ng/mL LPS + 10^{-5} M DHAP + 10 mM indomethacin + 10 μM 15dPGJ$_2$ group. (B) 1, 2, 3, 4, 5, 6 ,7 and 8 represent the above seven FACS analysis figures, respectively. Data were the mean × SD of 4 separate samples. *P<0.01 vs. LPS group. **P<0.01 vs. LPS + DHAP group. # P<0.01 vs. LPS + DHAP + indomethacin group. ▲P<0.01 in relation to LPS + indomethacin group.

LPS alone were firstly studied. After incubation with LPS for 18 h, the culture medium was collected and analyzed by HPLC. As shown in Fig. 1A–C, the retention time of $15dPGJ_2$ was 9.4 min. In control group, $15dPGJ_2$ was nearly undetectable. However, when cells were challenged with 50, 100 or 200 ng/mL LPS, $15dPGJ_2$ in supernatant increased obviously to 200 pg/mL (±23.34), 508 pg/mL (±43.92) and 688 pg/mL (±47.88), respectively.

And then, DHAP and LPS were administrated simultaneously. The results showed that none of $10^{-}7$ M, 10^{-6} M and 10^{-5} M DHAP could change the amount of $15dPGJ_2$ compared with control group. But in any of the groups exposed to 50 ng/mL, 100 ng/mL or 200 ng/mL LPS, all of 10^{-7} M, 10^{-6} M and 10^{-5} M DHAP obviously enhanced the production of $15dPGJ_2$ (Fig. 1D). At the same time, DHAP showed a dose-dependent manner in the cells treated with any given dose of LPS. In order to explore further why DHAP had different effects on LPS-activated or non-LPS-activated cells, in the following experiments, different doses of LPS with only 10^{-5} M DHAP were used.

3.2. Changes of cytokine levels

As well known, TNF-alpha and IL-10 are crucial pro-inflammatory and anti-inflammatory cytokine [17,18], then their contents in culture medium were chosen as the index to assess whether the production of $15dPGJ_2$ could bring about the pro-resolution pharmacological effect of DHAP *in vitro*. To this end, cells were treated with both 100 ng/mL LPS $+10^{-5}$ M DHAP and 10 mM indomethacin (a nonselective COX inhibitor), 100 nM SC-560 (a selective COX-1 inhibitor), or 100 μM NS-398 (a selective COX-2 inhibitor), respectively. Another group was served to study the direct effect of $15dPGJ_2$, in which LPS, DHAP, indomethacin and $15dPGJ_2$ were administrated simultaneously.

Consistent with what we had proved previously [8], DHAP could markedly inhibit the production of TNF-alpha in LPS-stimulated macrophage. However, DHAP could not change the level of IL-10 obviously (data not shown). As shown in Fig. 2, indomethacin attenuated this inhibitory effect of DHAP by ~70% ($P<0.01$), and this result was reversed, in part, by $15dPGJ_2$ ($P<0.01$). Experiments with SC-560 and NS-398 showed that only selective COX-2 inhibitor treated group had obvious higher TNF-alpha concentration compared with DHAP treated group ($P<0.01$).

We also established LPS+10 mM indomethacin group as a control, it showed an inhibitory effect on the production of TNF-alpha (from 267.33 pg/mL to 218.98 pg/mL), but not as efficient as DHAP (from 267.33 pg/mL to 102.23 pg/mL). The results showed that there was no significance difference between LPS+indomethacin group and LPS+DHAP+indomethacin group. Combining with above results, it demonstrated that when activity of COX was inhibited, DHAP could not exert its function to lessen the production of TNF-alpha.

3.3. Changes of cell apoptosis

Emerging data suggested that apoptosis of macrophages is a key event in the resolution of inflammation [19]. Thus apoptosis was also chosen as the index to study the role of $15dPGJ_2$ in the pro-resolving pharmacological effect of DHAP *in vitro*.

As seen in the results of flow cytometry analysis (Fig. 3A1,2), the untreated cells showed only 2.14% apoptotic rate while 100 ng/mL LPS made no obvious change in this

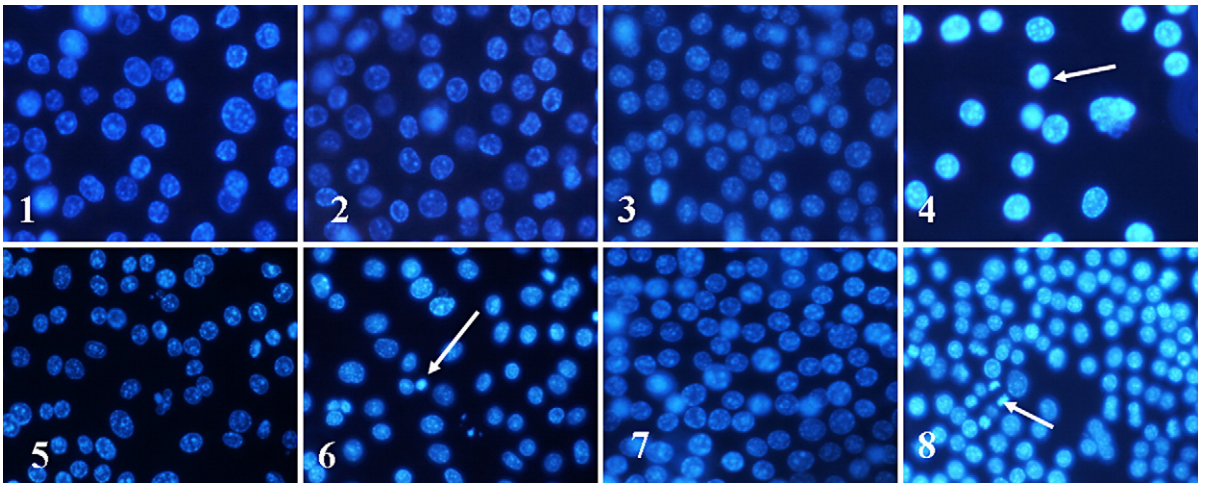

Fig. 4. Morphological changes in the nuclei (typical of apoptosis) of RAW 264.7 cells induced by DHAP. Cells were treated with indicated conditions for 24 h, stained with Hoechst 33258 and observed with a fluorescence microscope. Cells with condensed chromatin were defined as apoptotic RAW264.7 cells as marked with arrows. 1, Control group; 2, 100 ng/mL LPS group; 3, 100 ng/mL LPS+10 mM indomethacin group; 4, 100 ng/mL LPS+10^{-5} M DHAP group; 5, 100 ng/mL LPS+10^{-5} M DHAP+10 mM indomethacin group; 6, 100 ng/mL LPS+10^{-5} M DHAP+100 nM SC-560 group; 7, 100 ng/mL LPS+10^{-5} M DHAP+100 μM NS-398 group; 8, 100 ng/mL LPS+10^{-5} M DHAP+10 mM indomethacin+10 μM 15d-PGJ_2 group.

rate. Then we further studied the effect of DHAP on cell apoptosis. As a result, in the presence of LPS, 10^{-5} M DHAP markedly enhanced the percentage of apoptosis to 36.43% (±3.79) (Fig. 3A4). After different COX inhibitors were administrated simultaneously with LPS and DHAP, it was found that nonspecific inhibition of COX obviously reduced cell apoptotic percentage from 36.43%(±3.79) to 9.07% (±2.58), while it rose back to 27.01%(±3.98) by 15dPGJ$_2$ replacement (Fig. 3A5,8). COX-2 specific inhibitor NS-398, but not COX-1 specific inhibitor SD-560, could reduce cell apoptosis percentage to 13.99% (±2.56) which was obviously lower than LPS+DHAP group ($P<0.01$).

Apoptosis was further confirmed by analyzing the nuclear morphology of RAW 264.7 macrophages. Nuclear morphology was evaluated with membrane-permeable blue Hoechst 33258. Fig. 4 showed representative Hoechst 33258 fluorescence photomicrographs of cultured RAW 264.7 macrophages treated under indicated conditions. In control, LPS, LPS+indomethacin,

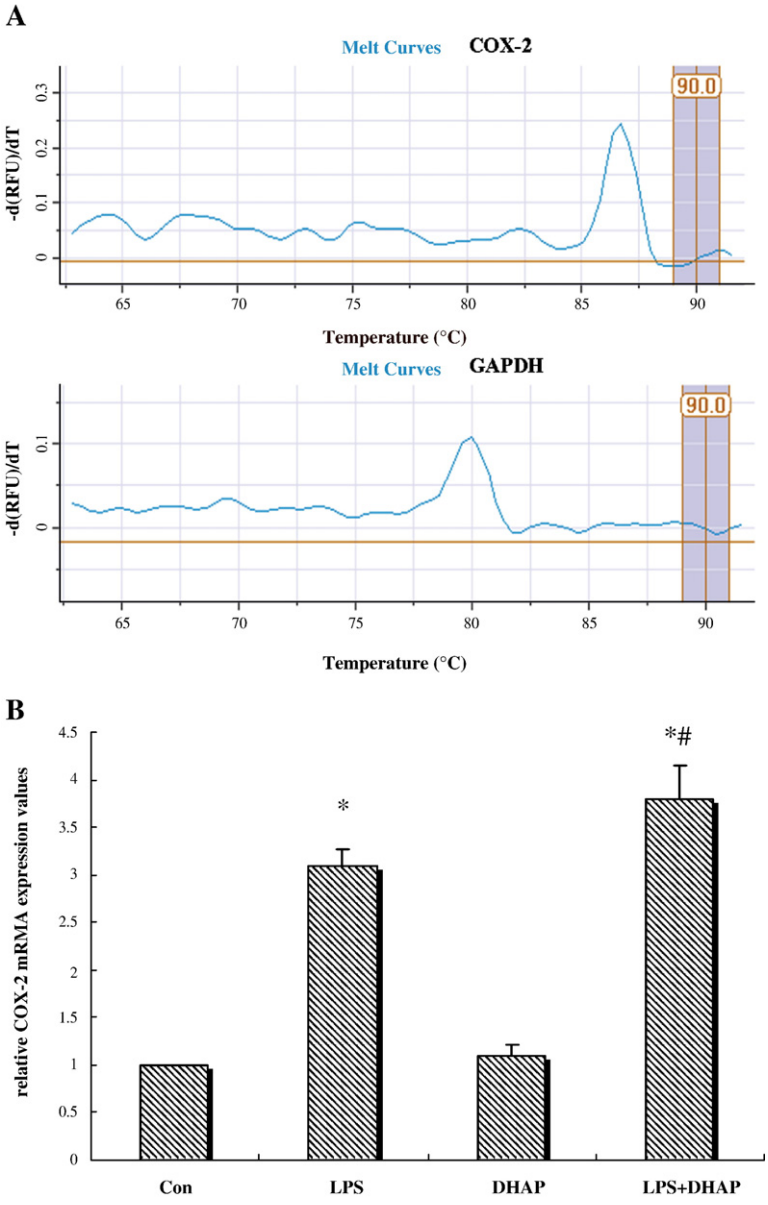

Fig. 5. DHAP increased COX-2 mRNA expression in LPS-activated RAW264.7 cells. A, The COX-2 and GAPDH melting curves. B, Data were expressed as fold compared with control. Cells were treated with indicated conditions for 18 h, and then total RNA was analyzed by quantitative real-time PCR (SYBR Green) using primers specific for COX-2. GAPDH was used as an internal standard. The value of control is 1. Values were expressed as mean±SD of 6 samples of each group. *$P<0.01$ vs. control group. #$P<0.05$ vs. LPS group.

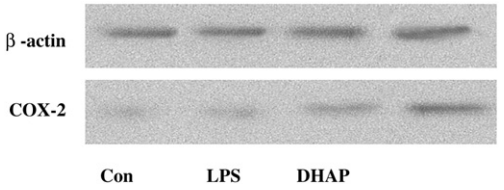

Fig. 6. DHAP increased COX-2 protein expression in LPS-activated RAW264.7 cells. Cells were treated with indicated conditions for 24 h, and then COX-2 protein level was analyzed by western blot. β-actin protein served as a loading control.

LPS+DHAP+indomethacin and LPS+DHAP+NS-398 groups (Fig 4.1,2,3,5,7), nuclei of RAW 264.7 cells appeared with regular contours and were round and large in size. Rarely RAW 264.7 cells with smaller nuclei and condensed chromatin were seen. In contrast, most nuclei of LPS+DHAP, LPS+DHAP+SD-560 and LPS+DHAP+indomethacin+15dPGJ$_2$ groups, RAW 264.7 cells appearing hypercondensed (brightly stained) were obviously increased (Fig. 4.4,6,8). Results from both flow cytometry analysis and nuclear morphology study confirmed that 15dPGJ$_2$ played a critical way in the pro-apoptotic effect of DHAP *in vitro*.

3.4. Changes of mRNA and protein of COX-2

Since COX-2 was crucial in the generation of 15dPGJ$_2$, we subsequently focused our study on the expression of COX-2 in different groups. The displayed efficiencies of two PCR assays were 96.80% for COX-2 and 98.75% for GAPDH, respectively. As shown in the melting curves, both curves were a single melting peak, indicating the specialty was good (Fig. 5A). The results of quantitation demonstrated that LPS-induced COX-2 gene expression increased about 3 folds, and DHAP increased LPS-induced COX-2 gene expression from relative value 3.1 (±0.17) to 3.8 (±0.35) (Fig. 5B).

We also assessed COX-2 protein levels by immunoblotting using polyclonal antibodies raised against COX-2. As expected, COX-2 protein was only detectable in control cells, both LPS and DHAP treatment alone could up-regulate its expression in cytoplasm, while, the COX-2 protein level in LPS+DHAP group was obviously higher than LPS group (seen in Fig. 6).

4. Discussion

Recently, there is an increasing notion in inflammation research field that the resolution phase of inflammation is equally well coordinated by "switching-off signals", such as the endogenous pro-resolution factors, including cyclopentenone prostaglandins (cyPGs) [15], lipoxin [20], heme-oxygenase-1(HO-1) [21], and angiopoietin-1 [22]. Since this innate inflammatory response is a beneficial defensive event [9], and most of the pro-resolving factors are triggered by mediators produced during the onset phase of inflammation [23], thus it isn't hard to understand that excessively dampening the onset phase of acute inflammation may ultimately inhibit resolution. These reasons may be responsible for the limitation of the current anti-inflammatory therapies to some extent.

The growth of herbal medicines or functional foods based on natural medicinal plants has gained rapid development in recent years around the globe [24]. Unfortunately, as for the research on the Chinese traditional anti-inflammatory herbs, relatively less attention has been focused on whether these herbs are associated with resolving of acute inflammation. In our previous work, it was showed that DHAP, an active component isolated from leaves of Tumaodongqing (*Ilex Pubescens Hook. Et Arn. Var glaber Chang*), exerted anti-inflammatory effect by reducing the TNF-alpha protein level in macrophages [8], thus, the present study was undertaken to explore whether it had potential pharmacological effects on the resolution stage of inflammation.

15dPGJ$_2$, a bioactive cyPGs, is physiologically formed by dehydration and isomerization of the PGD$_2$ [25]. Since 15dPGJ$_2$ preferentially prevents monocyte rather than polymorphonuclear leukocyte (PMN) from trafficking into the inflammatory site, therefore, it indicated that this cyPG does not control the onset phase in which PMNs predominated, instead, it tightly regulated the resolution phase in inflammatory response [10]. Thus this attractive pro-resolving lipid was chosen as our research target. In the present study, we first studied the effect of DHAP on the generation of 15dPGJ$_2$ in macrophages. Our results demonstrated that 10^{-5} M DHAP alone is insufficient to enhance the level of 15dPGJ$_2$ in cell culture medium, but it could obviously do this way with simultaneous treatment with LPS. Since LPS stimulation could commendably trigger the condition of inflammation, certainly, this result revealed the pro-resolving potential of DHAP *in vitro*.

To clarify the pharmacologic effect of DHAP on inflammatory resolution and its relation with 15dPGJ$_2$ more clearly, we further studied on some other important features. Decrease of pro-inflammatory cytokines and increase of anti-inflammatory cytokines are important changes in inflammatory resolution stage [10]. Thus we investigated whether DHAP could influence these cytokines. Consistent with what we had proved previously [8], DHAP could markedly inhibit the production of TNF-alpha in LPS-stimulated macrophage. However, in contrast, it could not change the level of IL-10 (data not shown). Then we questioned whether 15dPGJ$_2$ mediated this effect of DHAP to block the production of TNF-alpha. Since PGD$_2$ and its cyPG breakdown

product 15dPGJ$_2$ were generated in COX-2 pathway [10], then, different kinds of COX inhibitors were applied. As expected, indomethacin itself could partly inhibit the generation of TNF-alpha induced by LPS. That meant LPS-triggered TNF-alpha production was partly dependent on COX-2 pathway. When macrophages were co-treated with DHAP and above inhibitors, the results showed that DHAP could not depress the production of TNF-alpha so efficiently while COX-2 was inhibited by indomethacin or NS-398. That meant one or more of COX-2 derived prostaglandins might be involved in the inhibition of DHAP on TNF-alpha. Further more, the experiment of 15dPGJ$_2$ replacement by simultaneous administration of indomethacin and 15dPGJ$_2$ showed a direct mediating role of 15dPGJ$_2$ playing in this effect.

As being well documented, the fate of leukocytes should be efficient clearance after they had carried out their role [9], including systemic re-circulation and apoptosis [26,27]. As for the macrophages, being the phagocytotic executant to clear apoptotic leukocytes, they were always thought to be cleared by lymphatic drainage [28]. But *in vivo* data suggested that apoptosis of macrophages was also a key component of acute inflammatory resolution stage [19]. From then on, more attentions had been attracted in this field. Castrillo A et al. found 15dPGJ$_2$ exerted its pro-resolving function by inducing apoptosis of activated macrophages [15,29]. Reactive oxygen and nitrogen species all were thought to control the fate of macrophages in inflammation [30]. So, in the present study, apoptosis was also chosen as the important index to assess the pharmacologic effect of DHAP on inflammatory resolution. The results of both flow cytometry and nuclear morphologic analysis confirmed that DHAP could increase the apoptosis of macrophage when it was stimulated with LPS. This elevation was markedly inhibited by indomethacin and NS-398, while 15dPGJ$_2$ could partly reverse this change. So, we could get the conclusion that 15dPGJ$_2$ mediated, at least partly, the effect of DHAP to enhance the LPS-activated macrophages.

We further studied the direct effect of DHAP on COX-2 expression by real-time PCR and western blotting. DHAP was found to up-regulate the COX-2 gene in LPS-activated macrophages. This might help us to understand, at least to a certain extent, why DHAP could increase 15dPGJ$_2$ and then exert its pro-resolving potential.

Thus, above results demonstrated that DHAP had the pro-resolving pharmacological effect on LPS-triggered inflammation though up-regulation 15dPGJ$_2$, apoptosis and down-regulation of TNF-alpha of macrophages *in vitro*. As the regulative target of DHAP, 15dPGJ$_2$ was also partly mediated its function to inhibit TNF-alpha and enhance apoptosis. More and more researchers believe developing drugs based on endogenous mediators that are inherent to resolution might represent a new but attractive strategy in anti-inflammatory therapy [31,32] Unfortunately, such therapeutics, with the exception of those that regulate leukocyte apoptosis [33], is far from the clinic applications [9]. Thus, our results might give a clue for the future research on the pharmacologic effect of some anti-inflammatory Chinese Herbs.

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