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# Diphenyleneiodonium inhibits NF-κB activation and iNOS expression induced by IL-1β: involvement of reactive oxygen species

**A. Ferreira Mendes[1,2], A. Pato Carvalho[2], M. Margarida Caramona[1] and M. Celeste Lopes[1,2,CA]**

[1]Faculty of Pharmacy and [2]Centre for Neurosciences of Coimbra, Department of Zoology, University of Coimbra, Rua do Norte, 3000–295 Coimbra, Portugal

[CA]Corresponding Author
Tel: +351 239 480236/7
Fax: +351 239 480217
E-mail: mcflopes@imagem.ibili.uc.pt

***Aims*: In this work, we studied the mechanisms by which diphenyleneiodonium chloride (DPI) inhibits nitric oxide (NO) synthesis induced by the pro-inflammatory cytokine interleukin-1β (IL-1) in bovine articular chondrocytes. To achieve this, we evaluated the ability of DPI to inhibit the expression and activity of the inducible isoform of the NO synthase (iNOS) induced by IL-1. We also studied the ability of DPI to prevent IL-1-induced NF-κB activation and reactive oxygen species (ROS) production.**
***Results*: Northern and Western blot analysis, respectively, showed that DPI dose-dependently inhibited IL-1-induced iNOS mRNA and protein synthesis in primary cultures of bovine articular chondrocytes. DPI effectively inhibited NO production ($IC_{50}$ = 0.03 Ī 0.004 μM), as evaluated by the method of Griess. Nuclear factor-kappa B (NF-κB) activation, as evaluated by electrophoretic mobility shift assay, was inhibited by DPI (1–10 μM) in a dose-dependent manner. IL-1-induced ROS production, as evaluated by measurement of dichlorofluorescein fluorescence, was inhibited by DPI at concentrations that also prevented NF-κB activation and iNOS expression.**
***Conclusions*: DPI inhibits IL-1-induced NO production in chondrocytes by two distinct mechanisms: (i) by inhibiting NOS activity, and (ii) by preventing iNOS expression through the blockade of NF-κB activation. These results also support the involvement of reactive oxygen species in IL-1-induced NF-κB activation and expression of NF-κB-dependent genes, such as iNOS.**

**Key words:** Reactive oxygen species, Flavonoid-containing enzymes, Diphenyleneiodonium chloride, Pro-inflammatory cytokines, Chondrocyte, Arthritis

## Introduction

Interleukin-1β (IL-1), a potent pro-inflammatory cytokine, has been detected at elevated levels in the synovial fluid of patients with various forms of arthritis.[1,2] This cytokine has been shown to inhibit the synthesis of cartilage matrix proteins,[3,4] as well as to induce chondrocytes to produce matrix metalloproteinases[5] and inflammatory mediators, such as prostaglandins[6] and nitric oxide (NO).[7]

The large amounts of NO produced by chondrocytes in response to IL-1 result from activation of the gene expression and synthesis of the inducible isoform of the enzyme NO synthase (iNOS).[7,8] The iNOS protein synthesis is mainly regulated at the transcriptional level and the promoter region of the iNOS gene from different species has been reported to contain binding sites for several transcription factors, including nuclear factor-kappa B (NF-κB).[9,10] In most cell types, this transcription factor seems to be an essential requirement for iNOS induction in response to pro-inflammatory cytokines, such as IL-1 and tumor necrosis factor-α (TNF-α).[11–13] Inhibitors of NF-κB activation have been used to prevent iNOS expression and the subsequent NO production in response to a variety of stimuli.[10,14]

Several lines of evidence suggest that NO plays a significant role in the pathogenesis of arthritis. In humans, the production of nitrite (the stable end-product of NO) has been shown to be higher in the serum and synovial fluid from patients with rheumatoid arthritis and osteoarthritis than in age- and sex-matched controls.[15] Inhibitors of NO production have been shown to effectively reduce the severity of inflammatory arthritis and osteoarthritis induced in animals.[16–20] At the cellular level, NO has been shown to mediate some of the catabolic responses induced by IL-1 in chondrocytes; namely, suppression of proteoglycan synthesis,[4] induction of apoptosis,[21] activation of collagenase gene expression,[22] and inhibition of cell proliferation.[23]

Diphenyleneiodonium chloride (DPI) selectively inhibits flavonoid-containing enzymes by irreversibly binding to the flavin moiety of those enzymes, such as

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NADPH oxidase, mitochondrial NADH dehydrogenase, NADH oxidase, quinone oxidoreductase, cytochrome P450 reductase and NOS.[24–28] DPI has also been shown to suppress the expression of several genes induced by inflammatory cytokines in different cells. In chondrocytes, for instance, DPI inhibited IL-1-induced c-*fos* and collagenase expression,[22] whereas in mesangial cells it was found to inhibit NF-κB activation and the expression of the group IIA phospholipase $A_2$ induced by IL-1 and tumor necrosis factor-α.[29]

The present study aimed at elucidating the intracellular mechanisms by which DPI inhibits NO production induced by IL-1 in articular chondrocytes. The ability of DPI to prevent IL-1-induced iNOS expression and activity was investigated using primary cultures of bovine articular chondrocytes. To further characterize these cellular events, the ability of DPI to inhibit IL-1-induced NF-κB activation and reactive oxygen species (ROS) production was also evaluated.

## Material and methods

### Chemicals

Recombinant human IL-1β was purchased from R&D Systems (Abingdon, UK). DPI was from Calbiochem (San Diego, CA, USA). The anti-iNOS antibody was from Transduction Laboratories (Lexington, KY, USA). The anti-p65 antibody was from Serotec Ltd (Oxford, UK). The horseradish peroxidase-conjugated anti-rabbit antibody was from Dako A/S (Copenhagen, Denmark). The anti-actin antibody was from Boehringer Mannheim (Mannheim, Germany). The isotopes were from Amersham Pharmacia Biotech Inc. (Piscataway, NJ, USA). The oligonucleotide probe containing the specific consensus binding sequence for NF-κB was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). 2′,7′-Dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) was purchased from Molecular Probes Inc. (Eugene, OR, USA).

### Isolation and cell culture

Chondrocytes were isolated from bovine articular cartilage as described previously.[30] The cells were plated at $2 \times 10^6$ cells/ml in Ham's F-12 medium containing 3% antibiotic/antimycotic solution and 5% fetal bovine serum. The cells were allowed to recover for 24 h at 37°C in a humidified atmosphere supplemented with 5% $CO_2$. Prior to any treatments, the cells were serum-starved overnight and, thereafter, maintained in culture medium with 1% antibiotic/antimycotic solution and without serum.

### Northern blot analysis

Total RNA was isolated with the Trizol reagent (Gibco Brl, Grand Island, NY, USA), and quantitated by spectrophotometry at 260 nm. Denatured RNA samples (20 μg) were analyzed by gel electrophoresis in a 1% denaturing agarose gel, transferred to a nylon membrane and cross-linked by heating at 80°C for 30 min. The blots were hybridized with $^{32}$P-labeled iNOS c-DNA probe, subsequently stripped and reprobed with $^{32}$P-labeled glyceraldehyde dehydrogenase c-DNA as an internal standard to insure a roughly equal loading. The c-DNA probes were labeled with [α-$^{32}$P]dCTP by the klenow fragment using the Random Primed DNA Labeling kit from Boehringer Mannheim GmbH.

### Preparation of cytoplasmic and nuclear extracts

The cells were lysed in 400 μl of buffer 1 (10 mM of Tris-HCl, 10 mM of NaCl, 3 mM of $MgCl_2$, 0.5% Nonidet P-40, inhibitors cocktail at 1 : 7 dilution; pH 7.5) and incubated on ice for 15 min. The lysates were centrifuged at 2300 × *g*/5 min/4°C and the supernatants (cytoplasmic extracts) were collected and stored at −70°C. The pellets were resuspended in 30 μl of buffer 2 (20 mM of HEPES, 5 mM of $MgCl_2$, 0.2 mM of ethylenediamine tetraacetic acid, 1 mM of dithiothreitol (DTT), 300 mM of NaCl, 20% Glycerol, inhibitors cocktail at 1 : 7 dilution; pH 7.5). After a 20 min incubation on ice, the pellet lysates were centrifuged at 18,000 × *g*/20 min/4°C and the supernatants (nuclear extracts) were collected and stored at −70°C. The protein concentration of the extracts was measured using the bicinchoninic acid/copper (II) sulfate protein assay kit (Sigma Chemical Co., St. Louis, MO, USA).

### Western blot analysis

The cytoplasmic extracts (25 μg protein) were boiled in sodium dodecylsulfate (SDS) sample buffer (2.5% SDS, 0.0625 M Tris-HCl, 10% glycerol, 5% 2-mercaptoethanol, 0.05% bromophenol blue; pH 6.8). The proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes by electroblotting. The membranes were blocked with 5% skim milk in Tris-buffered saline-Tween (TBS-T) (20 mM of Tris-HCl, 150 mM of NaCl, 0.1% Tween-20) and then probed with the anti-iNOS antibody. After washing with TBS-T, the blots were incubated with a horseradish peroxidase-conjugated anti-rabbit antibody. The protein–antibody complexes were visualized by chemiluminescence using the ECL™ Western blotting detection reagents from Amersham Pharmacia Biotech Inc. The membranes were subsequently stripped and reprobed with an anti-actin antibody to ensure an equal protein loading.

### Electrophoretic mobility shift assay

The double-stranded NF-κB oligonucleotide probe was end-labeled with [γ-$^{32}$P]adenosine triphosphate

by the T4 polynucleotide kinase and purified through a Sephadex G-50 spin column. Nuclear extracts (10 μg of protein) were incubated for 40 min at 4°C in 20 μl of binding reactions containing 20 mM of HEPES, 50 mM of KCl, 1 mM of $MgCl_2$, 0.5 mM of DTT, 4% Ficoll 400, 2 μg of poly(dIdC), 20 μg of bovine serum albumen (pH 7.9) and 200,000 cpm/reaction of [$\gamma$-$^{32}$P]-labeled oligonucleotide probe. The DNA–protein complexes were resolved by electrophoresis on 4% native polyacrylamide gels. The DNA–protein complexes in the dried gel were visualized by autoradiography. For supershift analysis, nuclear extracts were incubated on ice for 2 h with 2 μg/reaction of anti-p65 antibody, before the addition of the radiolabeled oligonucleotide.

### Measurement of NO production

NO production was measured as the amount of nitrite released into the culture supernatant. Nitrite concentration was determined in cell-free culture supernatants using the spectrophotometric method based on the Griess reaction.[31]

### Assessment of cell viability

Chondrocyte cultures were treated with DPI (concentrations ranging from 0.001 to 10 μM) for 2 h prior to the addition of IL-1 (20 ng/ml) and then further incubated for 16 h. After collection of the cell-free supernatant for measurement of the nitrite concentration, the adherent cells were incubated, for 30 min at 37°C, with a solution of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) salt (500 μg/ml), which on reduction originates dark blue crystals of formazan. Formazan quantification was performed using an automatic plate reader (SLT, Austria) set at a test wavelength of 570 nm and a reference wavelength of 620 nm. The amount of formazan produced reflects the mitochondrial reducing capacity of the cells and their viability.[32]

### Measurement of reactive oxygen species production

The intracellular production of reactive oxygen species was measured using $DCFH_2$-DA as described previously[33,34] with modifications. $DCFH_2$-DA is a non-fluorescent probe that, on diffusion into the cells, is hydrolyzed by intracellular esterases to 2′,7′-dichlorodihydrofluorescein ($DCFH_2$), which is trapped within the cells. In the presence of reactive oxygen species, particularly peroxides, $DCFH_2$ is oxidized to the highly fluorescent compound 2′,7′-dichlorofluorescein.

Chondrocytes were loaded with 5 μM $DCFH_2$-DA in $Na^+$ saline solution (140 mM of NaCl, 5 mM of KCl, 1.5 mM of $CaCl_2$, 1.0 mM of $MgCl_2$, 1.0 mM of $NaH_2PO_4$, 5.6 mM of glucose, 20.0 mM of Hepes; pH 7.4) for 20 min at 37°C. After rinsing, the cells were treated with the indicated concentrations of DPI for 30 min. Then, IL-1 (20 ng/ml) was added to the corresponding wells and the cells further incubated for another 30 min. The fluorescence intensity of the cells was measured immediately at 37°C using a SPEX Fluorolog spectrometer, equipped with a thermostated waterbath, with excitation set at 502 nm and emission set at 550 nm.

## Results

The supernatants from chondrocyte cultures pretreated with DPI, in concentrations ranging from 0.001 to 10 μM, were used to evaluate NO production, whereas the adherent cells were used to assess the effect of DPI on cell viability. The viability of chondrocytes in culture was assessed by the MTT reduction assay, as described previously.[32] In this method, the optical density (O.D.) of the solution containing the formazan produced by reduction of the MTT salt is measured spectrophotometrically. The ability of IL-1-treated chondrocytes to reduce the MTT salt was identical to that of cells cultured in control conditions (0.379 ± 0.01 O.D. and 0.359 ± 0.02 O.D., respectively). The amount of formazan produced by cells treated with 1 μM (0.358 ± 0.02 O.D.) or 10 μM (0.361 ± 0.01 O.D.) of DPI did not change relatively to that obtained in control cells. Likewise, the reducing capacity of chondrocytes treated with 1 μM (0.372 ± 0.01 O.D.) and 10 μM (0.377 ± 0.03 O.D.) of DPI in the presence of IL-1 did not differ from that observed both in control and in IL-1-treated chondrocytes. These results show that DPI did not affect the mitochondrial reducing capacity of chondrocytes and, consequently, did not decrease the cell viability, even at the highest concentration tested (10 μM).

Pretreatment of chondrocyte cultures with DPI dose-dependently inhibited IL-1-induced NO production, as assessed by the amount of nitrites accumulated in the culture supernatants (Fig. 1A). Furthermore, DPI also effectively inhibited NO production when added to the cell cultures after IL-1 treatment (Fig. 1B). The concentrations required for half-maximal inhibition of NO production were identical irrespective of DPI being added before ($IC_{50}$ = 0.031 ± 0.004 μM) or after ($IC_{50}$ = 0.039 ± 0.003 μM) IL-1 treatment, as shown in Fig. 1A,B, respectively.

Pretreatment of chondrocyte cultures with DPI dose-dependently inhibited IL-1-induced iNOS mRNA (Fig. 2) and protein synthesis (Fig. 3). However, the concentrations of DPI required for inhibition of iNOS expression were higher than those that inhibited iNOS activity. At a concentration of 10 μM, DPI decreased iNOS mRNA and protein levels to approximately 10% of those obtained in cells treated with IL-1 alone (Figs. 2 and 3), whereas a concentration of 0.1 μM was

FIG. 1. Effect of DPI on IL-1-induced NO production. (A) Chondrocyte cultures were pretreated with the indicated concentrations of DPI for 2 h. Then, IL-1 (20 ng/ml) was added to the corresponding wells and the cells further incubated for 16 h. (B) Chondrocyte cultures were treated overnight with IL-1 (20 ng/ml). Then, the culture supernatant was removed and the cells washed three times with culture medium. New culture medium, containing the indicated concentrations of DPI, was added to the corresponding plates and the cells further incubated for 16 h. The nitrite concentration of each sample was determined as described in Materials and methods. Each bar represents the mean ± SD of six independent experiments, each performed in triplicate. * $p < 0.01$ relative to IL-1.

sufficient to completely abolish IL-1-induced NO production (Fig. 1). DPI alone did not affect iNOS mRNA and protein levels, nor nitrite concentration, relative to untreated cells (data not shown).

To further elucidate the mechanism by which DPI reduced iNOS expression, its effect on IL-1-induced NF-κB activation was evaluated. For this purpose, the chondrocyte cultures were previously treated with several concentrations of DPI (from 1 to 10 μM) as indicated in Fig. 4, for 2 h. Then, IL-1 (20 ng/ml) was added to the cell cultures, which were further incubated for 30 min. Electrophoretic mobility shift

FIG. 2. Effect of DPI on IL-1-induced iNOS mRNA expression. Chondrocyte cultures were pretreated with the indicated concentrations of DPI for 2 h. Then, IL-1 (20 ng/ml) was added and the cells further incubated for 6 h. iNOS and GAPDH mRNAs were detected by Northern blot, as described in Materials and methods. The results shown are representative of three independent experiments.

FIG. 3. Effect of DPI on IL-1-induced iNOS protein synthesis. Chondrocyte cultures were pretreated with the indicated concentrations of DPI for 2 h. Then, IL-1 (20 ng/ml) was added and the cells further incubated for 16 h. iNOS and actin levels were detected by Western blot, as described in Materials and methods. The results shown are representative of three independent experiments.

assay of an oligonucleotide probe containing the specific consensus binding sequence for NF-κB was used to detect active NF-κB dimers in the nuclear extracts obtained from cells treated with IL-1, either in the presence or absence of DPI. Nuclear extracts from cells pretreated with DPI (concentrations ranging from 1 to 10 μM) showed a dose-dependent decrease of the intensity of the bands corresponding to the NF-κB–DNA complexes (Fig. 4). At a concentration of 10 μM, DPI almost completely abolished IL-1-induced NF-κB activation. In competition assays, performed by incubation of the nuclear extracts from IL-1-treated cells with the [$^{32}$P]-labeled NF-κB oligonucleotide and a 100-fold excess of unlabeled probe, the formation of protein-[$^{32}$P]-labeled oligonucleotide complexes was completely abolished. Supershift analysis with an antibody against the p65 subunit of NF-κB revealed the presence of this protein in the protein–oligonucleotide complexes (Fig. 4).

Finally, $DCFH_2$, a probe that becomes fluorescent on oxidation,[33,34] was used to measure both the production of ROS in response to IL-1 and the ability of DPI to inhibit ROS production in chondrocyte cultures. The fluorescence intensity of chondrocytes loaded with $DCFH_2$-DA was measured as described in Materials and methods. The results in Fig. 5 show that IL-1 induced the production of ROS, which was inhibited, in a dose-dependent manner, by pretreatment of the chondrocyte cultures with DPI. Furthermore, the

FIG. 4. Effect of DPI on IL-1-induced NF-κB activation. Chondrocyte cultures were pretreated with the indicated concentrations of DPI for 2 h before the addition of IL-1 (20 ng/ml) for 30 min. The nuclear extracts, prepared as described in Materials and methods, were used to detect NF-κB binding to a specific oligonucleotide probe by EMSA. The results shown are representative of three independent experiments.

FIG. 5. Effect of DPI on IL-1-induced ROS production. The fluorescence intensity of chondrocyte cultures preloaded with $DCFH_2$-DA was measured after treatment with IL-1 (20 ng/ml), in the presence or absence of the indicated concentrations of DPI, as described in Materials and methods. Results are expressed as the percent increase of the fluorescence intensity relatively to control cells, which were loaded with $DCFH_2$-DA but otherwise left untreated. Each bar represents the mean ± SD of three independent experiments, each performed in triplicate.* $p < 0.05$ relative to IL-1.

concentrations of DPI that suppressed ROS production were also effective in inhibiting NF-κB activation and iNOS expression.

## Discussion

The results presented show that DPI effectively inhibited NO production induced by IL-1 in primary cultures of articular chondrocytes (Fig. 1A,B). The ability of DPI to suppress NO production was identical either when added to the chondrocyte cultures before or after IL-1 treatment (Fig. 1A,B), indicating that this compound inhibited the activity of the NOS enzyme. These results are in agreement with previous studies that demonstrated the ability of DPI to inhibit NOS activity by irreversibly binding to its flavin component.[26–28]

The results presented in Figs. 2 and 3 demonstrate that DPI (1–10 μM) also blocked, in a dose-dependent manner, IL-1-induced iNOS upregulation at both the mRNA and protein levels. Thus, DPI inhibits IL-1-induced NO production in primary bovine articular chondrocytes acting by two distinct mechanisms: (i) inhibition of IL-1-induced iNOS upregulation by acting at the transcriptional level; and (ii) inhibition of iNOS activity. Nevertheless, the concentrations of DPI required for inhibition of iNOS expression (Figs. 2 and 3) are higher than those sufficient to inhibit the enzyme activity (Fig. 1), indicating that the mechanism underlying induction of iNOS expression is less sensitive to inhibition by DPI than the iNOS enzyme itself.

Electrophoretic mobility shift assay (EMSA) analysis showed that DPI prevented IL-1-induced NF-κB activation in concentrations that also inhibited iNOS expression (Fig. 4). These results strongly suggest that DPI prevents IL-1-induced iNOS expression by inhibiting NF-κB activation. These results are in agreement with a recent report showing that DPI inhibited NF-κB activation and group IIA phospholipase $A_2$ expression induced by IL-1 and TNF-α in rat mesangial cells.[29]

In recent years, reactive oxygen species (ROS) have been increasingly recognized as signaling intermediates for many cytokine- and growth factor-induced responses in various cell types.[22,35,36] ROS have been shown to regulate many cellular events, including activation of transcription factors, namely NF-κB,[37–40] induction of gene transcription[22,35,41] and cell death.[42,43] In chondrocytes, IL-1 has been shown to induce the production of ROS, which were reported to mediate IL-1-induced expression of c-*fos* and collagenase.[22]

The flavonoid-containing enzymes sensitive to inhibition by DPI are sources of cellular ROS production.[24] Thus, most effects of DPI and other iodonium compounds have been attributed to the suppression of ROS production, usually interpreted as resulting from the inhibition of NADPH oxidase.[22,44–47]

The results presented in Fig. 5 show that DPI dose-dependently inhibited IL-1-induced ROS production, therefore indicating that flavonoid-containing enzymes are required for IL-1-induced ROS production in articular chondrocytes. Furthermore, our results also indicate that the ROS produced in response to IL-1 mediate and are required for the activation of NF-κB, which in turn is necessary for the transcriptional induction of iNOS expression in bovine articular chondrocytes (Figs. 2–4).

Whether the inhibitory effects of DPI result from the inhibition of NADPH oxidase and/or of other flavonoid-containing enzymes was not addressed in the present study. Nevertheless, we observed that the reducing capacity of chondrocyte's mitochondria, as evaluated by the MTT reduction assay, was not affected by DPI treatment, indicating that mitochondrial flavonoid-containing enzymes, such as NADH dehydrogenase, were not inhibited by DPI. Therefore, mitochondrial flavonoid-containing enzymes are not likely to contribute to IL-1-induced ROS production in articular chondrocytes, since the generation of ROS was completely inhibited by DPI (Fig. 5). In turn, this observation favors the involvement of plasma membrane-bound flavonoid-containing enzymes, such as NADPH oxidase, on IL-1-induced ROS production and on the subsequent activation of NF-κB and iNOS expression.

It is interesting to notice that the concentrations of DPI (1–10 μM) that were effective in inhibiting IL-1-induced ROS production (Fig. 5) were also sufficient to inhibit NF-κB activation (Fig. 4) and iNOS expression (Fig. 2), whereas much lower concentrations of DPI completely abolished the activity of iNOS (Fig. 1). Together, these results suggest that the flavonoid-containing enzymes, involved in IL-1-induced ROS production and underlying induction of NF-κB activation and iNOS expression, are less sensitive to inhibition by DPI than the iNOS enzyme itself.

Studies *in vivo* showed that DPI effectively suppressed the development of inflammatory arthritis in mice, which was attributed to its ability to simultaneously inhibit NADPH oxidase and NOS.[48] In the present study, the intracellular mechanisms that can explain, at least in part, the reported efficacy of DPI in the treatment of inflammatory arthritis were investigated for the first time. These mechanisms involve, on one hand, the ability of DPI to inhibit ROS production and, subsequently, the activation of NF-κB and the expression of NF-κB-dependent genes such as iNOS, and, on the other hand, the direct inhibition of iNOS activity. Therefore, adequate manipulation of the dosage regimen of DPI may allow for the selective inhibition of iNOS expression and/or activity.

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