European Journal of Pharmacology 633 (2010) 85–92

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Immunopharmacology and Inflammation

The flavonoid dioclein reduces the production of pro-inflammatory mediators *in vitro* by inhibiting PDE4 activity and scavenging reactive oxygen species

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ARTICLE INFO

Article history:
Received 15 September 2009
Received in revised form 11 January 2010
Accepted 20 January 2010
Available online 10 February 2010

Keywords:
Dioclein
Flavonoid
PDE4 inhibitor
Reactive oxygen species
Antioxidant
Inflammation

ABSTRACT

Reactive oxygen species, cytokines and chemokines produced at inflammatory sites are pivotal events in the progression of many diseases. Flavonoids are well-known for their antioxidant and anti-inflammatory activities. Here, we investigated the effects of the flavonoid dioclein on the production of mediators of inflammation *in vitro* and possible underlying mechanisms. Murine macrophages were pretreated with dioclein, rolipram, a PDE4 (cyclic nucleotide phosphosdiesterase type 4) inhibitor, or butylated hydroxytoluene (BHT), an antioxidant, and then activated with LPS or LPS/IFN-γ. The concentration of TNF-α, IL-6, CXCL1/KC, CCL2/JE, and nitric oxide (NO) was determined on culture supernatants. To evaluate potential mechanisms of action, dioclein was tested for inhibition of PDE4 activity and for antioxidant properties by chemiluminescence assays. Dioclein was efficient in reducing the production of cytokines, chemokines and NO in a concentration-dependent manner (from 5 to 50 μM). Dioclein was more effective than BHT and rolipram, while having similar inhibitory effects to the combination of BHT plus rolipram. Dioclein inhibited PDE4 activity with an approximate IC_{50} of 16.8 ± 1.4 μM and strongly reduced the concentration of reactive oxygen species in cell and cell-free systems, being more effective than the standard antioxidant BHT. The flavonoid dioclein possesses significant antioxidant and PDE4 inhibitory activity, showing that the substance may have substantial advantages over mechanisms of action already described for many flavonoids. Such effects account for the anti-inflammatory effects of dioclein, mainly by reducing the concentration of mediators of inflammation, such as cytokines, chemokines and reactive oxygen species by macrophages.

1. Introduction

Flavonoids constitute a large group of low molecular weight polyphenolic compounds derived from plants. Consumption of flavonoids in the diet has been shown to be inversely associated with morbidity and mortality from coronary heart disease (Hertog et al., 1993; Knekt et al., 1996). Flavonoids have strong antioxidant properties, which may delay the onset of atherogenesis by reducing peroxidative reactions, inflammatory mediators release and decreasing thrombotic tendency (Aviram and Fuhrman, 1998; Rice-Evans and Miller, 1997). In addition, some flavonoids have been described to inhibit directly or indirectly the activity of cyclic nucleotide phosphodiesterases (PDEs) (Ko et al., 2004; Middleton et al., 2000; Nichols and Morimoto, 2000; Orallo et al., 2005; Peluso, 2006).

Mammalian cells of the monocyte/macrophage lineage are critical in many inflammatory and immunoregulatory responses against infection (Matata and Galinanes, 2002; Sanlioglu et al., 2001). Macrophages are also thought to play an important role in the pathophysiology of several acute and chronic inflammatory diseases (Maus et al., 2003; Zhu et al., 2007). The role of macrophages in inflammatory diseases is secondary to the ability of macrophages to release a range of mediators of the inflammatory process (Brown et al., 2004; Condos et al., 2003; Drouet et al., 1991). For example, stimulation of macrophages with LPS (lipopolysaccharide) induces the release of reactive oxygen species,

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doi:10.1016/j.ejphar.2010.01.021

cytokines, including TNF-α and IL-6, and both CXC and CC chemokines (Chan et al., 2001; Dinarello et al., 1993; Hirano et al., 1996; Matata and Galinanes, 2002; Nishikawa et al., 1999; Sanlioglu et al., 2001; Xing et al., 1998). Agents that elevate cyclic AMP (adenosine 3',5' monophosphate) are effective inhibitors of macrophage function, including production of reactive oxygen species, cytokines and chemokines (Deng et al., 2006; Silva et al., 2001; Souness et al., 2000; Teixeira et al., 1997). Since the predominant PDE that metabolizes cyclic AMP in macrophages belongs to the PDE4 (cyclic nucleotide phosphosdiesterase type 4) family, PDE4 inhibitors suppress the production of cytokines, chemokines and reactive oxygen species by macrophages (Giembycz, 2002; Kuss et al., 2003; Miotla et al., 1998).

Dioclein is a flavonoid (flavanone family) isolated from the roots of *Dioclea grandiflora* Mart. ex Benth. (Bhattacharyya et al., 1995; Spearing et al., 1997). Recently we have reported an important vasodilator and hypotensive effect for dioclein (Almeida et al., 2002; Cortes et al., 2001; Lemos et al., 1999). Anti-inflammatory effects have not been described for this molecule. Hence, in this work we firstly evaluated the effect of dioclein on the production of mediators of inflammation *in vitro*. Since flavonoids have anti-oxidant activities and a few compounds have been described to exhibit some inhibitory activity on PDE4 (Ko et al., 2004; Nichols and Morimoto, 2000; Orallo et al., 2005; Peluso, 2006), we additionally evaluated the relevance of these two properties for the ability of dioclein to reduce the concentration of inflammatory mediators released by macrophages. The results showed here point to a new and not yet described combination of effects for flavonoids in the context of inflammation and their mediators.

2. Material and methods

2.1. Drugs and solutions

cAMP (cyclic adenosine 3',5' monophosphate), cGMP (cyclic guanosine 3',5'monophosphate), tris-(hydroxymethyl)-aminomethan (Tris), BHT (butylated hydroxytoluene), BSA (bovine serum albumin type V), ethylene glycol-bis (β-aminoethyl ether)-N, N, N',N'-tetraacetic acid (EGTA), LPS (lipopolysaccharide from *E. coli*), DMSO (dimethyl sulfoxide), luminol, lucigenin, MTT (methyl thiazol tetrazolium), rolipram, xanthine, xanthine oxidase, TG (sodium thyoglicolate) and zymosan were purchased from Sigma-Aldrich (St. Louis, MO, USA). [8-3H] cAMP (30 – 50 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA) and purified by thin layer chromatography on silica gel, using isopropanol:NH_4OH:H_2O (70:15:15) as a solvent. Dioclein (99% by HPLC) was synthesized according to (Spearing et al., 1997) and was dissolved in sterile DMSO (dimethyl sulfoxide, Sigma-Aldrich, St. Louis, MO, USA). The same vehicle was used for rolipram and luminol. Mouse recombinant IFN-γ was purchased from R&D Systems (Minneapolis, MN). The final concentration of DMSO did not exceed 0.3% in all experiments and had no effect when tested in control preparations. Lucigenin, LPS and BHT were dissolved in sterile PBS (Phosphate-buffered saline, pH 7.4) or in cell culture medium (Dulbecco´s Modified Eagle Medium, Sigma-Aldrich, St. Louis, MO, USA).

2.2. Animals

Animal care and experimental procedures were guided with the approval of the Ethics Committee in Animal Research of the Universidade Federal de Minas Gerais (CETEA/UFMG), published in the Brazilian College for Animal Experimentation (COBEA). Experiments were performed using 8 to 10 weeks male C57/BL6 mice, 20 to 25 g, from the Centro de Bioterismo (CEBIO/UFMG) and housed in an animal care facility of the Instituto de Ciências Biológicas at the Universidade Federal de Minas Gerais (ICB/UFMG). Food and water were available *ad libitum*. Each *in vitro* experiment used cells harvested from at least five animals.

2.3. Effect of dioclein on purified PDE4 isoforms

PDE4 was isolated by anion exchange chromatography from bovine aortic smooth muscle cytosolic fraction and the purified PDE was stored at -80 °C until use in small aliquots (Lugnier et al., 1986). PDE activity was measured by a radioenzymatic assay as previously described (Keravis et al., 1980) at a substrate concentration of 1 μM cAMP + 1 mM EGTA in the presence of 10,000 cpm [^{3}H]-cAMP as tracer. The buffer solution was composed of 50 mM Tris-HCl (pH 7.5), 2 mM magnesium acetate, and 1.25 mg BSA. Assays were carried out in the presence of 50 μM cGMP to prevent detection of PDE3 (cyclic nucleotide phosphosdiesterase type 3) activity. The concentration of the compound that produced 50% inhibition of substrate hydrolysis (approximate IC_{50}) was calculated by non-linear regression analysis from 3 independent concentration-response curves ± S.E.M (GraphPad Prism, San Diego, CA), and included 6 different concentrations of inhibitor.

2.4. Isolation and purification of peritoneal macrophages

To culture thyoglicolate-elicited peritoneal macrophages, mice were given an i.p. injection with 1.5 ml of sodium thyoglicolate (TG) 3% medium (Sigma-Aldrich, St. Louis, MO, USA). Four days after injection, mice (N = 5 to 6) were sacrificed by cervical dislocation and cells in the peritoneal cavity were harvested and washed with ice-cold incomplete DMEM (Dulbecco´s Modified Eagle Medium, Sigma-Aldrich, St. Louis, MO, USA). The pool of cells, containing at least 95% of macrophages, was collected, centrifuged and the cell pellet was resuspended in DMEM supplemented with 10% FBS (Fetal Bovine Serum, GIBCO), 100 U/ml penicillin and 100 μg/ml streptomycin. Macrophages were plated in a flat-bottom 96-well cell culture plate (Nunc Brand Products, Rochester, NY, USA) at a density of 2×10^6/ml. After incubation at 37 °C in 5% CO_2 for 3 h, the medium was aspirated to remove non-adherent cells, and the adherent macrophages were cultured in complete medium overnight or immediately used in the chemiluminescence assays. Using this protocol, we have obtained macrophages which provide reproducible responses, without significant activating levels, that are easily measurable with the protocols presented here.

2.5. In-vitro stimulation of macrophages

Macrophages cultured overnight, plated in a flat-bottom 96-well cell culture plate at a density of $2–3 \times 10^6$/ml, were treated with vehicle (DMSO 0,3% in medium or medium alone), dioclein (5, 10, 20, 40 and 50 μM), a standard antioxidant, BHT (10 mM) and with rolipram (10^{-6} M), a PDE4 inhibitor. The concentrations of BHT and rolipram used in this work were based in already published data (Au et al., 1998; Choi et al., 2007; Cooper et al., 1999; Podrez et al., 1999). After incubation with the chosen treatments for 10 min, macrophages were stimulated with 100 ng/ml of LPS. After incubation at 37 °C in 5% CO_2 for 24 h, the supernatants were aspirated and stocked for cytokines and chemokines evaluation by ELISA. For the evaluation o nitric oxide (NO) production, macrophages were stimulated with LPS (100 ng/ml) and IFN-γ (50 U/ml) after the chosen treatments. After incubation at 37 °C in 5% CO_2 for 48 h, the supernatant were aspirated and immediately used for the dosage of nitrite (NO_2^-) by Griess reaction.

2.6. Measurement of cytokines and chemokines in the cell culture medium

The concentration of murine cytokines TNF-α and IL-6, and chemokines CCL2/JE and CXCL1/KC, was measured by ELISA in the cell culture medium 24 h after pre-treatment with dioclein, vehicle, rolipram or BHT, and post LPS challenge (100 ng/ml), using commercially available antibodies according to the procedures supplied by the manufacturer (R&D Systems, Minneapolis, MN). Cell

culture supernatant was removed at the chosen time point and stocked at -20 °C until the time of the assay.

2.7. Measurement of nitrite concentration in cell culture medium

Accumulation of nitrite (NO_2^-) in the medium, as an index on nitric oxide (NO) production, was measured 48 h after pre-treatment with dioclein, vehicle, rolipram or BHT, and post LPS/IFN-γ challenge (100 ng/ml and 50 U/ml, respectively), by a colorimetric assay based on the Griess reaction (Jun et al., 1994). Briefly, aliquots of medium from each group were reacted with 1% sulfanilamide and 0.1% naphthyl ethylenediamine dihydrochloride (both purchased from Sigma-Aldrich, St. Louis, MO, USA) in 2.5% phosphoric acid at room temperature for 10 min NO_2^- concentration was determined by absorbance at 540 nm in comparison with a sodium nitrite ($NaNO_2$) curve as a standard.

2.8. Evaluation of respiratory burst

Luminol-enhanced and zymosan-induced chemiluminescence is a widely used protocol to assess cellular respiratory burst in intact cells (Fromtling and Abruzzo, 1985). A pool of cells was resuspended in colorless RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) and plated in triplicate at 3×10^5 cells/well in a Luminunc 96-well microwell plate (maxisorp, white, flat bottom, Nunc Brand Products, Rochester, NY). Luminol (5×10^{-5} mol/L) and zymosan (20 particles/cell) were added in each well after a 10 min exposure to dioclein, vehicle (DMSO 0,3%) or BHT. The zymosan-elicited luminescence was then recorded by a luminometer (LumiCount; Packard, Meriden, CT) for 120 min at 37 °C (60 cycles of measurement with intervals of 2 min). Chemilluminescence emission of each well was expressed as relative luminescence units (RLU). The area under the curve was calculated from the curves generated by the assay to evaluate the different patterns of luminol-enhanced chemiluminescence from each group.

2.9. Lucigenin-enhanced chemiluminescence in a cell-free system

The superoxide scavenging properties of dioclein was examined in a cell-free system comprising xanthine (100 μM) plus xanthine oxidase (0.03 U/ml), which allow us to evaluate the scavenging activity of the compound over a single reactive oxygen specie. The assay was carried out in a Luminunc 96-well microwell plate, maxisorp, white, flat bottom, containing 300 μl of reaction mixture, consisting on xanthine, xanthine oxidase, and lucigenin (5 μM) in PBS, pH 7.4, with or without dioclein. Because the superoxide signal produced by xanthine/xanthine oxidase (X/XO) was transient, the reaction was initiated by addition of xanthine oxidase into the mixture, and all counting commenced 1 min after xanthine oxidase addition. The lucigenin-enhanced chemiluminescence was then recorded by a luminometer for 120 min at 37 °C (60 cycles of measurement with intervals of 2 min). Chemiluminescence emission of each well was expressed as relative luminescence units (RLU). The area under the curve was calculated from the curves generated by the assay to evaluate the different patterns of lucigenin-enhanced chemiluminescence from each group.

2.10. Evaluation of cell viability

MTT assay was performed as previously described (Molinari et al., 2003). Briefly, at the experimental time-points cited above, medium from treated macrophages were removed and incomplete DMEM medium supplemented with MTT (5 mg/ml) was immediately added to each well in the same plate. The microplates were further incubated at 37 °C for 3 h at 5% of CO_2. The supernatant was then removed and lysing buffer, containing 20% w/v of SDS (sodium dodecyl sulphate, Sigma-Aldrich, St. Louis, MO, USA) in a solution of DMF (N,N-dimethyl formamide, Synth, Brazil) 50% in deionized water, were added to each well. After washing, the solution obtained were plated and read at 560 nm using a microtiter plate ELISA reader (SpectraMax 190, Molecular Devices, USA).

2.11. Statistical Analysis

Results are shown as the mean ± S.E.M. Analysis of variance (one-way ANOVA) was used followed by Student-Newman-Keuls post hoc analysis. The level of significance was set at $P<0.05$.

3. Results

3.1. Effects of dioclein on the production of TNF-α, IL-6, CXCL1/KC, CCL2/JE and nitric oxide (NO)

Activation of macrophages with LPS or LPS plus IFN-γ induced a significant production of cytokines/chemokines or NO, respectively, in the supernatant ($P<0.001$) (Fig. 1). Pretreatment with dioclein reduced the production of all mediators assessed in a concentration dependent manner (Fig. 1) without affecting cell viability, as assessed by MTT (data not shown). The maximal inhibitory effect of dioclein was 58% for TNF-α 73% for IL-6, 44% for CXCL1, 48% for CCL2/JE and 74% for NO. Levels of CCL5/RANTES and CXCL2/MIP-2 were not altered to any major extent upon treatment with dioclein (data not shown).

3.2. Effects of Dioclein on PDE4 activity

In order to evaluate the ability of dioclein to inhibit PDE4 isoenzymes, we tested a range of concentrations (1 - 100 μM) against partially purified PDE4. As seen in Fig. 2, dioclein inhibited PDE4 with an approximate IC_{50} value of 16.8 ± 1.4 μM. For comparison, rolipram, a standard inhibitor of PDE4, has an approximate IC_{50} in the range of 1 μM in this system (Bolger et al., 1996; Lugnier et al., 1986). Goncalves et al. (2009) have recently shown that the prototype PDE4 inhibitor rolipram inhibited purified PDE4 in the same system with an approximate IC_{50} of 0.7 μM.

3.3. Effects of dioclein as a scavenger of reactive oxygen species

Initial experiments evaluated the ability of dioclein to decrease the production of reactive oxygen species in intact cells. As seen in Fig. 3, activation of macrophages with zymosan induced a respiratory burst that peaked around 10 min after the addition of the stimulus. Treatment of cells with dioclein effectively inhibited the production of reactive oxygen species. When the area under the curve (AUC) was measured, maximal inhibition (90%) occurred with 50 μM of dioclein (Fig. 3A). Incubation of cells with BHT (30 mM) reduced by 53% (AUC) the production of reactive oxygen species (Fig. 3B).

In a cell free system in which reactive oxygen species, mainly superoxide, were produced by the reaction of xanthine with xanthine oxidase, addition of dioclein (50 μM) to the system blocked the production of free radicals by 54% (AUC) when compared to the vehicle group (Fig. 4). In the same system, a standard concentration BHT (30 mM) inhibited reactive oxygen species production by 28%, as calculated by the AUC (Fig. 4).

3.4. Comparative effects of dioclein versus rolipram with or without BHT on cytokine production

Rolipram (10^{-6} M) significantly reduced the production of IL-6, TNF-α and NO in the medium after challenge with LPS ($P<0.001$) (Fig. 5 A, B and E). The PDE4 inhibitor, rolipram, showed a minor inhibitory effect on the production of CXCL1/KC and CCL2/JE (Fig. 5 C and D). The antioxidant BHT (30 mM) also decreased the production of cytokines, chemokines and NO. BHT was less effective than

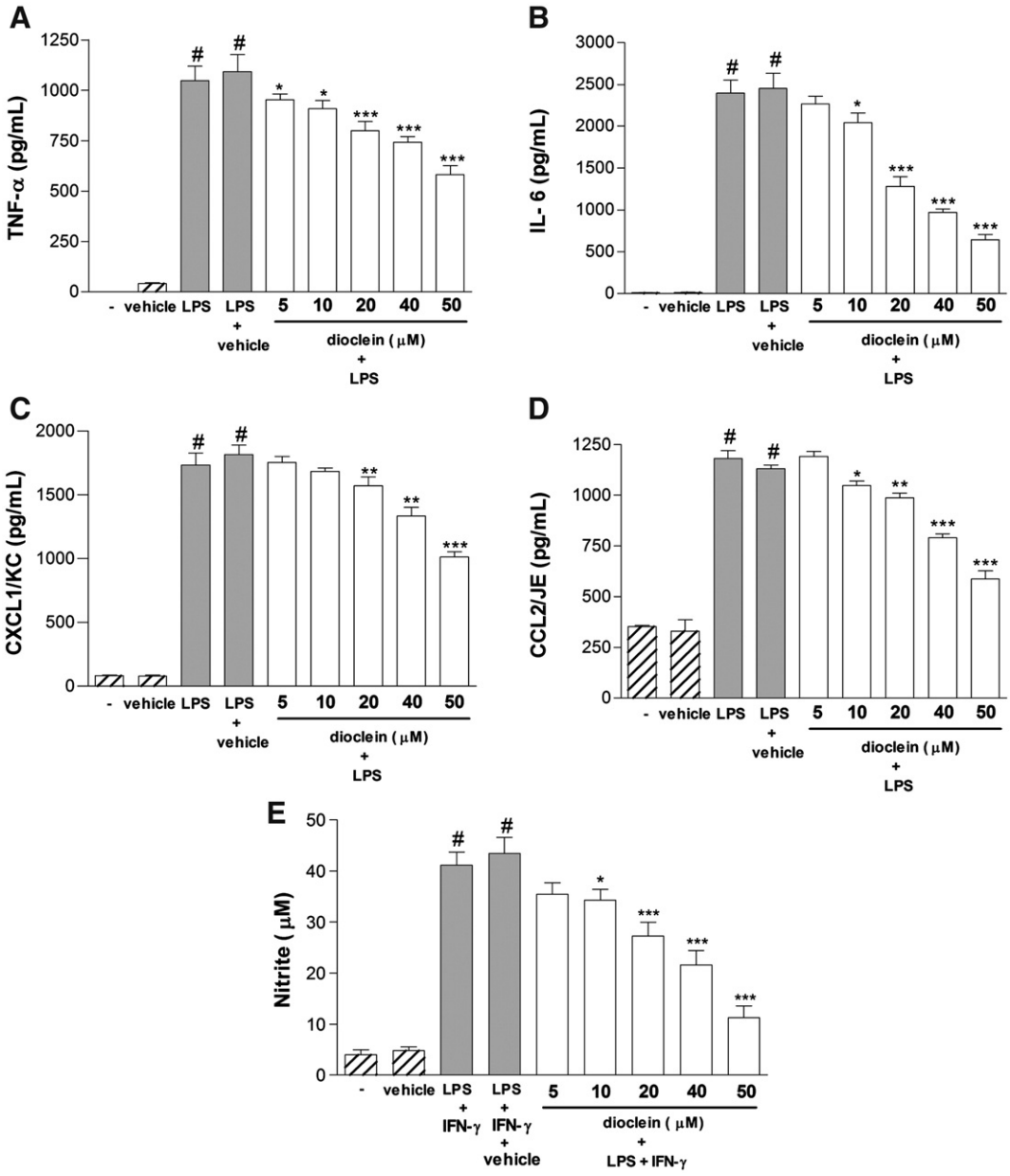

Fig. 1. Concentration-dependent effect of dioclein on the production of cytokines, chemokines NO by peritoneal murine macrophages. In panels A, B, C and D, the effect of dioclein on the production of TNF-α, IL-6, CXCL1/KC and CCL2/JE, respectively. The concentration of chemokines and cytokines were determined by ELISA 24 h post challenge with LPS (100 ng/ml). In panel E, effect of dioclein (μM) on the production of NO, assayed by Griess reaction, 48 h post challenge with LPS + IFN-γ (100 ng/ml and 50 U/ml). Results are shown as mean ± S.E.M. from at least 3 experiments. # $P<0.01$ when compared to medium/medium + vehicle. *$P<0.05$, **<0.01, ***<0.001 when compared to LPS + vehicle or LPS + IFN- γ.

rolipram at blocking IL-6 and TNF-α production ($P<0.001$ for both cytokines) but was more effective at blocking CXCL1/KC, CCL2/JE and NO ($P<0.05$, $P<0.01$ and $P<0.05$, respectively).

The combined treatment of BHT and rolipram was more effective than either treatment alone at blocking the production of IL-6, CCL2/JE and NO (Fig. 5). The combined treatment was as effective as rolipram alone at blocking TNF-α production and as BHT alone at blocking CXCL1/KC production (Fig. 5). Treatment with dioclein was as effective as the combined treatment with BHT and rolipram in all conditions tested (Fig. 5). Of notice, dioclein was also more effective than BHT or rolipram, separately, in the inhibition of IL-6, CCL2/JE and nitrite (Fig. 5). No alterations in cell viability, as evaluated by the MTT assay, were observed (data not shown).

4. Discussion

The major findings of the present study can be summarized as follows: (i) the flavonoid dioclein has significant suppressor activity on the production of the pro-inflammatory mediators IL-6, TNF-α, CXCL1/KC, CCL2/JE and NO, by LPS-stimulated macrophages *in vitro*; (ii) dioclein inhibits the function of isolated PDE4 isoenzyme and (iii) has significant reactive oxygen species scavenging activity both in macrophages and cell free systems; (iv) inhibition of PDE4 and reduction of the concentration of reactive oxygen species in the medium contributes to the inhibitory effects of dioclein. This combination of inhibitory effects that results in inhibition of inflammation is unique among flavonoids.

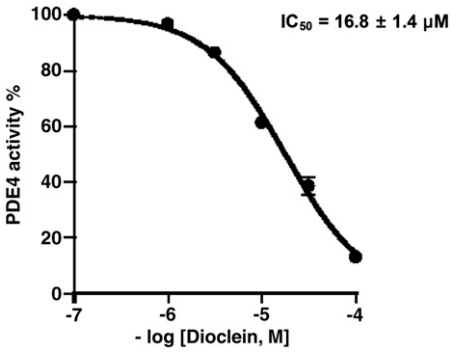

Fig. 2. Concentration-response curve of the effect of dioclein on the inhibition of purified-PDE4-isoform activity. The experiment was carried out using a radioenzymatic assay, with [^{3}H]-cAMP as radioactive tracer. Inhibition of substrate hydrolysis (**approximate IC_{50}**) was calculated by non-linear regression analysis from 3 independents concentration-response curves ± S.E.M and included 6 different concentrations of inhibitor.

Treatment of LPS-stimulated macrophages with the flavonoid dioclein greatly decreased the production of the inflammatory cytokines IL-6 and TNF-α, the chemokines CXCL1/KC and CCL2/JE, and NO, mediators mainly involved in the recruitment and activation of inflammatory cells, specially cells of the monocytic lineage and polymorphonuclear leukocytes (Alves-Filho et al., 2008; Harkin et al., 2001; Maus et al., 2003; Wilsson et al., 2008). Although our work focused on macrophages, other cell types are involved in a wide range of responses in the course of acute and chronic inflammatory process and should be considered in further experimental approaches with the

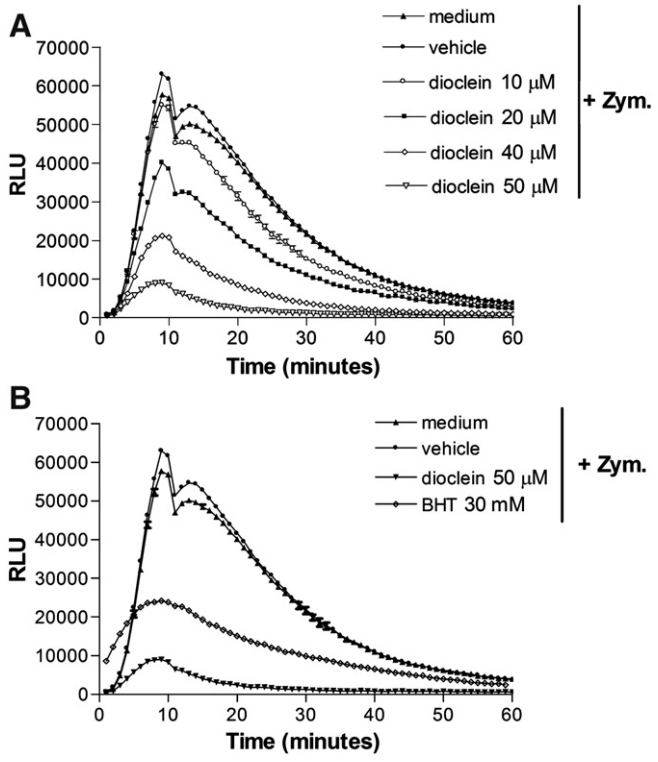

Fig. 3. Time course of luminol-enhanced chemilluminescence in mice peritoneal macrophages. Macrophages, pooled from five mice were plated (3×10^5 cells/well) and stimulated with zymosan (20 particles/cell). The relative luminescence units (RLU) were assayed in triplicate and results are representative of two different experiments. In panel A, concentration-dependent inhibition of dioclein over ROS production as expressed by decreased RLU after addition of zymosan. In panel B, comparative effect of vehicle, BHT and dioclein over ROS production as expressed by decreased RLU after addition of zymosan. ΔAUC: maximal inhibition of 90% with 50 μM of dioclein (panel A) and maximal inhibition of 53% with 30 mM of BHT (panel B).

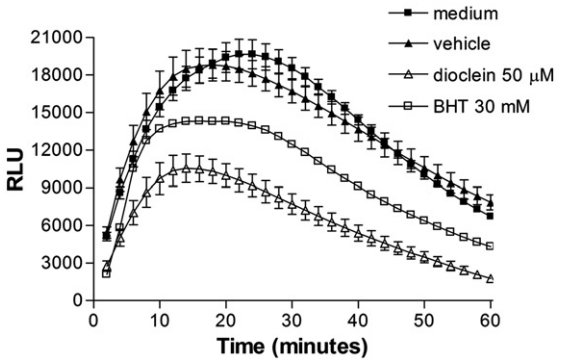

Fig. 4. Time course of lucigenin-enhanced chemilluminescence induced by the generation of superoxide (O_2^-) in the xanthine/xanthine oxidase cell-free system. Comparative inhibitory effect of vehicle, BHT and dioclein over O_2^- production, as expressed by decreased RLU after addition of xanthine oxidase. The relative luminescence units (RLU) were assayed in triplicate and results are representative of two different experiments. ΔAUC: maximal inhibition of 54% with 50 μM of dioclein and maximal inhibition of 28% with 30 mM of BHT.

flavonoid. These inhibitory effects of dioclein are consistent with other studies showing an inhibitory effect of flavonoids on the production of pro-inflammatory mediators *in vitro* and *in vivo* (Birrell et al., 2005; Cheng et al., 2007; Geraets et al., 2009; Porath et al., 2005). Because flavonoids differ in their ability to influence enzymatic systems, further studies were carried out to identify putative mechanisms of action of dioclein in the system. In particular, we focused on the possibility that dioclein inhibited PDE4 enzymes and possessed antioxidant properties.

The inhibition of PDEs, the enzymes that degrade and inactivate cyclic nucleotides, is one strategy to increase cAMP levels in leukocytes and to suppress inflammatory responses (Banner and Trevethick, 2004; Barnette and Underwood, 2000; Teixeira et al., 1997; Torphy, 1998). Recently, many flavonoids have been described as PDE4 inhibitors (Middleton et al., 2000; Nichols and Morimoto, 2000; Orallo et al., 2005; Peluso, 2006). Indeed, dioclein showed to be a PDE4 inhibitor, with an approximate IC_{50} value of 16.8 μM. The approximate IC_{50} for dioclein on PDE4 activity is compatible with the concentrations of dioclein which were necessary to reduce the concentration of reactive oxygen species in the medium and the production of cytokines, chemokines and NO. Moreover and in contrast to rolipram, dioclein is not a selective PDE4 inhibitor, as the compound has also been shown to block PDE1 isoform at low concentrations (Goncalves et al., 2009).

Flavonoids are well known as antioxidant compounds, with reactive oxygen species-scavenging activity reported in many *in vitro* and *in vivo* systems (Es-Safi et al., 2007; Rahman et al., 2006; Stevenson and Hurst, 2007). This scavenging activity of many classes of flavonoids is attributed to their chemical structure (Choi et al., 2002; Heijnen et al., 2001; Pavlovic and Santaniello, 2007; Wolfe and Liu, 2008; Zielinska-Przyjemska and Wiktorowicz, 2006), but the magnitude of its effects are quite variable. In an attempt to assay potential antioxidant properties for dioclein we induced a respiratory burst in macrophages by the addition of zymosan, which induces a high and sustained production of reactive oxygen species for at least 1 hour. This production is strictly linked to the activation of pro-oxidative systems (Fromtling and Abruzzo, 1985). The addition of dioclein to the medium led to a strong reduction on the relative luminescence induced by the interaction of luminol with reactive oxygen species, which was stronger than that induced by BTH, a well known antioxidant. Dioclein also reduced reactive oxygen species concentration in the medium in a cell-free xanthine/xanthine oxidase system. The scavenging action of dioclein was more effective than that of maximal concentrations of BHT. A previous study from our group has suggested this antioxidant property of dioclein in a model of myocardial ischemia and reperfusion, in which the flavonoid induced a cardioprotective effect during the reperfusion period by reducing

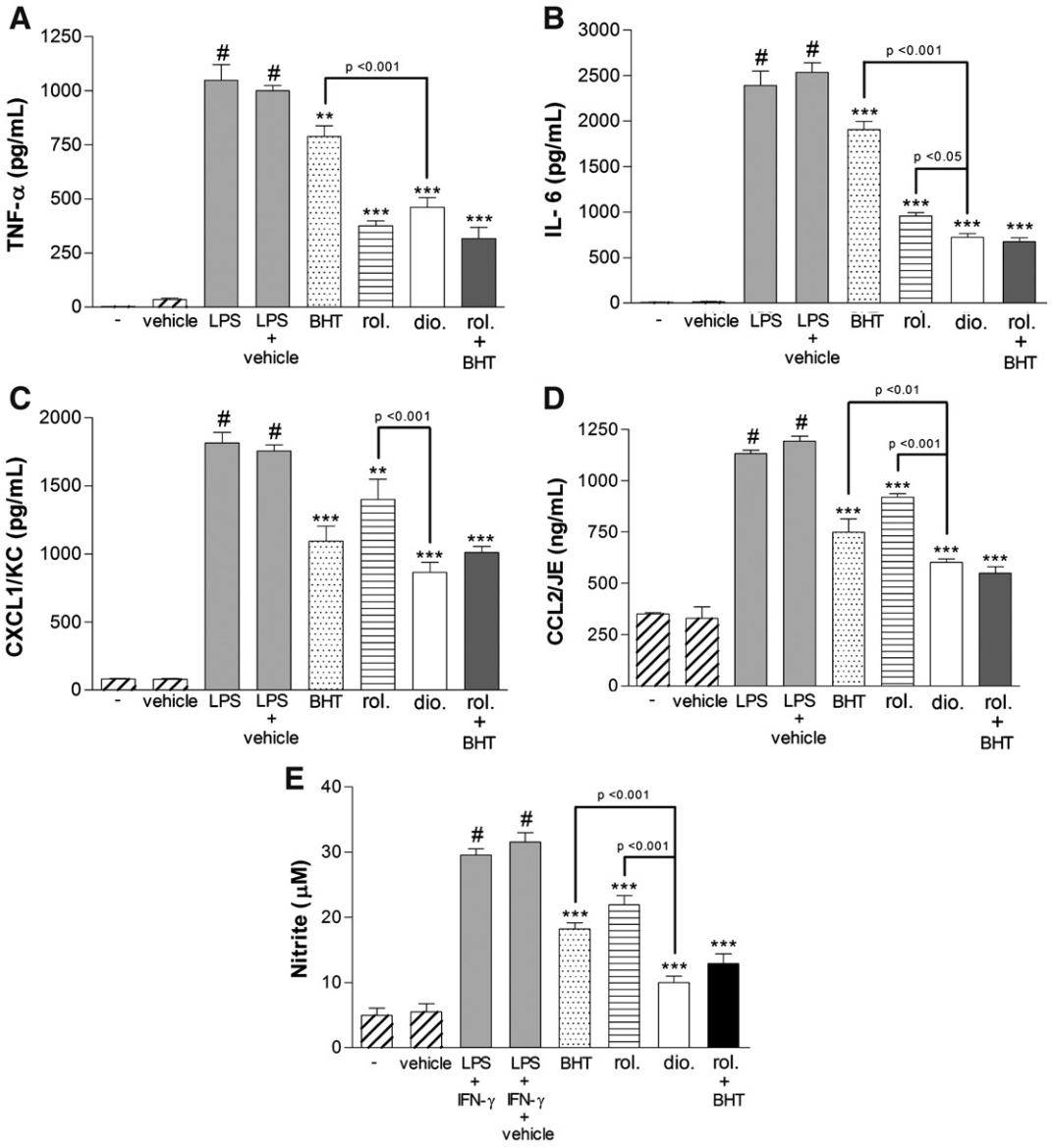

Fig. 5. Effects of BHT (30 mM), rolipram (rol.; 10^{-6} M), dioclein (dio.; 50 μM), or the combination of BHT + rolipram on the production of cytokines, chemokines and nitrite by peritoneal murine macrophages. In panels A, B, C and D, the effect of the compounds on the production of TNF-α, IL-6, CXCL1/KC and CCL2/JE, respectively. The concentration of chemokines and cytokines were determined by ELISA 24 h post challenge with LPS (100 ng/ml). In panel E, effect of the compounds on the production of nitrite, as assayed by Griess reaction, 48 h post challenge with LPS + IFN-γ (100 ng/ml and 50 U/ml). Results are shown as mean ± S.E.M. from at least 3 experiments. # $P<0.01$ when compared to medium/medium + vehicle. *$P<0.05$, **<0.01, ***<0.001 when compared to LPS + vehicle or LPS + IFN- γ. Black traces: differences between dioclein treatment and BHT or rolipram treatments.

the concentration of free radicals (Vianna et al., 2006). However, this is the first report of a strong scavenging activity of dioclein against a specific free radical anion, superoxide.

Inhibitors of PDE4 can also suppress the production of reactive oxygen species in addition to preventing other activation processes in leukocytes (Germain et al., 2001; Jacob et al., 2004). Indeed, the link between cAMP-elevating agents and reactive oxygen species reduction is well known (Brown et al., 2007). A series of experiments were then conducted to evaluate whether both the PDE4 inhibitory and reactive oxygen species scavenging activities of dioclein contributed to the ability of the compound to decrease cytokine production by stimulated macrophages. Treatment with BHT and rolipram was more effective than either drug alone against the production of IL-6, CCL2/JE and NO by LPS-stimulated macrophages. In contrast, full inhibitory activity against TNF-α and CXCL1/KC was observed when rolipram or BHT, respectively, was used alone. Under all conditions, dioclein was as effective as the combined treatment, suggesting that the inhibitory effect of dioclein on pro-inflammatory mediator production *in vitro* is associated to the free-radical scavenging and PDE4 inhibitor properties of the drug, which confirms that the reactive oxygen species-reducing activity of the compound cannot be solely ascribed to its antioxidant activity, the main property of many flavonoids in similar experimental approaches.

In addition to these anti-inflammatory effects, dioclein has vasodilator actions *in vitro* and induces hypotension *in vivo* (Almeida et al., 2002; Cortes et al., 2001; Lemos et al., 1999), reinforcing the wide range of effects for this compound. Moreover, further studies are needed to define the potential of dioclein in the context of atherosclerosis, which is associated with chemokine-driven inflammation in the vessel and, commonly, hypertension (Baltus et al., 2005; Koenen et al., 2009; Monaco et al., 2002; Riou et al., 2007). Recently published data of our group also showed that a complex prepared by inclusion of dioclein in β-cyclodextrin improves the hypotensive effect of the flavonoid by

increasing its bioavailability and enables dioclein to be effective after oral administration (Rezende et al., 2009), an interesting approach for future *in vivo* investigations.

In conclusion, our study shows that the flavonoid dioclein significantly inhibits or reduce the production of molecules associated with the recruitment and activation of macrophages during inflammatory processes. In this regard, dioclein may serve as template for the development of novel anti-inflammatory drugs, mainly based on its ability to control the inflammatory response at different levels, which is not a common attribute for many well studied flavonoids. Finally, it is interesting to note that the double actions of dioclein - PDE4 inhibition and reactive oxygen species scavenging - were more efficient than either action alone in preventing release of pro-inflammatory mediators, giving further support to the notion that drugs which act at multiple targets may be more effective at treating inflammation (Kuldo et al., 2005; Ribeiro and Horuk, 2005; Vidal et al., 2007).

Statement of conflicts of interest

The authors state no conflict of interest.

Acknowledgement

We thank Valdinéria Borges, Ilma Marçal, Eric Roma and Hélène Justiniano for technical assistance. This work received financial support from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) and from Centre National de Recherche Scientifique (CNRS, France).

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