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

Rodrigo Guabiraba [a], Ana Lucia Campanha-Rodrigues [b], Adriano L.S. Souza [a], Helton C. Santiago [c], Claire Lugnier [d], Jacqueline Alvarez-Leite [b], Virginia S. Lemos [e], Mauro M. Teixeira [a,*]

[a] *Laboratório de Imunofarmacologia, Departamento de Bioquímica e Imunologia – ICB, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627. Pampulha, 31270-901, Belo Horizonte - MG, Brazil*
[b] *Laboratório de Bioquímica Nutricional, Departamento de Bioquímica e Imunologia – ICB, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627. Pampulha, 31270-901, Belo Horizonte - MG, Brazil*
[c] *Laboratório de Gnotobiologia, Departamento de Bioquímica e Imunologia – ICB, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627. Pampulha, 31270-901, Belo Horizonte - MG, Brazil*
[d] *CNRS UMR, 7213, Biophotonique et Pharmacologie, Faculté de Pharmacie, Université de Strasbourg, 74 route du Rhin, BP 60024, 67401, Illkirch, France*
[e] *Laboratório de Fisiologia Cardiovascular, Departamento de Fisiologia e Biofísica – ICB, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627. Pampulha, 31270-901, Belo Horizonte - MG, Brazil*

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,

* Corresponding author. Tel./fax: +55 31 3409 2651.
E-mail address: mmtex@icb.ufmg.br (M.M. Teixeira).

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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