We have previously shown that a ditriazine derivative 4,10-dichloropyrido[5,6:4,5]thieno[3,2-\text{d}′,3,2-\text{d}]-1, 2, 3-ditriazine (DTD) modulates acute inflammation in murine models by inhibition of leukocyte functions and expression of inducible enzymes including nitric oxide synthase and cyclooxygenase-2 (COX-2). In the present work, we have demonstrated the anti-inflammatory effect of DTD after oral administration in the rat adjuvant-induced arthritis, by reduction of interleukin-1\(\beta\) and tumour necrosis factor-\(\alpha\) levels and COX-2 expression in the inflamed tissues. These mediators were also significantly decreased by DTD treatment in the angiogenesis-dependent murine air pouch granuloma model, where this agent exerted anti-inflammatory and antiangiogenic effects. In vitro experiments indicated that DTD is an inhibitor of the nuclear factor-\(\kappa\B) (NF-\kappa\B) pathway of cellular activation in macrophages, in parallel with the regulation of cytokine release. Our results suggest that the anti-inflammatory and antiangiogenic properties of DTD can be related to the inhibition of cytokine and PGE\(_2\) production by interfering with NF-\kappa\B activation. This compound thus offers a therapeutic potential for the treatment of chronic inflammatory diseases with an angiogenic component, such as rheumatoid arthritis.

**Keywords** Chronic inflammation · Angiogenesis · Cytokines · Cyclo-oxygenase-2 · Nuclear factor-\(\kappa\B\)

Inflammatory cytokines such as tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and interleukin-1\(\beta\) (IL-1\(\beta\)) play a critical role in the progression of rheumatoid arthritis. This complex autoimmune disease characterised by chronic inflammation, bone erosion and proliferation of the synovial lining is regulated by numerous mediators released by different cell types (Choy and Panayi 2001). TNF-\(\alpha\) can stimulate the release of other cytokines, including IL-1\(\beta\) and chemokines, as well as the expression of adhesion molecules and inducible enzymes (Osborn et al. 1989; Akahoshi et al. 1993; Arias-Negrete et al. 1995). Increased expression of cyclooxygenase-2 (COX-2) and large quantities of prostaglandin E\(_2\) (PGE\(_2\)) can be detected in rheumatoid synovial tissues, where they mediate erosion of cartilage and juxta-articular bone as well as angiogenesis, a vital process in the maintenance of newly formed granulomatous tissue (Koch 1998).

Angiogenesis is a very complex multistep process involving a variety of biologically active substances. Among them, TNF-\(\alpha\), IL-1\(\beta\) and PGS, which can induce several growth factors and proliferation of endothelial cells in vivo, may be crucial for enhancing neovascularization (Majima et al. 2000; Choy and Panayi 2001). Angiogenesis may be required in chronic inflammatory diseases, not only for the maintenance of tissue perfusion, but also to allow the increased cellular infiltration responsible for chronicity. Thus, inhibition of the implicated metabolites such as cytokines and PGS could have an obvious benefit in angiogenesis-dependent inflammatory diseases.

Nuclear factor-\(\kappa\B\) (NF-\kappa\B) comprises a family of inducible transcription factors that serve as important regulators of the host immune and inflammatory response. NF-\kappa\B has been implicated in the pathogenesis of chronic inflammatory diseases, such as asthma, inflammatory bowel diseases and rheumatoid arthritis (Tak and Firestein 2001), where the p50 and p65 subunits of NF-\kappa\B have been detected in the nuclei of synovial lining cells (Handel et al. 1995). Activation of NF-\kappa\B increases the expression of...
genes encoding many proinflammatory mediators, including cytokines, chemokines, receptors required for cell adhesion and migration, as well as inducible enzymes such as nitric oxide synthase (iNOS) and COX-2 (Pahl 1999). The most predominantly characterised NF-κB complex is a p50/p65 heterodimer, which is constitutively present in an inactive cytoplasmic complex by binding to inhibitor κB-α (IkB-α) and other IkB proteins. Stimulation results in phosphorylation of inhibitory proteins by IkB kinases followed by their degradation, which allows the translocation of NF-κB to the nucleus and binding to a NF-κB motif to act as transcriptional regulator. This pathway for saccharide (LPS) (Baldwin 1996; Abraham 2000). NF-κB activation is triggered by several agents such as TNF-α, IL-1β, phorbol derivatives or bacterial lipopolysaccharide (LPS) (Baldwin 1996; Abraham 2000). NF-κB plays an important role in the activation of the TNF-α and NF-κB pathway, cells were preincubated with compounds or vehicle for 10 min at 4°C and microsomal fraction was used to study COX-2 expression by Western blot analysis as described below. Supernatants were homogenized in 2.0 ml methanol and aliquots of supernatants were used to determine the content of PGE2 by radioimmunoassay.

Marine air pouch granuloma. Granulomatous tissue was induced in anaesthetized female albino Swiss CD-1 mice (25–30 g) by the injection of 3 ml air into the dorsal subcutaneous tissue on day −1, followed by the intrapouch injection of 0.5 ml 1% v/v croton oil in Freund’s complete adjuvant on day 0 (Gross et al. 1991). Animals with oedema values of 1.1 ml larger than normal paws were included in the experiment (day 25) for the determination of PGE2 and thromboxane B2 (TXB2). After death, hind paws were amputated above the ankle and homogenized in 2.5 ml saline. After centrifugation at 10,000×g for 15 min at 4°C, supernatants were used for the determination of PGE2, TNF-α and IL-1β levels, by radioimmunoassay (Moroney et al. 1988) and fluoroimmunoassay (Pennanen et al. 1995), respectively. Supernatants were centrifuged at 100,000×g for 100 min at 4°C and the microsomal fraction was used to assess COX-2 expression by Western blot analysis as described below. Supernatants were homogenized in 2.0 ml methanol and aliquots of supernatants were used to determine the content of PGE2 by radioimmunoassay.

Assessment of vascularity. On day 6 animals were anaesthetized and warmed to 40°C for peripheral vasodilation. A vascular cast was made by the intravenous injection of 1 ml 10% carmine red solution in 5% gelatine. The carcasses were chilled at 2°C for 3 h and the granulomatous air pouch linings dissected. The removed tissues were oven dried at 56°C for 2 days, weighed and digested for 2 days at 56°C in 2 ml buffer (2 mM dithiothreitol, 20 mM Na3HPO4, 1 mM EDTA, 12 U/ml papain) according to Fandale et al. (1986). The dye was then dissolved by the addition of 4 N NaOH and the digest was centrifuged at 2,000×g for 15 min. The dye content of samples was assayed spectrophotometrically at 490 nm against a carmine red standard curve. The results were then expressed as vascular index (V.I., in milligrams dye/gram dry tissue).

Measurement of PGE2, cytokines levels and COX-2 expression in granulomatous tissues. Tissue extracts were obtained by homogenization of granulomas in 0.5 ml 5 mM KH2PO4/0.1 g wet tissue and after centrifugation at 2,000×g for 10 min at 4°C, aliquots of the supernatants were used to measure PGE2 levels by radioimmunoassay (Moroney et al. 1988) and TNF-α and IL-1β levels by time-resolved fluoroimmunoassay (Pennanen et al. 1995). Supernatants were further centrifuged at 100,000×g for 100 min at 4°C and microsomal fraction was used to study COX-2 expression by Western blot analysis as described below. Protein was measured by the Bradford method using bovine serum albumin as standard.

Materials and methods

Adjuvant arthritis. All studies were performed in accordance with European Union regulations for the handling and use of laboratory animals. The protocols were approved by the institutional Animal Care and Use Committee. Adjuvant arthritis was elicited in female Lewis rats (175–200 g) by injection of 0.1 ml Mycobacterium butyricum (10 mg/ml) in mineral oil into the base of the tail (Taurog et al. 1988). Paw volumes were measured at the beginning of the experiment by using a water displacement plethysmometer. Animals were housed in propylene cages with food and water ad libitum. The light cycle was automatically controlled (on 0700 hours; off 1900 hours) and the room temperature thermostatically regulated to 21±1°C. The magnitude of the inflammatory response was evaluated by measuring the volume of both hind paws at day 17. Animals with oedema values of 1.1 ml larger than normal paws were then randomized into treatment groups. 1.0 ml DTD (10 mg/kg) or vehicle (propylene glycol, distilled water: 1/1, v/v) was administered p.o. once daily and the oedema in hind paws was measured on days 17–24. Serum was collected on the last day of the experiment (day 25) for the determination of PGE2 and thromboxane B2 (TXB2). Cell culture. The mouse macrophage cell line RAW 264.7 was cultured in DMEM medium containing 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum. Macrophages were removed from the tissue culture flask using a cell scraper and resuspended at a concentration of 2.5×106 cells/ml. Cells were co-incubated with Ershicheria coli LPS (1 μg/ml) at 37°C for 20 h in the presence of compounds or vehicle in 24-well culture plates and TNF-α and IL-1β levels measured in the supernatants by time-resolved fluoroimmunoassay (Pennanen et al. 1995). The adherent cells were used to discard the possible cytotoxic effect of the tested compounds by the mitochondrial reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) to formazan (Gross et al. 1991). For experiments related to the NF-κB pathway, cells were preincubated with compounds or vehicle for 15 min and then stimulated with LPS (1 μg/ml) for different times.

Electrophoretic mobility shift assay (EMSA). Nuclear and cytoplasmic extracts from RAW 264.7 macrophages were prepared as described (López-Collazo et al. 1998). Protein was determined by the DC Bio-Rad protein reagent (Bio-Rad, Hercules, Ca., USA).