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ORIGINAL ARTICLE

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Therapeutic administration of 3,4,5-trimethoxy-4'-fluorochalcone, a selective inhibitor of iNOS expression, attenuates the development of adjuvant-induced arthritis in rats

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Abstract We have previously investigated the effects of a series of dimethoxy- and trimethoxychalcone derivatives, with various patterns of fluorination, on nitric oxide production in LPS-stimulated murine RAW 264.7. The present study was designed to determine if 3,4,5-trimethoxy-4'-fluorochalcone (CH 17) could modulate the production of NO and/or prostaglandins in vivo. On the mouse macrophage cell line RAW 264.7 CH 17 inhibited dose-dependently NO production, with an IC_{50} value in the nanomolar range, and reduced PGE_2 levels by a 58% at 10 μM. This compound had no direct inhibitory effect on iNOS and COX-2 activities. NO reduction was the consequence of inhibition of the expression of iNOS. In vitro experiments indicated that CH 17 is an inhibitor of the nuclear factor-κB (NF-κB) pathway of cellular activation in macrophages. This compound exhibited in vivo an inhibitory behaviour correlated with its in vitro results on nitrite and PGE_2 accumulation. In the rat adjuvant-induced arthritis, oral administration of CH 17 (25 mg/kg) on days 17–24 after adjuvant injection, significantly inhibited paw oedema, protected from weight loss and reduced the levels of inflammatory mediators (nitrites and PGE_2) in paw homogenates, without affecting PGE_2 levels in stomach homogenates. The profile and potency of this compound, a selective inhibitor of iNOS expression that interferes with NF-κB activation, may have relevance for the inhibition of the inflammatory response, representing a new approach to the modulation of different inflammatory pathologies.

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Introduction

Nitric oxide (NO) is a multifunctional molecule generated by nitric oxide synthase (NOS), and isoforms of NOS have been characterized (Forstermann et al. 1995). Constitutively expressed NOS (cNOS), which is mainly found in neurons and endothelial cells, it transiently produces small amounts of NO, which appears to have beneficial effects on many physiological processes (Forstermann et al. 1995). In contrast, the inducible isoform of NOS (iNOS) by cytokines and/or endotoxin is found in many cells including endothelial cells, macrophages, neutrophils, chondrocytes, and synoviocytes, and generates much larger amounts of NO over longer periods of time, and contributes to the cytotoxic and cytostatic effects of cytokines and/or endotoxin.

Recently, excessive production of NO generated by iNOS has been described in rheumatoid arthritis (Clancy et al. 1998; Borderie et al. 1999). Synovium and cartilage are important sources of increased NO production in patients with inflammatory arthritis. Increased local production of NO may contribute to the pathogenesis of inflammatory arthritis by increasing synovial blood flow and by modulating cellular function within synovium and articular cartilage (Grabowski et al. 1997). NO also seems to play an important role in the pathogenesis of arthritis in animal models including adjuvant induced arthritis in rats (Ialenti et al. 1993; Cannon et al. 1996), and treatment with inhibitors of NOS has been shown to significantly suppress the progression of arthritis in laboratory animals (McCartney Francis et al. 1993; Connor et al. 1995).

Pharmacological, genetic and biochemical analysis of the pathways involved in the expression of iNOS showed that activation of nuclear factor-κB (NF-κB) is an essential requirement for the expression of this enzyme (Xie and Nathan 1994). Moreover, transient activation of

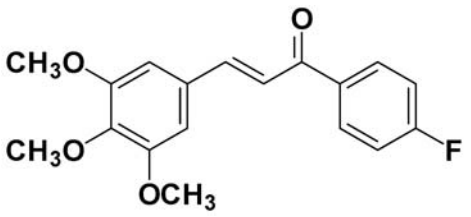

Fig. 1 Chemical structure of CH 17

NF-κB constitutes an important step in the course of several immune and inflammatory responses and plays a key role in the regulated expression of genes encoding many pro-inflammatory mediators, including cytokines, chemokines, receptors required for cell adhesion and migration, as well as, inducible enzymes (Pahl 1999). NF-κB has been implicated in the pathogenesis of chronic inflammatory diseases, such as asthma, inflammatory bowel diseases and rheumatoid arthritis (Tak and Firestein 2001). Because of this pivotal role of NF-κB activation in a variety of inflammatory conditions, this transcription factor is a relevant target for the pharmacological action of anti-inflammatory molecules (Baeuerle and Baichwal 1997).

We have previously studied several chalcone derivatives that exert different effects on a wide range of enzymes and metabolites implicated in the inflammatory process (Herencia et al. 1998). Some of them are able to control NO, superoxide and prostaglandin (PG) production in vitro as well as in vivo, having a potential role in modulating the inflammatory process (Herencia et al. 1999, 2001). Recently, we have investigated the effects of a series of dimethoxy- and trimethoxychalcone derivatives, with a various patterns of fluorination, on nitric oxide production in LPS-stimulated murine RAW 264.7 (Rojas et al. 2002). In the present work, we have assessed the possible beneficial effect of one of these chalcone derivatives, 3,4,5-trimethoxy-4´-fluorochalcone (CH 17) (Fig. 1) in the treatment of the rat adjuvant-induced arthritis, a chronic inflammatory process, and we have also determined if CH 17 could modulate the production of NO and/or PGs in vivo and if it exerts any action on NF-κB activation in vitro.

Materials and methods

Materials. CH 17 (Fig. 1) was prepared using the Claisen-Schmidt condensation, which has been previously reported (Li et al. 1995). [5,6,8,11,12,14,15(n)-^{3}H]PGE_2 was from Amersham Iberica. The rest of reagents were from Sigma (St. Louis, MO, USA).

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. Cells were resuspended at a concentration of 2×10^6/ml and co-incubated with *Escherichia coli* LPS (1 μg/ml) at 37°C for 20 h in the presence of test compounds or vehicle. The nitrite concentration as reflection of NO release and PGE_2 levels were determined in culture supernatants fluorometrically (Misko et al. 1993) and by RIA (Moroney et al. 1988), respectively. On the other hand, the NO donor sodium nitroprussiate (SNP) at 100 μM was added 30 min before LPS to determine if exogenous NO could modulate the activity of CH 17. The mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan was used to assess the possible cytotoxic effects of compounds.

iNOS and COX-2 activity in intact cells. RAW 264.7 macrophages stimulated for 20 h with LPS were washed and Hank's buffer supplemented with L-arginine (0.5 mM) and arachidonic acid (10 μM) was added for 2 h incubation with test compounds to determine their effects on iNOS and COX-2 activities. Supernatants were collected for the measurement of nitrite and PGE_2 accumulation for the last 2 h as above. In a different set of experiments, culture medium of 20 h LPS-stimulated macrophages in the presence of test compound was removed and medium supplemented with L-arginine (0.5 mM) and arachidonic acid (10 μM) was added for 2 h incubation without test compound. Supernatants were collected for the measurement of nitrite and PGE_2 accumulation for the last 2 h as above.

COX-1/COX-2 activity in intact human monocytes. Human monocytes were obtained as previously described (Rioja et al. 2002). To assess the effects of the compound on COX-2 activity, aspirin-treated human monocytes were incubated with LPS (1 μg/ml) for 24 h to induce COX-2. Cultured medium was then changed and CH 17, indomethacin, NS398 or vehicle were added for 15 min pre-incubation at 37°C. Arachidonic acid (10 μM) was then added and the cells were incubated for a further 2 h. In parallel experiments, aspirin-untreated monocytes were preincubated for 15 min with test compounds or vehicle. Afterwards, arachidonic acid (10 μM) was then added and the cells were incubated for to 2 h at 37°C to assess the effects of compounds on COX-1 activity. After the incubation period, supernatants were collected for the measurement of PGE_2 levels as above.

Western blot assay. Cellular lysates from cell line RAW 264.7 (10^6 cells/well) incubated for 18 h with LPS were obtained with lysis buffer (1% Triton X-100, 1% deoxycholic acid, 20 mM NaCl, and 25 mM Tris, pH 7.4). Following centrifugation (10,000×*g*, 15 min), supernatant protein was determined and 25 μg protein were loaded on 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes for 90 min at 125 mA. Membranes were blocked in PBS-Tween 20 containing 3% w/v unfatted milk. For iNOS, membranes were incubated with anti-iNOS polyclonal antibody (1/1,000 dilution); for COX-2, membranes were incubated with specific anti-COX-2 polyclonal antiserum (1/1,000); for β-actin, membranes were incubated with specific anti-β-actin polyclonal antiserum (1/1,000). Blots were washed and incubated with peroxidase-conjugated goat anti-rabbit IgG (1/20,000 dilution; Dako, Glostrup, Denmark). The immunoreactive bands were visualized using an enhanced chemiluminescence system (ECL; Amersham Iberica, Madrid, Spain).

Electrophoretic mobility shift assay. RAW 264.7 macrophages were preincubated with compounds or vehicle for 15 min and then stimulated with LPS (1 μg/ml) for 1 h. Nuclear and cytoplasmic extracts from cells were prepared as described (López-Collazo et al. 1998). Protein was determined by the DC Bio-Rad protein reagent (Bio-Rad, Hercules, CA, USA). The double-stranded oligonucleotide containing the consensus NF-κB sequence (Promega, Madison, WI, USA) was end-labelled using T4 polynucleotide kinase (Amersham Pharmacia Biosciences, Madrid, Spain) and [γ-^{32}P]ATP, followed by purification using G-25 microcolumns (Amersham Biosciences, Madrid, Spain). Incubations were performed on ice with 6 μg nuclear extract, 100,000 c.p.m. labelled probe, 2 μg poly(dI-dC), 5% v/v glycerol, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 100 mM NaCl and 10 mM TRIS-HCl buffer (pH 8.0) for 15 min. Complexes were analyzed by non-denaturing 6% polyacrylamide gel electrophoresis in 0.5×TRIS-borate buffer followed by autoradiography of the dried gel. Supershift assays were carried out after incubation of the nuclear extracts with 2 μg of Ab for 1 h at 4°C, followed by electrophoretic mobility shift assay (not shown).

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 male Lewis rats (175–200 g) by injection of 0.1 ml of *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 07.00 h; off 19.00 h) 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 randomised into treatment groups. Twenty-five milligrams per kilogram of CH 17 or 2 mg/kg of dexamethasone or vehicle (propylene glycol, glycerol, distilled water: 4/2/4, v/v/v) was administered p.o. twice daily and the body weight and oedema in hind paws were measured on days 17–24. On the last day of experiment (day 25), rats were placed on a radiographic box at a distance of 90 cm from X-ray source. Radiographic analysis of arthritic hind paws was performed by X-ray machine (Univet LX 160, Multimage, Italy) with a 40 kW exposition for 0.01 s. Serum was collected on day 25 for the determination of PGE_2 and TXB_2. After death, hind paws were amputated above the ankle and homogenized in 2.5 ml of saline. After centrifugation at 10,000×*g* for 15 min at 4°C, supernatants were used for the determination of nitrite and PGE_2 levels as above. Aliquots of supernatants were sonicated and centrifuged at 10,000×*g* for 20 min at 4°C and the supernatants were used to assess iNOS and COX-2 expression by Western blot analysis as described above. Stomachs were homogenized in 2.0 ml of methanol and aliquots of supernatants were used to determine the content of PGE_2 by RIA.

Statistical analysis. Statistical evaluation included one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons. *P*-values of $p<0.05$ (*) or $p<0.01$ (**) were taken as significant. Results are shown as mean ± SEM for *n* experiments. Inhibitory concentration 50% (IC_{50}) values were calculated from at least four significant concentrations.

Results

Effect of CH 17 on production of nitrite and PGE_2 in LPS-stimulated RAW 264.7 cells

LPS stimulation of RAW 264.7 macrophages for 20 h induced iNOS and COX-2 with the consequent generation of large quantities of NO and PGE_2. As shown in Fig. 2, CH 17 inhibited the generation of NO dose-dependently, with IC_{50} values of 0.033 (0.009–0.084) µM. Under these conditions, this chalcone derivative reduced PGE_2 production by a 58% at 10 µM. At the highest concentration tested (10 µM), CH 17 did not exert cytotoxic effects (<5%) during the 20 h incubation period as indicated by MTT reduction (data not shown). To determine if the inhibition of nitrite and PGE_2 production was either due to interference with the enzyme induction by LPS or due to direct action of this compound on NOS or COX activity, the following experiments were performed. CH 17 was incubated for 2 h with cells after the induction of the enzyme by LPS. No significant reduction of nitrite or PGE_2 production during these 2 h was observed (Table 1). Nevertheless, 1400 W a known inhibitor of iNOS activity, caused a very significant reduction of nitrite production, whereas the selective COX-2 inhibitor NS398 caused a marked inhibition of PGE_2 generation. In another set of experiments, CH 17 was present during the 20 h LPS treatment of cells. After washing with fresh medium, cells were incubated for a further 2 h with L-arginine (0.5 mM) and arachidonic acid (10 µM). The nitrite and PGE_2 production during these 2 h was significantly reduced by this chalcone derivative (Table 1). This suggests that the presence of CH 17 during the induction period can affect iNOS and/or COX-2 expression levels.

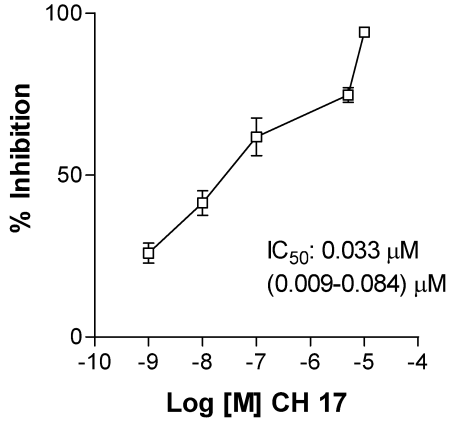

Fig. 2 Dose-response curve of nitrite inhibition by CH 17 in LPS-stimulated RAW 264.7 macrophages. Cells were incubated with LPS in the presence of CH 17 for 20 h and supernatants were used to determine mediator levels. Each value represents the mean ± SEM for more than three triplicate experiments

Table 1 Effects of CH 17 and reference compounds on iNOS and COX-2 activity in RAW 264.7. In A, cells were stimulated with LPS for 20 h. After washing the cells, test compounds were added and incubated for 2 h in the presence of L-arginine (0.5 mM) and arachidonic acid (10 µM). In B, cells were co-incubated with LPS and test compounds for 20 h. After washing the cells, incubation proceeded for 2 h in the presence of L-arginine (0.5 mM) and arachidonic acid (10 µM). *ND* not determined

	A		B	
	NO_2^- (ng/ml)	PGE_2 (ng/ml)	NO_2^- (ng/ml)	PGE_2 (ng/ml)
20 h without LPS	8.4±0.2*	4.8±1.6*	7.2±0.8*	6.9±0.9*
Control	115.8±10.2	44.0±2.0	127.3±9.6	53.3±4.0
CH 17 (10 µM)	109.1±9.5	40.7±2.5	31.8±2.1*	25.2±2.4*
CH 17 (1 µM)	111.4±8.2	40.2±2.9	65.7±5.3*	43.6±3.2
1400 W (1 µM)	30.8±0.1*	ND	19.4±1.0*	N.D.
NS398 (1 µM)	N.D.	3.0±0.6*	N.D.	11.2±1.5*

Results are the mean ± SEM; n=6; *$p<0.01$ vs. control group

Effect of CH 17 on COX-1/COX-2 activity in human monocytes

COX-2 was induced in aspirin-treated monocytes by 24 h LPS-stimulation. Cultured medium was then changed and after 15 min pre-incubation of vehicle or test compounds, arachidonic acid was added and cells were incubated for a further 2 h. In a parallel experiment COX-1 activity was studied in non-induced cells after 2 h incubation with arachidonic

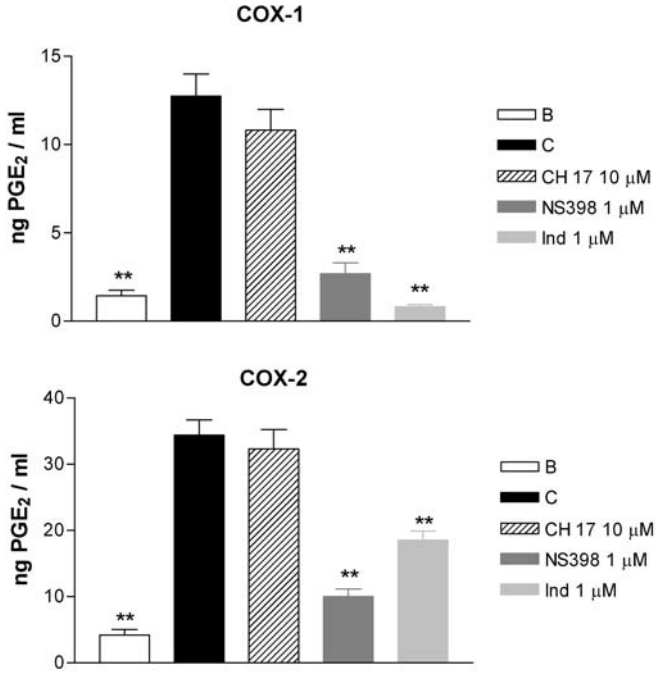

Fig. 3 Effects of CH 17 and reference compounds on COX-1 and COX-2 activity on human monocytes. *B* non-stimulated cells, *C* AA-stimulated cells. Results are the mean ± SEM. n=6. $^{**}p<0.01$ vs. control group (*C*)

acid. As shown in Fig. 3, COX-1 activity and COX-2 activity were not affected by CH 17. As expected, the reference compound indomethacin was more potent as COX-1 inhibitor, whereas NS398 selectively inhibited COX-2 activity.

Effect of a NO donor on the inhibition of PGE_2 level by CH 17

The inhibitory activity of CH 17 on PGE_2 production by LPS treatment was challenged with an NO donor (SNP). However, the inhibition of PGE_2 generation induced by the chalcone derivative was partially reverted by treatment with the NO donor (Fig. 4).

Effect of CH 17 on iNOS and COX-2 protein expression in LPS-stimulated RAW 264.7 cells

Western blot analysis was carried out on lysates of macrophages obtained as described. LPS induced iNOS and COX-2 expression, which correlated with an increase in nitrite accumulation in the medium (Fig. 5). The addition of CH 17 and the reference compound, dexamethasone, reduced iNOS expression as well as nitrite levels; CH 17 did not affect significantly COX-2 expression, as it did dexamethasone.

Effect of CH 17 on LPS-induced NF-κB DNA binding in RAW 264.7

To investigate if CH 17 may act on the NF-κB pathway of cell activation, analysis of DNA binding was performed by EMSA in nuclear extracts from RAW 264.7 macrophages stimulated with 1 μg/ml LPS for 1 h, in the absence or presence of CH 17. As shown in Fig. 6 this compound inhibited the LPS-induced binding activity of NF-κB to DNA at 10 μM. The inhibitor of proteasome function MG132 (5 μM) was used as reference compound.

Effect of CH 17 on adjuvant arthritis

We have investigated if CH 17 was able to exert anti-inflammatory effects in a chronic inflammatory disease model, the rat adjuvant-induced arthritis. As shown in Figs. 7 and 8, oral administration of CH 17 (25 mg/kg) on days 17–24 after adjuvant injection, significantly reduced paw oedema and protected on weight loss when compared with control group. As expected, dexamethasone inhibited paw oedema but it was not able to protect on weight loss. At the end of the experiment (day 25) paw swelling was reduced in CH 17 treated animals by 57% relative to the paw volume of vehicle treated animals (control) and the levels of inflammatory mediators (nitrite and PGE_2) in paw homogenates were significantly inhibited (Fig. 9). In serum, PGE_2 content was also significantly reduced by CH 17, which

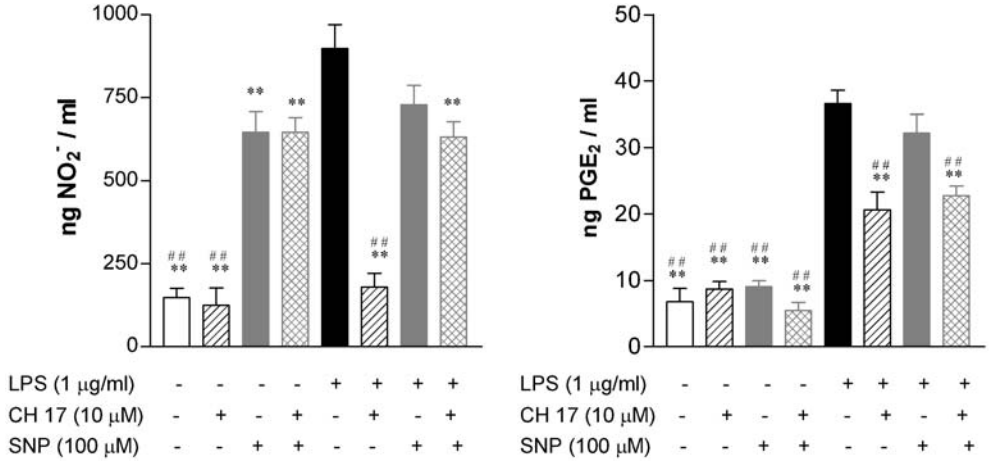

Fig. 4 Effects of a NO donor (*SNP*) on nitrite and PGE_2 production in RAW 264.7 cells. RAW 264.7 macrophages were preincubated with SNP (100 μM), CH 17 (10 μM) or vehicle for 30 min and then stimulated with LPS for 20 h. Results are the mean of three different experiments. $^{**}p<0.01$ vs. LPS group, $^{\#\#}p<0.01$ vs. LPS + SNP group

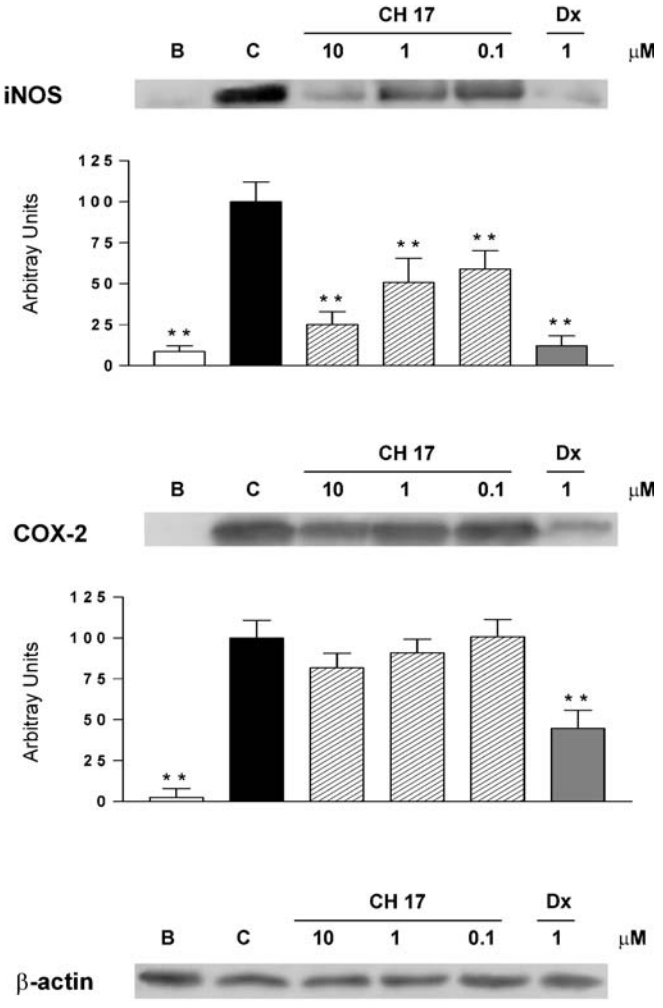

Fig. 5 Effect of CH 17 (10, 1 and 0.1 μM) and dexamethasone (1 μM) on iNOS, COX-2 and β-actin expression on 20 h LPS-stimulated RAW 264.7 macrophages. The figure is representative of three similar experiments. *B* non-stimulated cells, *C* LPS-stimulated cells. ***p*<0.01 vs. LPS-stimulated cells (*C*)

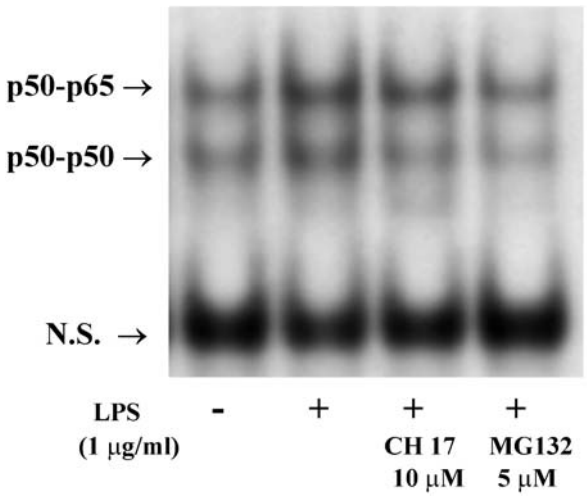

Fig. 6 Effect of CH 17 on NF-kB-DNA binding in nuclear extracts. RAW 264.7 cells were preincubated with CH 17 (10 μM) or MG132 (5 μM) for 15 min before LPS stimulation for 1 h. Supershift assays with Abs against proteins of the c-Rcl family (not shown) identified p50-p50 and p50-p65 as the complexes present in the lower and upper bands, respectively. Results are representative of three independent experiments. *N.S.* non-specific

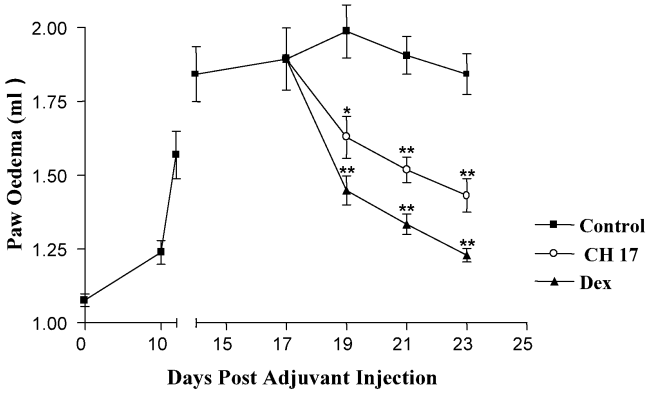

Fig. 7 Effect of CH 17 (25 mg/kg; p.o.) and dexamethasone (2 mg/kg; p.o.) on the development of adjuvant-induced arthritis in male Lewis rats. Animals were injected with *M. butyricum* on day 0 and arthritic rats were randomised into treatment groups on day 17. Compounds were administered twice daily on days 17–24 and the oedema in both paws was measured every day to assess the magnitude of the inflammatory response. Means ± SEM; *n*=6 animals per group; **p*<0.05; ***p*<0.01 vs. vehicle-treated arthritic rats (*Control*)

failed to modify TXB_2 levels in serum (345.5±11.8 ng/ml in control group and 357.1±5.7 ng/ml in CH 17-treated rats) and PGE_2 in stomach homogenates (Fig. 10).

Effect of CH 17 on iNOS and COX-2 protein expression in arthritic paws

We examined if the decreased levels of these mediators (nitrite and PGE_2) in paw homogenates from CH 17-treated arthritic rats were associated with the inhibition of iNOS and COX-2 expression. As shown by Western Blot (Fig. 11) treatment with CH 17 potently reduced iNOS protein expression without affecting COX-2 expression measured in supernatants of homogenated arthritic paws on day 25, with respect to the control group. As expected, dexamethasone inhibited the protein expression of both enzymes.

Radiographic analysis of the effect of CH 17 on adjuvant arthritis

A radiographic examination of hind paws from rats 25 days post adjuvant injection revealed bone matrix resorption and osteophyte formation at the joint margin (Fig. 12a). CH 17 and dexamethasone markedly reduced the degree of bone resorption, soft tissue swelling and osteophyte formation (Fig. 12b, c).

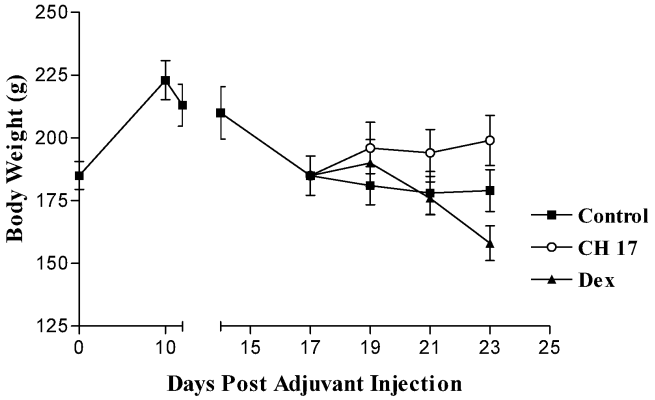

Fig. 8 Effect of CH 17 (25 mg/kg; p.o.) and dexamethasone (2 mg/kg; p.o.) on body weight gain in adjuvant-induced arthritis in male Lewis rats. Animals were injected with *M. butyricum* on day 0 and arthritic rats were randomised into treatment groups on day 17. Compounds were administered twice daily on days 17–24 and the body weight was measured every day. Means ± SEM; n=6 animals per group

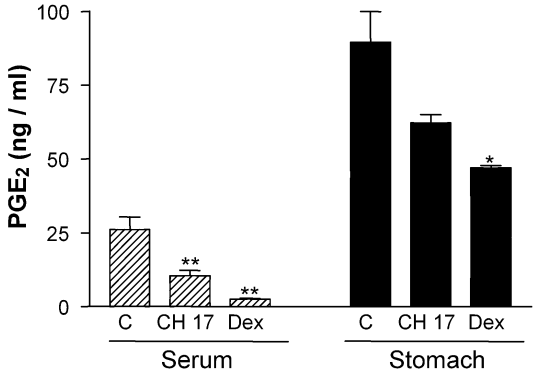

Fig. 10 Effect of CH 17 (25 mg/kg) and dexamethasone (2 mg/kg) on PGE_2 levels in serum and stomach of adjuvant-induced arthritic rats. Compounds were administered twice daily on days 17–24 and serum and stomach were recovered on day 25 post-adjuvant injection for analysis. Data represent mean ± SEM; n=6 animals per group. *p<0.05, **p<0.01 vs. vehicle-treated arthritic rats (*C*)

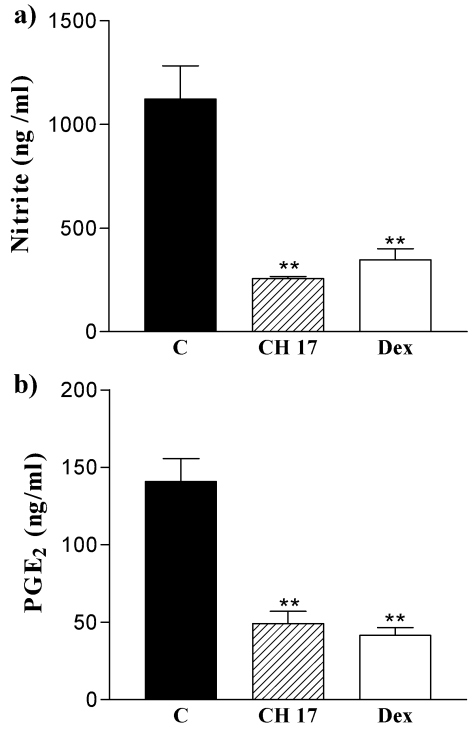

Fig. 9a, b Effect of CH 17 (25 mg/kg) and dexamethasone (2 mg/kg) on nitrite and PGE_2 levels in rat paws. Compounds were administered twice-daily on days 17–24 and paw tissues were recovered on day 25 post-adjuvant injection for analysis. Data represent mean ± SEM; n=6 animals per group. **p<0.01 vs. vehicle-treated arthritic rats (*C*)

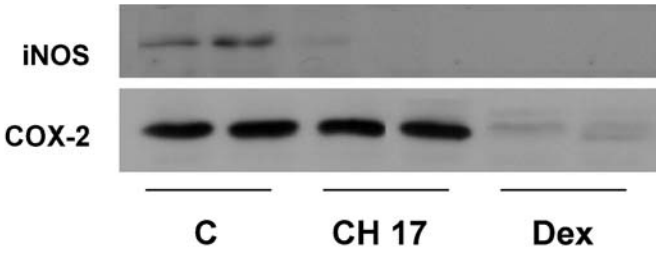

Fig. 11 Western blot analysis of adjuvant-induced iNOS and COX-2 expression in rat paws after treatment with vehicle (*C*) or CH 17 (25 mg/kg; p.o.) or dexamethasone (2 mg/kg; p.o.) on days 17–24. Paw tissues were recovered on day 25 post-adjuvant injection for analysis. The figure is representative of three similar experiments (n=6 animals per group)

Discussion

NO is synthesized by 3 genetically distinct NOS isoforms (Nathan 1992; Nathan and Xie 1994). The constitutive low-output endothelial NOS isoform is a major modulator of vascular tone and blood flow; the constitutive low-output neuronal NOS isoform modulates synaptic plasticity; and the inducible high-output iNOS isoform functions as an effector component of the cell-mediated immune response and may be important in inflammatory responses.

In the present study, we have shown that CH 17 potently inhibited the production of NO in LPS-stimulated RAW 264.7 macrophage cells (IC_{50}=0.033 µM) and reduced PGE_2 levels by a 58% at 10 µM, without any evidence of a cytotoxic effect. This compound had no direct inhibitory effect on iNOS and COX-2 activities. It can be suggested that NO reduction was the consequence of inhibition of the expression of iNOS, whereas PGE_2 reduction was not due to a direct inhibitory action on COX-2 activity or expression. Besides, CH 17 did not reduce the level of PGE_2 production in human monocytes depending on COX-1 or COX-2 activity. On the other hand, CH 17 did not modify arachidonic acid release by a direct action on phospholipase A_2 activity (data not shown). Several investigators have suggested that NO affects the level of COX-2 and that PGE_2 affects NO production. Thus, the CH 17-induced reduction in NO may suppress PGE_2 production indirectly. In checking this possibility, we found that when SNP (a NO donor) was added to CH 17-treated

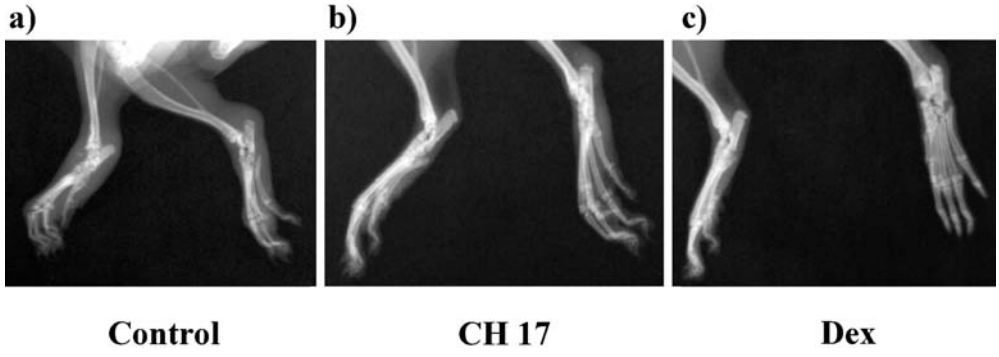

Fig. 12a–c Radiographic progression of adjuvant-induced arthritis in the tibiotarsal joint of rats. **a** The hind paws from control rats (25 days) demonstrated bone resorption and joint erosion. **b**, **c** CH 17 (25 mg/kg) and dexamethasone (2 mg/kg) suppressed joint pathology and soft tissue swelling in the rat hind paw. The figure is representative of three similar experiments (n=6 animals per group)

RAW 264.7 cells, it did not restore completely PGE_2 level. Nevertheless, we have observed that the presence of this chalcone derivative during the induction process (20 h LPS treatment-RAW 264.7 macrophage cells) can modify the activity of COX-2. NO may act in an autocrine manner to modulate the cellular response to inflammation and inhibition of NO production would result in inhibition of PG generation (Salvemini et al. 1993). Our results suggest that CH 17 was able to reduce PGE_2 levels by different mechanism; on the one hand, CH 17 inhibited iNOS expression and NO production and, as a consequence, reduced, at least in part, PGE_2 level and on the other hand, CH 17 modified the induction process or affected on some way the activity of COX-2.

NF-κB is the major transcription factor responsible for inducting iNOS gene expression induced by LPS and inflammatory cytokines (Xie et al. 1994). We have shown that CH 17 reduced LPS-induced NF-κB-DNA binding activity at 10 μM, which also inhibited the LPS-stimulated increase in NO synthesis and iNOS expression, suggesting that an interference with the NF-κB pathway may account for the inhibitory action of CH 17 at this level. On the other hand, activation of the NF-κB transcription factor system has been implicated in the induction of COX-2 gene expression in many cell types (Yamamoto and Gaynor 2001). However, CH 17 did not inhibit the expression of this enzyme in RAW 264.7. In this way, it has been reported that inhibition of NF-κB activation, has no repressive effect on transcriptional activation at the COX-2 promoter in LPS-stimulated RAW 264.7 macrophages, suggesting a lack of a requirement for NF-κB activity in COX-2 induction (Wadleigh et al. 2000).

There is increasing evidence that an enhanced formation of NO by iNOS contributes to the inflammatory process (Cuzzocrea et al. 1999; Pryor and Squadrito 1995; Salvemini et al. 1996; Wei et al. 1995) and that the production of ROS such as hydrogen peroxide, superoxide and hydroxyl radicals at the site of inflammation contributes to tissue damage (Cuzzocrea et al. 1998; Salvemini et al. 1998). In addition to PGs and NO, peroxynitrite is also generated in several models of experimental arthritis (Szabo et al. 1998; Cuzzocrea et al. 2000a, 2000b). Pro-inflammatory cytokines such as interleukin-1 and tumour necrosis factor-α cause activation of the iNOS pathway in bone cells and NO derived from this pathway potentiates cytokine and inflammation induced bone loss. These actions of NO are relevant to the pathogenesis of osteoporosis in inflammatory diseases such as rheumatoid arthritis, which are characterized by increased NO production and cytokine activation (van't Hof and Ralston 2001). The increase in NO production following adjuvant injection is not merely a non-specific inflammatory response to the oil vehicles and/or mycobacterial components used, but it is rather related to the specific immune and/or inflammatory responses that drive adjuvant-induced arthritis in rats (Cannon et al. 1996).

The model of adjuvant-induced arthritis in the rat has been used for many years for evaluation of anti-arthritic/anti-inflammatory agents (Winder et al. 1969) and is well characterized. Previous reports have implied that iNOS has a role in the development of inflammation based on the prophylactic and therapeutic effects of different inhibitors of NOS (Ialenti et al. 1993; Stefanovic-Racic et al. 1993; Connor et al. 1995; Brahn et al. 1998); however, other authors (Fletcher et al. 1998) suggest that iNOS may be involved with the initial stages of the immune response to adjuvant injection, but its product, NO, does not mediate the chronic inflammation and joint destruction which occurs during the late phase in this model.

Our data indicate that CH 17 is effective, by oral route, in the treatment of experimental chronic inflammation and radiographic signs of protection against bone resorption and osteophyte formation were present in the joints of CH 17-treated rats. The inhibition of joint inflammation by CH 17 treatment was accompanied by reduction in NO and PGE_2 levels in arthritic paw homogenates, as well as in iNOS protein expression. This compound also reduced serum PGE_2 levels without affecting serum TXB_2 production and PGE_2 generation in stomach of arthritic rats, which are depending on COX-1 activity.

The interactions between the iNOS and COX pathways can lead to controversial results depending on the cell system and experimental conditions. In some systems, both pathways are induced by inflammatory stimuli but they seem to act in an independent manner, as observed in rat aorta in culture stimulated with LPS (Bishop-Bailey et al. 1997) or in the response to zymosan in the rat air pouch (Payá et al. 1997). On the contrary, the possibility exists of an interaction between NO and COX. In vitro, it has been reported that NO attenuates the synthesis of PGE_2 in chondrocytes from osteoarthritic patients (Amin et al. 1997), and it has been suggested that NO is involved in the negative regulation of COX-2 expression in rat peritoneal

macrophages (Habib et al. 1997). Nevertheless, there are reports that NO potentiates cytokine-induced PGE_2 production in a number of cell systems such as vascular smooth muscle cells (Inoue et al. 1993), rat mesangial cells (Tetsuka et al. 1994), human microglial cells (Janabi et al. 1996) and human airway epithelial cells (Watkins et al. 1997). This effect of NO could be due to an amplification of COX-2 expression (Tetsuka et al. 1996). However, in a murine model of air pouch granulomatous inflammation, it is suggested that NO produced by iNOS seemed to inhibit the induction of COX-2, and low levels of NO appeared to activate COX (Vane et al. 1994). A regulatory role of NO in the in vivo production of prostanoids has been observed in rats treated with LPS (Salvemini et al. 1995a) and in the carrageenin rat air pouch (Salvemini et al. 1995b). In a model of zymosan mouse air pouch, the administration of aminoguanidine (selective iNOS inhibitor) largely reduced COX activity and the content of PGE_2 in exudate, without affecting the expression of COX-2 (Posadas et al. 2000). As a consequence, inhibitors of iNOS may reduce inflammation by the dual inhibition of NO and PGs (Salvemini et al. 1995a, 1995b).

Besides, after administration of CH 17 rats exhibited an important protection on weight loss when compared with the respective control group or with the reference dexamethasone group.

In conclusion, our results demonstrated that CH 17 exerts chronic anti-inflammatory effects which may be related with the inhibition of iNOS expression and the NF-κB activation, and the resulting reduction of NO and PG production. The profile and potency of this compound may have relevance for the inhibition of the inflammatory response, representing a new approach to the modulation of different inflammatory pathologies.

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