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European Journal of Pharmacology 465 (2003) 183–189
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ttCH, a selective inhibitor of inducible nitric oxide synthase expression with antiarthritic properties

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Received 5 December 2002; received in revised form 5 February 2003; accepted 11 February 2003

Abstract

In a previous work, we investigated the effects of a series of dimethoxy- and trimethoxychalcone derivatives, with various patterns of fluorination, on nitric oxide production in lipopolysaccharide-stimulated murine RAW 264.7 cells. The present study was designed to determine if 2,4,6-trimethoxy-2′-trifluoromethylchalcone (ttCH) could modulate the production of nitric oxide (NO) and/or prostaglandins in vitro and in vivo. On the mouse macrophage cell line RAW 264.7, ttCH inhibited dose-dependently NO and prostaglandin E_2 production, with IC_{50} in the micromolar range. This compound had no direct inhibitory effect on inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 activities. NO reduction was the consequence of inhibition of the expression of iNOS. This compound also exhibited in vivo an inhibitory behaviour on nitrite and prostaglandin E_2 levels. We have assessed the effect of ttCH in the treatment of acute and chronic inflammatory processes such as the mouse carrageenan paw oedema and the rat adjuvant-induced arthritis. The present study demonstrated that ttCH exerts acute and chronic anti-inflammatory effects that may be related with the inhibition of iNOS expression.

Keywords: Chalcones; Nitric oxide (NO); Prostaglandin E_2; RAW 264.7; Zymosan-injected mouse air pouch; Adjuvant-induced arthritis

1. Introduction

Nitric oxide (NO) may play a key role in mediating bone and tissue damage in inflammatory conditions associated with cytokine activation, such as rheumatoid arthritis. Other actions of NO which are relevant to the pathogenesis of joint inflammation include changes in the vascular permeability of inflamed tissues, potentiation of tumor necrosis factor-α and interleukin-1β release by leukocytes, and stimulation of angiogenic activity by human monocytes (Ralston, 1997). Inhibitors of NO synthase suppress the development of disease in animal models of inflammatory arthritis (McCartney Francis et al., 1993; Ialenti et al., 1993). Diminution of NO correlates with improvement in clinical and laboratory measures of disease activity (Ralston, 1997).

NO and prostaglandin E_2 are two pleiotropic inflammatory mediators overproduced in arthritis-affected joints. The inducible isoform of nitric oxide synthase (iNOS) and cyclooxygenase-2 are found both in the synovial tissue and in the cartilage. Their expression is regulated by catabolic cytokines, such as interleukin-1β and tumor necrosis factor-α. These inflammatory mediators play a profound role in the pathogenic processes that arise in the pannus of rheumatoid arthritis (Amin et al., 1999).

Chalcone skeleton has been considered the biological precursor of flavonoids. This family of natural products is formed by a huge number of compounds possessing a wide array of biological effects (Middleton, 1998), including anti-inflammatory, analgesic, antioxidant, antibacterial, antifungal and antiprotozoal activities (Haraguchi et al., 1998; Hsieh et al., 1998). We have previously studied several chalcone derivatives as potential anti-inflammatory agents (Herencia et al., 1998, 1999, 2001) and reported that some of them are able to control NO (Herencia et al., 2002), superoxide and prostaglandin production in vitro as well as in vivo, having a potential role in modulating the inflammatory process. Recently, we have investigated the effects of a series of dimethoxy- and trimethoxychalcone derivatives, with various patterns of fluorination, on nitric oxide production in lipopolysaccharide-stimulated murine RAW 264.7 (Rojas et al., 2002). The present study was designed to determine if one of these chalcone derivatives, 2,4,6-trimethoxy-2′-trifluoromethylchalcone (ttCH) (Fig. 1), could modulate the

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doi:10.1016/S0014-2999(03)01457-2

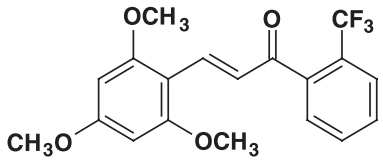

Fig. 1. Chemical structure of ttCH.

production of NO and/or prostaglandins in vitro and in vivo. We have also assessed the effect of ttCH in the treatment of acute and chronic inflammatory processes such as the mouse carrageenan paw oedema and the rat adjuvant-induced arthritis.

2. Materials and methods

2.1. Chemicals

Chalcone derivative was prepared according to procedures previously described (Rojas et al., 2002). [5,6,8,11,12,14,15(*n*)-^{3}H]prostaglandin E_2 was from Amersham Iberica (Madrid, Spain). iNOS and cyclooxygenase-2 polyclonal antiserum were purchased from Cayman Chemicals (Michigan, USA). The peroxidase-conjugated goat anti-rabbit IgG was purchased from Dako (Copenhagen, Denmark). *M. butyricum* was obtained from Difco Chemicals (Michigan, USA) and the rest of reagents were from Sigma (Missouri, USA).

2.2. Cell culture

The mouse macrophage cell line RAW 264.7 was cultured in Dulbecco's modified Eagle's medium (DMEM) 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* lipopolysaccharide (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 prostaglandin E_2 levels were determined in culture supernatants fluorometrically (Misko et al., 1993) and by radioimmunoassay (RIA) (Moroney et al., 1988), respectively. 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.

2.3. iNOS and cyclooxygenase-2 activity in intact cells

RAW 264.7 macrophages stimulated for 20 h with lipopolysaccharide were washed and Hanks' 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 cyclooxygenase-2 activities. Supernatants were collected for the measurement of nitrite and prostaglandin E_2 accumulation for the last 2 h as above.

2.4. Western blot assay

Cellular lysates from cell line RAW 264.7 (10^6/well) incubated for 18 h with lipopolysaccharide were obtained with lysis buffer (1% Triton X-100, 1% deoxycholic acid, 20 mM NaCl, 25 mM Tris, pH 7.4). Following centrifugation (10,000 × *g*, 15 min), supernatant protein was determined and 25 μg protein was loaded on 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes for 90 min at 125 mA. Membranes were blocked in phosphate buffer saline (PBS)-Tween 20 containing 3% w/v nonfat milk. For iNOS, membranes were incubated with anti-iNOS polyclonal antibody (1:1000 dilution); for cyclooxygenase-2, membranes were incubated with specific anti-cyclooxygenase-2 polyclonal antiserum (1:1000). 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).

2.5. Mouse air pouch

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. Air pouch was performed in female Swiss mice (25–30 g) as previously described (Escrig et al., 1997). Six days after the initial air injection, 1 ml of sterile saline or 1 ml of 1% w/v zymosan in saline was injected into the air pouch. Test compound or dexamethasone (0.1 μmol/pouch) was injected 1 h before and 8 h after zymosan injection. Twenty-four hours after zymosan administration, the animals were killed by cervical dislocation, and the exudate in the pouch was collected with 1 ml of saline. Leukocytes present in exudate in the pouch were measured using a Coulter counter. After centrifugation of exudates (1200 × *g* at 4 °C for 10 min), the supernatants were used to measure nitrite and prostaglandin E_2 levels as described above.

2.6. Carrageenan paw oedema

The anti-inflammatory activity of ttCH was assessed by the carrageenan paw oedema test (Sugishita et al., 1981). ttCH (25 mg/kg), indomethacin (5 mg/kg) or vehicle (propylene glycol/glycerol/water (4:1:5), v/v) was administered orally 1 h before injection of carrageenan (0.05 ml; 3% w/v in saline) into the subplantar area of the right hind paw. The volumes of the injected and contralateral paws were measured at 1, 3 and 5 h after induction of oedema by using a plethysmometer (Ugo Basile, Comerio, Italy). The volume

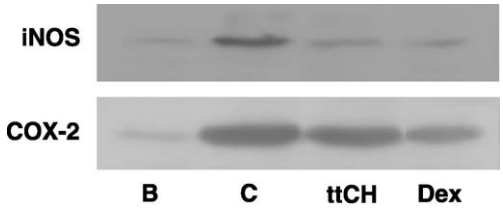

Fig. 2. Effect of ttCH (10 μM) and dexamethasone (1 μM) on iNOS and cyclooxygenase-2 expression on 20 h lipopolysaccharide-stimulated RAW 264.7. B = without LPS; C = control (with LPS). The figure is representative of three similar experiments.

of oedema was expressed for each animal as the difference between the carrageenan-injected and contralateral paws.

2.7. Adjuvant arthritis

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 0700 h; off 1900 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 randomized into treatment groups. 25 mg/kg of ttCH or vehicle (propylene glycol/glycerol/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, 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, Cavaria, Italy) with a 40-kW exposition for 0.01 s. 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 prostaglandin E_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 cyclooxygenase-2 expression by Western blot analysis as described above.

2.8. Statistical analysis

Statistical evaluation included one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons. *P* values <0.05 (*) or <0.01 (**) were taken as significant. Results are shown as mean ± S.E.M. for *n* experiments. Inhibitory concentration 50% (IC_{50}) values were calculated from at least four significant concentrations.

3. Results

3.1. Effect of ttCH on NO and prostaglandin E_2 levels in lipopolysaccharide-stimulated RAW 264.7 cells

Lipopolysaccharide stimulation of RAW 264.7 macrophages for 20 h induced iNOS and cyclooxygenase-2 with the consequent generation of large quantities of NO and prostaglandin E_2. Under these conditions, ttCH inhibited the generation of these mediators dose-dependently, with IC_{50} values of 1.02 (0.81–1.70) μM and 0.31 (0.10–0.70) μM for nitrite and prostaglandin E_2, respectively. At the highest concentration tested (10 μM), ttCH did not exert cytotoxic effects during the 20-h incubation period as indicated by MTT reduction (data not shown).

3.2. Effect of ttCH on iNOS and cyclooxygenase-2 activity in lipopolysaccharide-stimulated RAW 264.7 cells

To determine if the inhibition of nitrite and prostaglandin E_2 production was either due to interference with the enzyme induction by lipopolysaccharide or due to direct action of this compound on NOS or cyclooxygenase activity, ttCH was incubated for 2 h with cells after the induction of the enzyme by lipopolysaccharide. No significant reduction of nitrite or prostaglandin E_2 production during these 2 h was observed (data not shown).

Table 1
Effect of ttCH and reference compound on the 24-h zymosan-injected air pouch

	Migration[a]	NO_2^-[a]		Prostaglandin E_2[a]	
	10^6 cells/ml	ng/ml	% Inhibition	ng/ml	% Inhibition
Saline	1.98 ± 0.2	0.1 ± 0.1[b]	–	0.4 ± 0.2	–
Zymosan	23.78 ± 2.9	289.1 ± 26.4	–	41.2 ± 7.3	–
ttCH (0.1 μmol/pouch)	14.39 ± 0.54[c]	133.2 ± 28.2[b]	53.9 ± 5.3	20.4 ± 1.8[b]	50.5 ± 2.1
Dexamethasone (0.1 μmol/pouch)	23.47 ± 3.8	13.3 ± 1.1[b]	95.4 ± 8.9	3.3 ± 0.7[b]	92.0 ± 2.7

Cellular migration, nitrite and prostaglandin E_2 levels are measured in exudates.
[a] Results are expressed as mean ± S.E.M. ($n = 6–12$).
[b] $P<0.01$ compared with the zymosan group.
[c] $P<0.05$ compared with the zymosan group.

3.3. Effect of ttCH on iNOS and cyclooxygenase-2 protein expression in lipopolysaccharide-stimulated RAW 264.7 cells

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

3.4. Effect of ttCH on NO and prostaglandin E_2 levels in the mouse air pouch

After 24 h following zymosan injection, nitrite and prostaglandin E_2 levels were greatly increased in the mouse pouch exudates. Intrapouch administration of ttCH at 0.1 μmol/pouch resulted in a significant reduction of both metabolites in the exudates, affecting in a lesser extent cell accumulation (Table 1). Dexamethasone, assayed as reference compound, potently reduced nitrite and prostaglandin E_2 levels without affecting cell accumulation.

3.5. Effect of ttCH on carrageenan-induced mouse paw oedema

Oral administration of ttCH (25 mg/kg) significantly reduced carrageenan-induced mouse paw oedema, showing the greatest effect at 3 h, as shown in Fig. 3. As expected, indomethacin at 5 mg/kg was effective in controlling the paw swelling.

3.6. Effect of ttCH on rat adjuvant arthritis

We have investigated if ttCH was able to exert anti-inflammatory effects in a chronic inflammatory disease model, the rat adjuvant-induced arthritis. As shown in Fig. 4, oral administration of ttCH (25 mg/kg) on days 17–24 after adjuvant injection significantly protected weight loss (a) and reduced paw oedema (b) when compared with control group. At the end of the experiment (day 25), paw swelling was reduced in ttCH-treated animals by 60% relative to the paw volume of vehicle-treated animals (control) and the levels of inflammatory mediators (nitrites and prostaglandin E_2) in paw homogenates were significantly inhibited (Fig. 5).

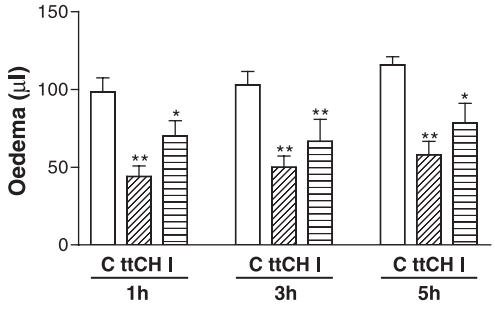

Fig. 3. Effect of ttCH and indomethacin on carrageenan mouse paw oedema, 1, 3 and 5 h after the induction of inflammation. ttCH administered orally at 25 mg/kg; I = indomethacin at 5 mg/kg. Data represent mean ± S.E.M. ($n = 6$ animals). *$P < 0.05$, **$P < 0.01$.

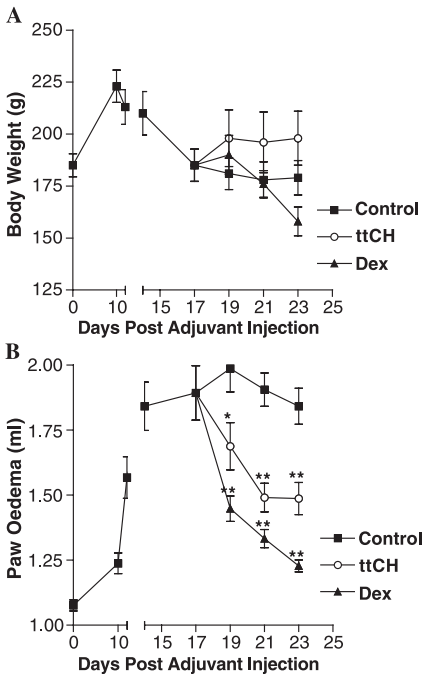

Fig. 4. Effect of ttCH (25 mg/kg, p.o.) and dexamethasone (2 mg/kg, p.o.) on body weight gain (A) and on paw oedema (B) in adjuvant-induced arthritis in Lewis rats. Compounds were administered twice daily on days 17–24 and the body weight and oedema in both paws were measured every day. Data represent mean ± S.E.M. ($n = 6$ animals per group). *$P < 0.05$, **$P < 0.01$.

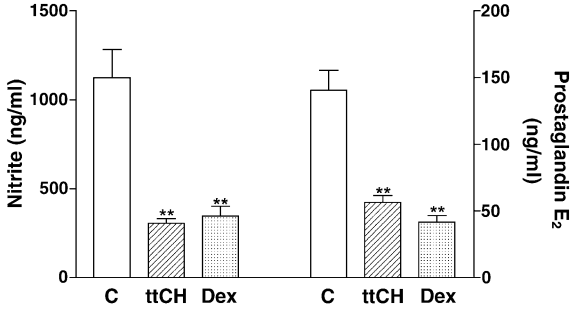

Fig. 5. Effect of ttCH (25 mg/kg, p.o.) and dexamethasone (2 mg/kg, p.o.) on nitrite and prostaglandin E_2 levels in rat paws. Compounds were administered twice daily on days 17–24 and paw tissues were recovered on day 25 post-adjuvant injection for analysis. Data represent mean ± S.E.M. ($n = 6$ animals per group). *$P < 0.05$, **$P < 0.01$.

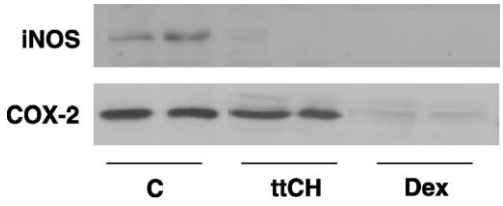

Fig. 6. Effect of ttCH (25 mg/kg, p.o.) and dexamethasone (2 mg/kg, p.o.) on iNOS and cyclooxygenase-2 expression in rat paws. Compounds were administered twice daily on days 17–24 and paw tissues were recovered on day 25 post-adjuvant injection for analysis. The figure is representative of three similar experiments ($n = 6$ animals per group).

3.7. Effect of ttCH iNOS and cyclooxygenase-2 protein expression in arthritic paws

We examined if the decreased levels of these mediators (nitrite and prostaglandin E_2) in paw homogenates from ttCH-treated arthritic rats were associated with the inhibition of iNOS and cyclooxygenase-2 expression. As shown by Western Blot (Fig. 6), treatment with ttCH potently reduced iNOS protein expression without affecting cyclooxygenase-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.

3.8. Radiographic analysis of the effect of ttCH 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. 7A). ttCH and dexamethasone markedly reduced the degree of bone resorption, soft tissue swelling and osteophyte formation (Fig. 7B,C).

4. Discussion

The induction of NO synthase and cyclooxygenase-2 greatly increases the synthesis of NO and prostaglandins, which contribute to the pathophysiology of various inflammatory processes. Inducible NO-synthase inhibition results in modulation of the inflammatory response in different models such as subcutaneous granuloma formation in rats (Iuvone et al., 1994) and delayed paw swelling induced by carrageenan in mice (Ianaro et al., 1994). In humans, the production of NO by activated macrophages or neutrophils can be an index of bronchial inflammation and a mechanism for increasing asthmatic inflammation (Alving et al., 1993). Furthermore, NO has been shown, in in vitro and in vivo studies, to increase the production of pro-inflammatory prostaglandins (Salvemini et al., 1993, 1995). On the other hand, overproduction of prostaglandins by cyclooxygenase-2 expression in vivo has been reported for chronic inflammatory conditions such as rheumatoid arthritis (Kang et al., 1996) and experimental models of inflammation (Seibert et al., 1994; Vane et al., 1994).

In the present study, we have shown that ttCH inhibited the production of NO and prostaglandin E_2 in lipopolysaccharide-stimulated RAW 264.7 macrophage cells. The inhibition was dose-dependent without any evidence of a cytotoxic effect. This compound had no direct inhibitory effect on iNOS and cyclooxygenase-2 activities. It can be suggested that NO reduction was the consequence of inhibition of the expression of iNOS, whereas prostaglandin E_2 reduction was not due to a direct inhibitory action on cyclooxygenase-2 activity or expression. In our previous studies, it was shown that it is not cyclooxygenase-1 or phospholipase A_2 inhibitor (data not shown). Besides, the addition of exogenous substrates does not seem to affect its inhibitory effect. Nevertheless, we have observed that the presence of this compound during the induction process (20-h lipopolysaccharide treat-

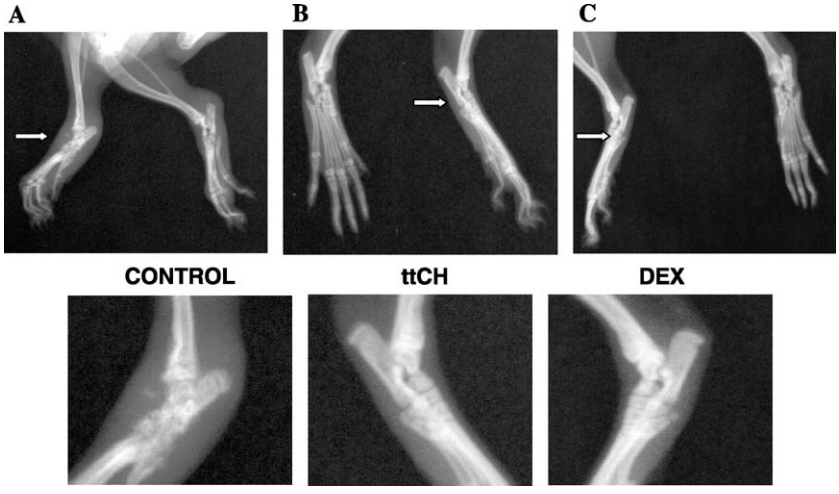

Fig. 7. Radiographic progression of adjuvant-induced arthritis in the tibiotarsal joint of rats. The hind paws from control rats (25 days) demonstrated bone resorption and joint erosion (A). ttCH (25 mg/kg, p.o.) and dexamethasone (2 mg/kg, p.o.) suppressed joint pathology and soft tissue swelling in the rat hind paw (B, C). The figure is representative of three similar experiments ($n = 6$ animals per group).

ment) can modify the activity of cyclooxygenase-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 prostaglandin generation (Salvemini et al., 1993). In vivo inhibitory effects on nitrite and prostaglandin E_2 levels were also observed for ttCH. This compound exhibited an inhibitory behaviour similar to its in vitro results on nitrite and prostaglandin E_2 accumulation; in the 24-h zymosan-stimulated mouse air pouch, ttCH reduced nitrite and prostaglandin E_2 levels as well as in the rat adjuvant arthritis. In another model of inflammation, the mouse paw oedema induced by carrageenan, ttCH also exerted potent inhibitory effects.

Previous reports have implied that iNOS has a role in the development of inflammation based on the prophylactic effects of different inhibitors of NOS (Ialenti et al., 1993; Stefanovic-Racic et al., 1993; Connor et al., 1995); 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 occur during the late phase in this model. In this regard, our results suggest that iNOS is one of the critical agents in the development of the inflammation process and joint destruction. Therapeutic administration of ttCH, by oral route, is effective in the treatment of experimental chronic inflammation, and the inhibition of joint inflammation by ttCH treatment was accompanied by reduction in NO and prostaglandin E_2 levels as well as in iNOS protein expression. Besides, after administration of ttCH, rats exhibited an important protection on weight loss when compared with the respective control group or with the reference dexamethasone group.

In summary, the present study demonstrated that ttCH exerts acute and chronic anti-inflammatory effects that may be related with the inhibition of iNOS expression and the resulting reduction of NO and prostaglandin production. The profile and potency of this compound may have relevance for the inhibition of the inflammatory response, representing a new approach for the modulation of different inflammatory pathologies.

Acknowledgements

This work was supported by grants from CICYT (SAF2001-2639), Spanish Ministerio de Ciencia y Tecnología and 06-30-4544-99 from CDCH-UCV, Venezuela. The collaborative work was performed under the auspices of the CYTED Program (Programa Iberoamericano de Ciencia y Tecnología para el Desarrollo), subprogram X.6 (PIBARTRI). Javier Rojas was the recipient of a Research Fellowship from the ALFA Program of The European Union at the University of Valencia.

References

Alving, K., Weitzberg, E., Lundberg, J.M., 1993. Increased amount of nitric oxide in exhaled air of asthmatics. Eur. Respir. J. 6, 1368–1370.

Amin, A.R., Attur, M., Abramson, S.B., 1999. Nitric oxide synthase and cyclo-oxygenases: distribution, regulation, and intervention in arthritis. Curr. Opin. Rheumatol. 11, 202–209.

Connor, J.R., Manning, P.T., Settle, S.L., Moore, W.M., Jerome, G.M., Webber, R.K., Tjoeng, F.S., Currie, M.G., 1995. Suppression of adjuvant-induced arthritis by selective inhibition of inducible nitric oxide synthase. Eur. J. Pharmacol. 273, 15–24.

Escrig, V., Ubeda, A., Ferrándiz, M.L., Darias, J., Sánchez, J.M., Alcaraz, M.J., Payá, M., 1997. Variabilin: a dual inhibitor of human secretory and cytosolic phospholipase A_2 with anti-inflammatory activity. J. Pharmacol. Exp. Ther. 282, 123–131.

Fletcher, D.S., Widmer, W.R., Luell, S., Christen, A., Orevillo, C., Shah, S., Visco, D., 1998. Therapeutic administration of a selective inhibitor of nitric oxide synthase does not ameliorate the chronic inflammation and tissue damage associated with adjuvant-induced arthritis in rats. J. Pharmacol. Exp. Ther. 284, 714–721.

Haraguchi, H., Ishikawa, H., Mizutani, K., Tamura, Y., Kinoshita, T., 1998. Antioxidative and superoxide scavenging activities of retrochalcones in *Glycyrrhiza inflata*. Bioorg. Med. Chem. 6, 339–347.

Herencia, F., Ferrándiz, M.L., Ubeda, A., Dominguez, J.N., Charris, J.E., Lobo, G.M., Alcaraz, M.J., 1998. Synthesis and anti-inflammatory activity of chalcone derivatives. Bioorg. Med. Chem. Lett. 8, 1169–1174.

Herencia, F., Ferrándiz, M.L., Ubeda, A., Guillén, I., Dominguez, J.N., Charris, J.E., Lobo, G.M., Alcaraz, M.J., 1999. Novel anti-inflammatory chalcone derivatives inhibit the induction of nitric oxide synthase and cyclo-oxygenase-2 in mouse peritoneal macrophages. FEBS Lett. 453, 129–134.

Herencia, F., Ferrándiz, M.L., Ubeda, A., Guillén, I., Dominguez, J.N., Charris, J.E., Lobo, G.M., Alcaraz, M.J., 2001. 4-Dimethylamino-3′,4′-dimethoxychalcone downregulates iNOS expression and exerts anti-inflammatory effects. Free Radic. Biol. Med. 30, 43–50.

Herencia, F., Lopez-Garcia, M.P., Ubeda, A., Ferrándiz, M.L., 2002. Nitric oxide-scavenging properties of some chalcone derivatives. Nitric Oxide 6, 242–246.

Hsieh, H.K., Lee, T.H., Wang, J.P., Wang, J.J., Lin, C.N., 1998. Synthesis and anti-inflammatory effect of chalcones and related compounds. Pharm. Res. 15, 39–46.

Ialenti, A., Moncada, S., Di Rosa, M., 1993. Modulation of adjuvant arthritis by endogenous nitric oxide. Br. J. Pharmacol. 110, 701–705.

Ianaro, A., O'Donnell, C.A., Di Rosa, M., Liew, F.Y., 1994. A nitric oxide synthase inhibitor reduces inflammation, down-regulates inflammatory cytokines and enhances interleukin-10 production in carrageenin-induced oedema in mice. Immunology 82, 370–375.

Iuvone, T., Carnuccio, R., Di Rosa, M., 1994. Modulation of granuloma formation by endogenous nitric oxide. Eur. J. Pharmacol. 265, 89–92.

Kang, R.Y., Freire Moar, J., Sigal, E., Chu, C.Q., 1996. Expression of cyclo-oxygenase-2 in human and an animal model of rheumatoid arthritis. Br. J. Rheumatol. 35, 711–718.

McCartney Francis, N., Allen, J.B., Mizel, D.E., Albina, J.E., Xie, Q.W., Nathan, C.F., Wahl, S.M., 1993. Suppression of arthritis by an inhibitor of nitric oxide synthase. J. Exp. Med. 178, 749–754.

Middleton, E.J., 1998. Effect of plant flavonoids on immune and inflammatory cell function. Adv. Exp. Med. Biol. 439, 175–182.

Misko, T.P., Schilling, R.J., Salvemini, D., Moore, W.M., Currie, M.G., 1993. A fluorometric assay for the measurement of nitrite in biological samples. Anal. Biochem. 214, 11–16.

Moroney, M.A., Alcaraz, M.J., Forder, R.A., Carey, F., Hoult, J.R.S., 1988. Selectivity of neutrophil 5-lipoxygenase and cyclo-oxygenase inhibition by an anti-inflammatory flavonoid glycoside and related aglycone flavonoids. J. Pharm. Pharmacol. 40, 787–792.

Ralston, S.H., 1997. The Michael Mason Prize Essay 1997. Nitric oxide and bone: what a gas! Br. J. Rheumatol. 36, 831–838.

Rojas, J., Payá, M., Dominguez, J.N., Ferrándiz, M.L., 2002. The synthesis and effect of fluorinated chalcone derivatives on nitric oxide production. Bioorg. Med. Chem. Lett. 12, 1951–1954.

Salvemini, D., Misko, T.P., Masferrer, J.L., Seibert, K., Currie, M.G., Needleman, P., 1993. Nitric oxide activates cyclo-oxygenase enzymes. Proc. Natl. Acad. Sci. U. S. A. 90, 7240–7244.

Salvemini, D., Manning, P.T., Zweifel, B.S., Seibert, K., Connor, J., Currie, M.G., Needleman, P., Masferrer, J.L., 1995. Dual inhibition of nitric oxide and prostaglandin production contributes to the antiinflammatory properties of nitric oxide synthase inhibitors. J. Clin. Invest. 96, 301–308.

Seibert, K., Zhang, Y., Leahy, K., Hauser, S., Masferrer, J., Perkins, W., Lee, L., Isakson, P., 1994. Pharmacological and biochemical demonstration of the role of cyclo-oxygenase 2 in inflammation and pain. Proc. Natl. Acad. Sci. U. S. A. 91, 12013–12017.

Stefanovic-Racic, M., Stadler, J., Evans, C.H., 1993. Nitric oxide and arthritis. Arthritis Rheum. 36, 1036–1044.

Sugishita, E., Amagaya, S., Ogihara, Y., 1981. Anti-inflammatory testing methods: comparative evaluation of mice and rats. J. Pharmacobio-Dyn. 4, 565–575.

Taurog, J.D., Argentieri, D.C., McReynolds, R.A., 1988. Adjuvant arthritis. Methods Enzymol. 162, 339–355.

Vane, J.R., Mitchell, J.A., Appleton, I., Tomlison, A., Bishop-Bailey, D., Croxtall, J., Willoughby, D.A., 1994. Inducible isoforms of cyclo-oxygenase and nitric-oxide synthase in inflammation. Proc. Natl. Acad. Sci. U. S. A. 91, 2046–2050.