Novel anti-inflammatory chalcone derivatives inhibit the induction of nitric oxide synthase and cyclooxygenase-2 in mouse peritoneal macrophages

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Abstract In a previous work, we tested a series of chalcone derivatives as possible anti-inflammatory compounds. We now investigate the effects of three of those compounds, CH1, CH8 and CH12, on nitric oxide and prostanoid generation in mouse peritoneal macrophages stimulated with lipopolysaccharide and in the mouse air pouch injected with zymosan, where they showed a dose-dependent inhibition with inhibitory concentration 50% values in the μM range. This effect was not the consequence of a direct inhibitory action on enzyme activities. Our results demonstrated that chalcone derivatives inhibited de novo inducible nitric oxide synthase and cyclooxygenase-2 synthesis, being a novel therapeutic approach for inflammatory diseases.

Key words: Chalcone; Inducible nitric oxide synthase; Cyclooxygenase-2; Mouse peritoneal macrophage; Lipopolysaccharide; Mouse air pouch

1. Introduction

Macrophages participate in host defense, immunity and inflammatory responses, where they are potently activated resulting in the production of cytokines, oxygen and nitrogen species and eicosanoids. These inflammatory and immunomodulatory mediators cause diverse biological effects. In macrophages, bacterial lipopolysaccharide (LPS) alone or in combination with cytokines like interferon-γ (IFNγ) is one of the best-characterized stimuli to induce the transcription of genes encoding pro-inflammatory proteins, resulting in cytokine release and synthesis of enzymes such as cyclooxygenase-2 (COX-2) [1] and inducible nitric oxide synthase (iNOS) [2]. The inducible isoform COX-2 would be responsible for the high prostanoid generation during inflammatory responses [3], although the participation of COX-1, constitutively expressed in mammalian cells, has also been suggested [4].

Reactive nitrogen intermediates such as nitric oxide (NO) are shown to play a central role in inflammatory and immune reactions. Apart from their vascular effects, NO and derived oxidants, such as peroxynitrite, contribute to tissue injury. In addition, NO can enhance the production of a variety of other inflammatory mediators, including tumor necrosis factor-α (TNFα), interleukin-1 and reactive oxygen intermediates which participate directly and indirectly in the macrophage-dependent inflammatory response [5].

Activated macrophages appear to be the main cellular source of iNOS and these cells contribute significantly to the induction of iNOS and generation of NO after LPS administration to mice [6]. LPS activation of murine macrophages leads to co-induction of iNOS and COX-2 [7,8], which involves common mechanisms such as tyrosine phosphorylation [9]. A number of studies have indicated that iNOS and COX-2 pathways are also co-induced in vivo in different inflammation models [10–14].

Recently, we have investigated the effects of a series of chalcone derivatives on human neutrophil functions in vitro as well as on eicosanoid release and TNFα production in mice [15]. The present study was designed to determine if three of these chalcone derivatives (Fig. 1) could modulate the production of NO and/or prostaglandins (PGs) in vitro using mouse peritoneal macrophages, as well as in vivo, in the mouse air pouch.

2. Materials and methods

2.1. Materials

[5,6,8,11,12,14,15(*n*)-^{3}H]PGE_2 and L-[2,3,4,5-^{3}H]arginine monohydrochloride were from Amersham Iberica (Madrid, Spain). [^{35}S]methionine was from ICN (CA, USA). iNOS and COX-2 polyclonal antiserum and NS398 were purchased from Cayman Chem. (MI, USA). The rest of the reagents were from Sigma (MO, USA).

2.2. Isolation and culture of murine peritoneal macrophages

Female Swiss mice weighing 25–30 g were used to obtain highly purified peritoneal macrophages. Cells were harvested by peritoneal lavage 4 days after intraperitoneal injection of 1 ml 10% thioglycolate broth and were resuspended in culture medium (120 mM NaCl, 4.7 mM KCl, 1.2 mM $CaCl_2 \cdot 7H_2O$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM HEPES, 1 mM L-arginine and 10 mM glucose), supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and incubated at 37°C for 2 h [16]. The adherent cells were used to perform the following experiments. 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 test compounds on murine peritoneal macrophages [17].

2.3. Nitrite (NO_2^-) and PGE_2 production

Murine peritoneal macrophages (2×10^5/well) were co-incubated with test compounds and *Escherichia coli* LPS (serotype 0111:B4)

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Abbreviations: NO, nitric oxide; iNOS, inducible NO synthase; LPS, lipopolysaccharide; COX, cyclooxygenase; PG, prostaglandin; IFNγ, interferon-γ; NF-κB, nuclear factor κB; TNFα, tumor necrosis factor-α; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IC_{50}, inhibitory concentration 50%; AG, aminoguanidine; DEX, dexamethasone; NADPH, nicotinamide adenine dinucleotide phosphate reduced form

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(10 μg/ml) at 37°C for 24 h. Nitrite and PGE_2 accumulation were measured in 24 h supernatants.

In a different set of experiments, culture medium of 24 h LPS-stimulated macrophages (4×10^5/well) in the absence or presence of test compounds was removed and medium supplemented with L-arginine (0.5 mM) and arachidonic acid (10 μM) was added for a further 2 h incubation with or without test compounds, respectively. Supernatant was collected for the measurement of nitrite and PGE_2 accumulation for the last 2 h. The nitrite concentration as reflection of NO release was assayed fluorometrically [18]. The amount of nitrite was obtained by extrapolation from a standard curve with sodium nitrite as a standard. PGE_2 levels were assayed by a radioimmunoassay [19].

2.4. NOS and COX activity in broken cell preparations

After stimulation with LPS (10 μg/ml) for 24 h, murine peritoneal macrophages (10^7/ml) were collected and after centrifugation, the cell pellet was sonicated and centrifuged as previously described [13]. High speed supernatant and pellet were used for NOS and COX assays, respectively. NOS activity was determined by assessing the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline in the cytosolic cellular fraction, as described by Mitchell et al. [20]. COX-2 activity was assayed as previously described [21] and PGE_2 levels were determined by a radioimmunoassay [19]. The protein content was quantified by the Bradford technique [22].

2.5. Immunoprecipitation assay

Cellular lysates were obtained from mouse peritoneal macrophages incubated with LPS (10 μg/ml) and IFNγ (10 U). At the indicated times and doses, cells (1.7×10^6/well) were lysed with lysis buffer (1% Triton X-100, 1% deoxycholic acid, 20 mM NaCl and 25 mM Tris, pH 7.4). For immunoprecipitation assays, macrophages were stimulated with appropriate concentrations of LPS and/or IFNγ for different times in the absence or presence of test compounds. Then, cells were shifted to methionine-free RPMI 1640 medium and pulse-labelled for 3 h with 25 μCi/ml [^{35}S]methionine (*trans* ^{35}S-label). Radiolabelled iNOS and COX-2 isoenzymes present in the cellular lysate were immunoprecipitated with specific polyclonal antibodies against recombinant iNOS and COX-2 and subjected to SDS-PAGE under reducing conditions. After fluorography, radioactive bands were excised and quantified in a β-counter.

2.6. Mouse air pouch model

An air pouch was performed in female Swiss mice (25–30 g) as previously described [21]. Chalcone derivatives and dexamethasone (0.1 and 0.01 μmol/pouch) were injected 1 h before and 8 h after zymosan. 24 h 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 exudates were measured using a Coulter counter. After centrifugation of exudates ($1200\times g$ at 4°C for 10 min), the supernatants were used to measure nitrite and PGE_2 levels as described above.

2.7. Statistical analysis

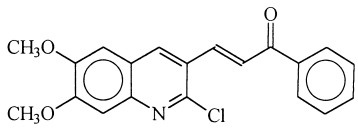
Statistical evaluation included one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons. *P*-values of $P<0.05$ (*) or $P<0.01$ (**) were taken as significant. Results are shown as mean ± S.E.M. for *n* experiments. Inhibitory concentration 50% (IC_{50}) values were calculated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

3. Results

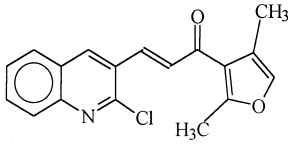
3.1. Effects of chalcone derivatives on nitrite and PGE_2 production in mouse peritoneal macrophages

In preliminary experiments, all chalcones belonging to the series reported by Herencia et al. [15] were screened at the concentration of 10 μM for their activity on nitrite and PGE_2 production in mouse peritoneal macrophages stimulated by LPS (data not shown). The most active chalcone derivatives (CH1, CH8 and CH12) were selected for further studies. These compounds inhibited nitrite and PGE_2 production dose-dependently (Fig. 2) with IC_{50} values in the μM range. CH8 and CH12 exhibited a similar behavior, with a higher potency on PGE_2. In contrast, CH1 was more potent on the nitrite production. As expected, aminoguanidine reduced the nitrite accumulation ($IC_{50}=25.1$ μM) without a significant effect on PGE_2 levels at 100 μM. NS398 inhibited to a great extent PGE_2 production ($IC_{50}=3.1$ nM), whereas dexamethasone reduced very significantly both metabolites ($IC_{50}=35.8$ nM and $IC_{50}=1.0$ nM for nitrite and PGE_2, respectively). Essentially the same inhibition patterns were observed when the cells were stimulated with LPS+IFNγ (data not shown). These compounds did not affect the cellular viability, as assessed by mitochondrial reduction of MTT after 24 h treatment (data not shown), indicating that they were not cytotoxic.

CH 1



CH 8



CH 12

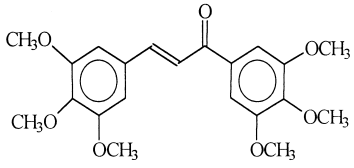


Fig. 1. Structures of chalcone derivatives CH1, CH8 and CH12.

3.2. Effects of chalcone derivatives on NOS and COX activities in mouse peritoneal macrophages

To determine if the inhibition of nitrite and PGE_2 production was either due to an interference with the enzyme induction by LPS or due to a direct action of these compounds on NOS and COX activities, the following experiments were performed. Chalcone derivatives were incubated with cells after the induction of these enzymes by LPS. Cells were treated with LPS for 24 h to allow the induction and expression of iNOS and COX-2 and then, were washed with culture medium. Test compounds were added at 10 μM and incubated for 2 h in fresh culture medium supplemented with L-arginine and arachidonic acid. No significant reduction of either nitrite nor PGE_2 production during these 2 h was observed for the chalcone derivatives (Table 1). Nevertheless, aminoguanidine, a known inhibitor of NOS activity, caused a very significant reduction of nitrite production (50%) without affecting the PGE_2 level, whereas the selective COX-2 inhibitor NS398 caused a marked inhibition of PGE_2 production (78%). The

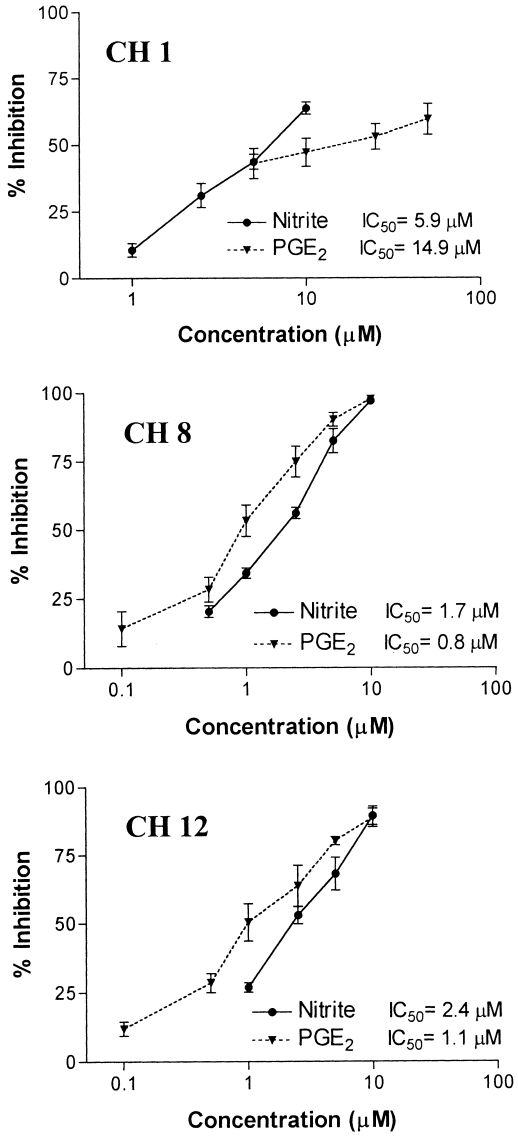


Fig. 2. Dose-response curves of nitrite and PGE_2 inhibition by tested compounds in stimulated peritoneal macrophages. Cells were incubated with LPS in the presence of inhibitors for 24 h and supernatants were used to determine mediator levels. Each value represents the mean ± S.E.M. for more than three triplicate experiments.

corticosteroid dexamethasone, an inhibitor of iNOS and COX-2 gene expression, failed to significantly modify the levels of both metabolites.

In another set of experiments, chalcone derivatives were present during the 24 h LPS treatment of cells. After washing with fresh medium, macrophages were incubated for a further 2 h. The nitrite and PGE_2 production during these 2 h was significantly reduced by chalcones and dexamethasone (Table 1). This suggests that the presence of chalcone derivatives during the induction period can affect iNOS and COX-2 expression levels.

To corroborate our results on enzyme activity, we examined the effects of chalcone derivatives on broken cell preparations. The high speed supernatant fraction of 24 h LPS-stimulated cells was used as the source of iNOS. These compounds at 10 μM were inactive on citrulline generation. In contrast, aminoguanidine reduced the levels of citrulline by 56% (Table 2). The assay performed in absence of calcium gave no significant differences, but in the absence of NADPH, there was a significant reduction of the citrulline production (by 39%). The latter effect confirms that this enzyme activity shows a good correlation with the well-characterized properties of iNOS. The microsomal cellular fraction (enriched in COX enzyme) was used to assay the PGE_2 production. Only NS398 caused a significant inhibition of the generation of PGE_2 in this subcellular preparation.

3.3. *Effects of chalcone derivatives on iNOS and COX-2 protein expression in mouse peritoneal macrophages*

Data described above suggest that the inhibition of NO and PGE_2 production by some of these chalcones in peritoneal macrophages occurs at the level of enzyme expression.

As expected [2], a synergy between LPS (10 μg/ml) and IFNγ (10 U) was obtained in our experimental system and, thus, it was selected for further studies. To characterize the time-course of enzyme synthesis, mouse peritoneal macrophages were stimulated with LPS+IFNγ during 6, 12 and 24 h in medium free of methionine, containing [^{35}S]methionine (25 μCi/ml) for the last 3 h of the experiment. Cells were lysed and the supernatants were used to determine iNOS and COX-2 by immunoprecipitation with specific antibodies. In parallel experiments, we assayed nitrite and PGE_2 accumulation in different time-courses of cell stimulation. Fig. 3a shows a peak in de novo iNOS synthesis at 12 h. At this time, signifi-

Table 1
Effects of chalcone derivatives and reference compounds on NOS and COX activity in peritoneal macrophages

	A (2 h treatment)		B (24 h treatment)	
	NO_2^- (ng/ml)	PGE_2 (ng/ml)	NO_2^- (ng/ml)	PGE_2 (ng/ml)
24 h without LPS	1.9 ± 0.3**	2.1 ± 0.2**	6.8 ± 0.5**	1.2 ± 0.2**
Control	59.2 ± 6.2	8.6 ± 0.6	53.0 ± 3.1	6.7 ± 0.3
CH1 (10 μM)	58.9 ± 6.9	7.1 ± 0.7	17.9 ± 1.2**	5.1 ± 0.6*
CH8 (10 μM)	50.6 ± 8.6	7.4 ± 0.7	10.8 ± 1.5**	3.2 ± 0.6**
CH12 (10 μM)	51.3 ± 4.8	7.5 ± 0.5	6.2 ± 0.2**	2.2 ± 0.2**
Aminoguanidine (100 μM)	30.5 ± 3.5**	7.8 ± 1.0	17.5 ± 1.9**	6.3 ± 0.4
NS398 (10 μM)	77.1 ± 6.5	3.5 ± 0.5**	43.0 ± 2.3	1.4 ± 0.1**
Dexamethasone (1 μM)	39.7 ± 3.8	6.9 ± 0.7	6.4 ± 0.2**	0.9 ± 0.1**

A: Cells were stimulated with LPS for 24 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).
B: Cells were co-incubated with LPS and test compounds for 24 h. After washing the cells, incubation proceeded for 2 h in the presence of L-arginine (0.5 mM) and arachidonic acid (10 μM).
Results are the mean ± S.E.M. of at least three independent experiments assayed in triplicate. *$P < 0.05$ and **$P < 0.01$ compared with the control.

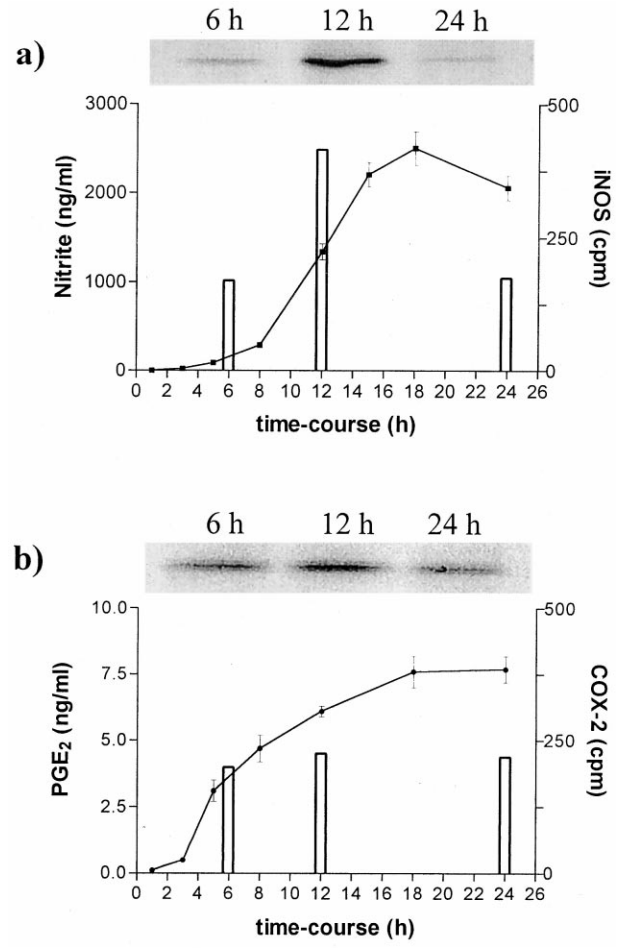


Fig. 3. Time-course of nitrite and PGE_2 production and iNOS and COX-2 protein synthesis by macrophages stimulated with LPS+IFNγ. Aliquots of supernatants were assayed for nitrite (a) and PGE_2 (b) production at indicated times. Bars indicate de novo synthesis of enzymes iNOS (a) and COX-2 (b) in immunoprecipitates. Cells were washed 3 h before the indicated times with fresh methionine-free medium and medium containing 25 μCi [^{35}S]methionine was added for the last 3 h of the experiment. Cellular lysate was used to evaluate the immunoprecipitation assay. The quantification data and insert are representatives of three different experiments.

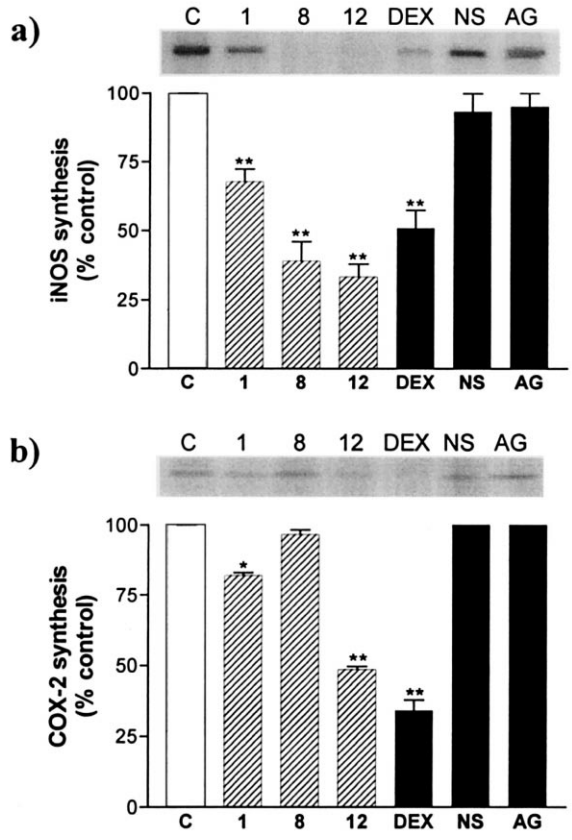


Fig. 4. De novo protein synthesis, (a) iNOS and (b) COX-2, by mouse peritoneal macrophages stimulated for 12 h with LPS+IFNγ in the presence of test compounds (chalcones at 10 μM, dexamethasone at 10 μM, NS398 at 10 μM and aminoguanidine at 100 μM). Cells were washed 3 h before the indicated time with fresh methionine-free medium and medium containing 25 μCi [^{35}S]methionine was added for the last 3 h of the experiment. Cellular lysate was used to evaluate the immunoprecipitation assay. Quantification data are mean ± S.E.M. of three different experiments. DEX = dexamethasone, NS = NS398, AG = aminoguanidine, $*P < 0.05$ and $**P < 0.01$.

cant levels of nitrite were detected and the maximal accumulation of nitrite was reached at 18 h. As shown in Fig. 3b, COX-2 protein synthesis was similar during all periods assayed and protein synthesis and PGE_2 levels were detected at an earlier time than iNOS and nitrite.

We then quantified de novo iNOS and COX-2 protein synthesis in the presence of the three chalcone derivatives (10 μM) and reference inhibitors dexamethasone (10 μM), NS398 (10 μM) and aminoguanidine (100 μM), in 12 h LPS+IFNγ-stimulated cells. Results obtained by fluorography (see figure inserts) and its protein quantification (Fig. 4a and b) showed that CH12 affected iNOS (66.9% inhibition) and COX-2 (51.4%) synthesis, whereas CH8 only affected iNOS (54.9%) and CH1 showed a lower inhibition of iNOS (32.1%)

Table 2
Effects of chalcone derivatives and reference compounds on NOS and COX activities in broken cells preparations

	Citrulline (pmol/mg)	PGE_2 (ng/mg)
Without LPS		3.2 ± 0.1**
Control	775.6 ± 58.8	9.6 ± 0.3
CH1 (10 μM)	679.0 ± 40.4	8.9 ± 0.8
CH8 (10 μM)	796.6 ± 47.2	9.9 ± 0.4
CH12 (10 μM)	645.2 ± 44.2	9.3 ± 0.3
Aminoguanidine(100 μM)	343.4 ± 19.8**	N.D.
NS398 (1 μM)	N.D.	7.2 ± 0.3*

Microsomal and cytosolic fractions from 24 h LPS-stimulated cells were used to determine NOS and COX activities as citrulline (pmol/mg protein) and PGE_2 (ng/mg protein) generated, respectively, as indicated in Section 2.
Results are the mean ± S.E.M. of at least three independent experiments assayed in triplicate. $*P < 0.05$ and $**P < 0.01$ compared with the control. N.D., not determined.

Table 3
Effects of chalcone derivatives and dexamethasone on cell accumulation, nitrite and PGE_2 levels in exudates of a 24 h zymosan-injected mouse air pouch

	Doses (μmol/pouch)	Cell accumulation ($\times 10^6$ cells/ml)	Nitrite (ng/ml)	PGE_2 (ng/ml)
Saline		3.2 ± 0.3**	2.3 ± 0.6**	0.5 ± 0.1**
Control		17.4 ± 1.3	100.1 ± 13.9	14.5 ± 1.1
CH1	0.1	16.7 ± 1.3	36.5 ± 6.5**	5.1 ± 0.7**
	0.01	16.5 ± 1.9	54.9 ± 6.9*	5.7 ± 0.8**
CH8	0.1	11.1 ± 0.7**	40.9 ± 6.9**	3.2 ± 0.7**
	0.01	12.8 ± 0.6*	58.4 ± 4.7*	5.4 ± 1.1**
CH12	0.1	14.9 ± 0.7	45.8 ± 6.9**	4.8 ± 1.1**
	0.01	15.5 ± 0.7	56.1 ± 5.6*	5.3 ± 0.7**
Dexamethasone	0.1	10.4 ± 1.0**	28.3 ± 3.3**	0.9 ± 0.1**
	0.01	12.5 ± 0.8*	40.5 ± 7.2*	4.1 ± 0.5**

Compounds were administered at 0.1 and 0.01 μmol/pouch, at times indicated in Section 2. Results are the mean ± S.E.M. for 6–12 animals. *$P < 0.05$ and **$P < 0.01$ compared with the control (zymosan-injected air pouches).

and COX-2 (18.0%). As expected, dexamethasone reduced the iNOS (49.2%) and COX-2 (65.9%) synthesis, whereas neither NS398 nor aminoguanidine, at the dose assayed, affected significantly the protein synthesis, as clear evidence that their action does not depend on this mechanism.

3.4. *In vivo effects of chalcone derivatives (mouse air pouch model)*

To corroborate in vivo the inhibitory effect observed in isolated macrophage cells, we administered the compounds intrapouch and determined the cell accumulation and mediators release in the zymosan-injected mouse air pouch. Chalcone derivatives (at 0.1 and 0.01 μmol/pouch) did not affect the cellular migration except CH8. Nevertheless, they reduced to a great extent nitrite and PGE_2 levels in the exudate of the pouch (Table 3). As expected, dexamethasone caused a marked reduction in those metabolites and besides, reduced the cellular accumulation.

4. Discussion

NO produced by iNOS has been implicated in the pathophysiology of inflammatory and immune diseases. It is known that iNOS participates in the production of pathological changes in adjuvant arthritis and its inhibition would attenuate these changes [23]. iNOS inhibition also results in the control of the inflammatory response in other models including granuloma formation by subcutaneous implantation in rats of carrageenin-soaked sponges [24], chronic granulomatous colitis in rats [25] or the delayed paw swelling induced by carrageenin in mice [26]. In humans, the production of NO by activated macrophages or neutrophils can be an index for human bronchial inflammation and a mechanism for amplifying asthmatic inflammation [27]. There is also evidence of an increased NO production in infectious gastroenteritis [28] and rheumatoid arthritis [28,29], where peroxynitrite has also been detected [30]. NO is also implicated in human ostheoarthritis, classically considered as a non-inflammatory disease, where it may be involved in cartilage destruction [31].

We have shown that three novel chalcone derivatives inhibit dose-dependently NO and PGE_2 production in vitro, in mouse peritoneal macrophages stimulated by LPS and also in vivo, in the mouse air pouch injected with zymosan, with some differences in potency and selectivity. Our results indicate that in vitro, CH12 and CH8 are more potent on PGE_2 than on nitrite levels, whereas CH1 is more potent as inhibitor of nitrite. These compounds had no direct inhibitory effect on iNOS or COX-2 activities, as judged either in intact cells after induction of these enzyme or by assays with broken cells. In our previous studies, it was shown that they are not COX-1 or phospholipase A_2 inhibitors [15]. Besides, the addition of exogenous substrates does not seem to affect their inhibitory effects in a significant way. Our results thus suggest that these chalcones modify the induction of iNOS and COX-2.

Characterization of the synthetic process of these proteins led us to select LPS+IFNγ as stimulus and 12 h as the time for maximal detection of synthesis. The data presented here demonstrate that inhibition of de novo synthesis of iNOS may be the major mechanism responsible for the observed inhibition of NO production by the three chalcone derivatives. These compounds could selectively inhibit the enhanced expression of this enzyme in inflammation. Inhibition of COX-2 synthesis may account for the inhibitory effect on PGE_2 generation of CH12 and at least in part of CH1. In contrast, our results on the synthesis of COX-2 exclude this mechanism for CH8. Nevertheless, we have observed that the presence of this compound during the induction process (24 h LPS treatment) 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 [7]. Nevertheless, this is a matter of debate since the results obtained vary according to the cell type considered and experimental conditions[8,32–35]. The in vivo situation can be more difficult to evaluate, specially if non-selective inhibitors are used [36]. COX metabolites can also influence the NO production in some cases, since PGE_2 can exert a negative feedback by inhibition of iNOS expression [37] due to an interference with nuclear factor κB (NF-κB) activation [38]. In our experimental protocol, in murine peritoneal macrophages, we found no evidence for a direct iNOS-COX interaction, as previously shown in cell lines of murine macrophages [34,35]. It is thus unlikely that such a mechanism can be relevant for the inhibitory effects of these chalcones in LPS-stimulated murine peritoneal macrophages.

NO overproduction can be controlled by NOS inhibitors. Nevertheless, at doses normally used, they inhibit frequently constitutive isoforms, which leads to hypertension and a decreased local blood flow [39] or progressive leukocytosis and inflammation in the ileum after chronic treatment [40]. In addition, chronic inhibition of NOS can result in an increased iNOS expression followed by overproduction of NO upon

removal of the inhibitor [41]. These chalcone derivatives may selectively inhibit the enhanced expression of iNOS to control high production of NO during inflammatory or immune conditions. It is known that activation of NF-κB is crucial for the increase in iNOS gene expression and some anti-oxidants are able to control this redox-sensitive step, although mechanisms other than interaction with free radicals may be implicated [42]. Whether these chalcones inhibit de novo protein synthesis by modification of nuclear factors or by other mechanisms remains to be determined.

In vivo, inhibitory effects on nitrite and PGE_2 levels were also observed for the three chalcones. CH12 and CH8 exhibited an inhibitory behavior correlated with their in vitro results on nitrite and PGE_2 accumulation in 24 h LPS-stimulated mouse peritoneal macrophages, whereas CH1 was more potent than expected and reduced both metabolites in a similar way. In this model, the mouse air pouch injected with zymosan, iNOS and COX-2 is induced in several cell types, including migrating leukocytes and also macrophages lining the pouch [14], raising the possibility that the activity of this compound can be different according to the cell involved. On the other hand, CH8 was able to decrease leukocyte migration in vivo. It is interesting to note that this compound showed an inhibitory action on human 5-lipoxygenase and it also reduced TNFα levels in mice [15], which suggests that different mechanisms could be involved in its anti-inflammatory effect.

COX activity is the main therapeutic target for non-steroidal anti-inflammatory drugs. Nevertheless, COX inhibition can result in an increased COX-2 expression [37] and suppression of treatment with reversible COX-2 inhibitors would result in a transient overproduction of PGs [43]. Furthermore, some inhibitors of COX-1 and COX-2 would be less effective at more inflamed sites, since the supply of arachidonic acid can determine their effectiveness [44]. These chalcone derivatives able to control NO and PG production by mechanisms other than enzyme inhibition have a potential role in modulating the inflammatory process.

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