Effects of carprofen (*R* and *S* enantiomers and racemate) on the production of IL-1, IL-6 and TNF-α by equine chondrocytes and synoviocytes

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Chondrocytes and synoviocytes harvested from the joints of healthy horses were maintained in tissue culture. Production of the cytokines interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) in response to lipopolysaccharide (LPS), and the effects of addition of carprofen (racemate and *R* and *S* enantiomers) were determined. Lipopolysaccharide failed to stimulate TNF-α activity in both cell types but concentrations of IL-1 and IL-6 were both increased in a concentration and time-related manner. Both carprofen enantiomers and the racemic mixture attenuated the increase in IL-6 induced by LPS in synoviocytes, and S carprofen exerted a similar effect on chondrocytes. Neither enantiomer nor the racemate of carprofen suppressed the increase in IL-1 release produced by LPS in chondrocytes and synoviocytes. An action of carprofen to suppress IL-6 release might contribute to the actions which occur *in vivo*.

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INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the principal form of therapy for osteoarthritis (OA) in animals. The traditional rationale for NSAID use is their ability to inhibit cyclooxygenase (COX) and hence suppress pain and the inflammatory process. However, the effects of these drugs on articular cartilage have also been investigated and it is now recognized that many NSAIDs can directly modify synthesis and degradation of proteoglycans by chondrocytes *in vitro* (Collier & Ghosh, 1991; Benton *et al.*, 1997; Armstrong & Lees, 1999).

Carprofen is a 2-aryl propionic acid NSAID used extensively by veterinarians for the treatment of OA in horses, dogs and cats. It is a chiral compound and hence exists as two mirror-image enantiomers (*R* and *S* carprofen). The drug is commercially available as a 50:50 racemic mixture of the two enantiomers. As the body is a chiral environment, the two enantiomers are likely to have different pharmacokinetic and pharmacodynamic properties, and must therefore be considered as separate drugs. Indeed, studies in this laboratory have shown significant pharmacokinetic differences with *R* carprofen predominating over *S* carprofen in a range of body fluids (plasma, inflammatory exudate, interstitial fluid and synovial fluid). Moreover, the extent of *R* enantiomer predominance has been shown to vary between species (McKellar *et al.*, 1994; Lees *et al.*, 1996; Taylor *et al.*, 1996; Armstrong *et al.*, 1999).

Reports that carprofen produces anti-inflammatory effects *in vivo*, at concentrations which have moderate or slight inhibitory effect on COX, have led to the suggestion that the anti-inflammatory mechanism of action of carprofen may be at least partly because of mechanisms other than COX inhibition (Lees *et al.*, 1994; Delatour *et al.*, 1996; Lees *et al.*, 1996). We have recently demonstrated that the *S* enantiomer and racemic carprofen stimulate proteoglycan synthesis by equine chondrocytes in culture (Armstrong & Lees, 1999). The mechanism by which carprofen produces this stimulation was not determined.

There have been several reports that certain NSAIDs may, in addition to inhibiting COX, modify cytokine production (Chang *et al.*, 1990; Lozanski *et al.*, 1992; Bahl *et al.*, 1994; Gonzalez *et al.*, 1994). Concentrations of interleukin 1 (IL-1), interleukin 6 (IL-6) and tumour necrosis factor-α (TNF-α) in synovial fluid are known to increase in joint diseases including OA. Also, these cytokines have been shown to induce cartilage damage and bone resorption and to inhibit proteoglycan synthesis *in vitro* (May, 1992; Westacott & Sharif, 1996). Inhibition of these cytokines by NSAIDs may be an alternative mechanism of action. Therefore, we investigated the effects of carprofen (*R* and *S* enantiomers, and racemate) on the production of IL-1, IL-6 and TNF-α by equine cultured chondrocytes and synoviocytes.

MATERIALS AND METHODS

Source of materials

All chemicals were obtained from Sigma Chemical Co. (Poole, Dorset, UK), except culture media and antibiotics (Life

Technologies, Paisley, Scotland, UK), foetal calf serum (Harlan Sera Lab., Loughborough, Leics, UK), pronase (British Drig Houses, Poole, Dorset, UK), cytokines (R and D Systems, Abingdon, Oxon, UK). The D10 cells were purchased from Deutsche Sammlung von Mikroorganismen und Zelkulturen, Braunschweig, Germany. Dr C. Bird supplied the B9 cells and Dr P. McLaughlin provided the L929 cells.

Chondrocyte and synoviocyte culture

Equine articular cartilage and synovium were obtained from the metacarpophalangeal joints of healthy horses aged between 8 and 18 years which had been presented to a local abattoir. For each experiment, tissue from four to six limbs from at least two different animals was combined and mixed together. Cartilage was sliced from the subchondral bone and 2×2 cm squares of synovial membrane were cut from the opened joint. Both tissues were placed into flasks containing complete medium (Dulbecco's modified Eagle medium containing 10% foetal calf serum and 1% penicillin-streptomycin). In each experiment, the cartilage and synovial membrane from four to six horses were combined to minimize interindividual variation.

The cartilage was finely chopped, then incubated with pronase (1% in medium) for 30 min followed by incubation with bacterial collagenase (2% in medium) for 3 h. Digested cartilage was then filtered through a 70-mm cell strainer, centrifuged at $320 \times g$ for 10 min, resuspended in complete medium and seeded into tissue culture plates at 120 000 cells/1mL-well. The synovial membrane was chopped roughly and then incubated in bacterial collagenase (2% in medium) for 3 h. Synoviocytes were isolated using a 70-mm cell strainer, centrifuged at $320 g$ for 10 min, resuspended in complete medium and seeded into tissue culture plates at 70 000 cells/1mL-well. All cultures were maintained in complete medium for 2–4 days to allow cells to adhere to the plates.

Experimental design

Initial experiments were necessary to determine the concentration of bacterial (*Echerichia coli*) lipopolysaccharide (LPS) and duration of culture required to induce significant production of each cytokine (IL-1, IL-6 and TNF-α) by both chondrocytes and synoviocytes.

Experiment 1

Once cell monolayers had adhered to the culture plate, the existing medium was replaced with complete medium containing a range of concentrations of LPS (1–500 µg/mL). Each LPS concentration was repeated in quadruplicate. After 4 h of incubation (37 °C, 5% CO_2), the media were removed and stored at −20 °C awaiting IL-1, IL-6 and TNF-α bioassay.

Experiment 2

Monolayers of chondrocytes and synoviocytes from another group of horses were cultured until adherent to plates, then the existing medium was replaced with complete medium containing LPS at a concentration chosen after analysis of the results obtained in the first experiment (100 µg/mL for chondrocytes and 200 µg/mL for synoviocytes in the IL-1 study, 10 µg/mL for chondrocytes and 200 µg/mL for synoviocytes in IL-6 study). The cells were incubated with this medium for a range of time periods (2–24 h) after which the medium was removed and stored at −20°C awaiting bioassay. Each incubation time was repeated in quadruplicate.

Experiment 3

The effect of carprofen on IL-1 and IL-6 production by chondrocyte and synoviocyte monolayers was then assessed. Chondrocytes and synoviocytes from a third group of horses were obtained and adherent monolayers were grown as described previously. The media were replaced with fresh complete media containing both LPS and carprofen. The concentration of LPS was as determined in experiment 1 (150 µg/mL LPS for IL-1 production by chondrocytes; 200 µg/mL LPS for IL-1 production by synoviocytes; 10 µg/mL LPS for IL-6 production by chondrocytes; 150 µg/mL LPS for IL-6 production by synoviocytes). Carprofen enantiomers (a gift from Pfizer Central Research, Sandwich, Kent, UK) were dissolved in absolute ethanol to produce stock solutions of 10 mg/mL of each enantiomer and 20 mg/mL racemate. Stock solutions were diluted in the complete medium containing the appropriate concentration of LPS to produce media containing a range of concentrations of carprofen (*R* and *S* enantiomers: 0.23–92 µM, racemate: 0.46–183 µM). Each carprofen concentration was repeated in quadruplicate. The duration of culture in these new media was that which produced optimal cytokine production in experiment 2 (4 h for IL-1 production by chondrocytes and synoviocytes; 24 h for IL-6 production by chondrocytes; 12 h for IL-6 production by synoviocytes). Control cultures contained either no LPS, LPS but no carprofen or LPS and dexamethasone (0.5 µg/mL) as a positive control.

Following incubation, the medium was harvested and stored at −20°C awaiting bioassay. The chondrocyte and synoviocyte monolayers were digested in papain solution (0.1 M sodium acetate, 2.4 mM EDTA, 5 mM L-cysteine, 10 U papain/mL) and the DNA content was calculated as described previously (Armstrong & Lees, 1999).

Bioassays

Cytokine activity was determined using bioassays. Although sensitive immunoassays are available to detect human cytokines, we have demonstrated that such assays do not detect equine cytokines (unpublished data). The three bioassays used in this study have been well documented and are highly sensitive and specific (Espevik & Nissen-Meyer, 1986; Van Oers *et al.*, 1988; Hopkins & Humphreys, 1989). Therefore, it was not necessary to demonstrate this using blocking antibodies. Furthermore, all standard curves included samples with no cytokine to establish baseline cell proliferation.

Interleukin-1 bioassay

The bioassay for IL-1 was based on IL-1 stimulated proliferation of the mouse T-lymphocyte cell line D10(N4)M. A volume of 100 μL diluted medium from chondrocyte or synoviocyte cultures was added to microtitre plates and 100 μL D10 cells (2×10^5 cells/mL) were added and incubated for 72 h. Cell proliferation was determined by adding 20 μL (3-[4,5-di-methylthiazol-2–3]-2,5)-diphenyltetrazolium bromide salt (MTT, 2.5 mg/mL) and incubating for 2 h. The cells were lysed using 100 μL 20% sodium dodecyl sulphate (SDS) in 50% dimethylformamide and absorbance was read at 560 nm. A standard curve for IL-1 was prepared using recombinant human (rh)-IL-1β.

Interleukin-6 bioassay

The bioassay for IL-6 was based on an IL-6 dependent murine hybridoma cell line B9. A volume of 100 μL of diluted medium from chondrocyte/synoviocyte cultures was added to microtitre plate wells and 100 μL B9 cells (5×10^4 cells/mL) were added and incubated for 72 h. Cell proliferation was determined by adding 20 μL MTT (2.5 mg/mL) and incubating for 4 h. The cells were lysed by the addition of 100 μL of 20% SDS in 50% dimethylformamide and absorbance at 560 nm was read. A standard curve containing rh-IL-6 was prepared in the same manner.

Tumour necrosis factor-α bioassay

The bioassay for TNF-α was based on the cytotoxic effect of TNF-α on the murine fibroblast cell line L929. A volume of 100 μL L929 cells (5×10^5 cells/mL) was added to microtitre plate wells and incubated for 4 h to allow cells to adhere. A volume of 100 μL undiluted or diluted medium from the chondrocyte/synoviocyte cultures was added to each well and plates incubated overnight. Cell growth/death was determined by adding 20 μL MTT (5 mg/mL), incubating for 2 h, decanting off the medium and dissolving the colour by adding 100 μL DMSO/well. The absorbance was then read at 560 nm. A standard curve was produced using rh-TNF-α.

Purified equine cytokines were not available. Recombinant human cytokines were therefore used to prepare standard curves for all three bioassays. Therefore, calculated concentrations are expressed as cytokine-like activity. Values are also adjusted for DNA content of the chondrocyte or synoviocyte cultures. Furthermore, in the drug studies, cytokine activity is expressed as a percentage of activity in control cultures exposed to LPS, but no drug.

Statistical analysis

Comparisons between cultures exposed to different concentrations of LPS, cultures exposed to LPS for different time periods and between control and drug treated groups, were performed using analysis of variance. To determine between which groups

differences occurred, a Bonferroni/Dunn post hoc test was performed. The level of significance was $P < 0.05$.

RESULTS

Interleukin-1

The magnitude of the results obtained for the IL-1 bioassay often varied between repeats of the assay. This was probably because of variation in the sensitivity of different passages of the D10 cell line, also, the fact that chondrocytes and synoviocytes were obtained from different animals each time could have contributed to this effect. However, despite this variation in the absolute values for IL-1-like activity, the relative effects (i.e. increase or decrease) of adding LPS or carprofen were consistent and repeatable.

Interleukin-1-like activity in medium from chondrocyte cultures increased with increasing concentrations of LPS, however, activity was significantly greater than non-LPS treated controls only when the LPS concentration was 150 μg/mL or more (Fig. 1). A peak of IL-1-like activity was observed following 4 h stimulation (Fig. 2). Interleukin-1-like activity in medium from synoviocytes increased as LPS concentrations increased up to 200 μg/mL, whilst 500 μg/mL LPS caused excessive cell death, as determined by the DNA assay (Fig. 1). A peak in IL-1-like activity in medium from synoviocyte cultures occurred after 4 h of stimulation (Fig. 2).

In the drug studies, LPS significantly increased IL-1-like activity in both the chondrocyte and synoviocyte cultures (Figs 3 and 4). In either case, dexamethasone, in the presence of LPS, significantly reduced IL-1-like activity to levels below those produced by non-LPS, non-drug treated control cells. Addition of carprofen had a similar effect in the two cell types. The two enantiomers produced a bell shaped response, i.e. IL-1-like activity peaked with a concentration of 23 μM of enantiomer at levels significantly higher than those produced by non-LPS treated cells. The highest concentration of racemic carprofen (183 μM) also significantly increased IL-1-like activity above levels produced by non-LPS treated cells.

Interleukin-6

Addition of LPS (10–500 μg/mL) produced a significant increase in IL-6-like activity in medium from chondrocytes, and this response was maximal with a concentration of 20 μg/mL (Fig. 5). Interleukin-6-like activity peaked after 24 h incubation with LPS (Fig. 6). However, 150 μg/mL LPS was the only concentration to produce a significant increase in IL-6-like activity in synoviocyte cultures (Fig. 5). The response of synoviocytes peaked after 12 h incubation (Fig. 6).

In the drug study using chondrocyte cultures, addition of S enantiomer carprofen at concentrations of 23 μM or above significantly reduced IL-6 production to baseline (non-LPS stimulated) levels (Fig. 7). Although not statistically significant, the R enantiomer at all concentrations and racemate at higher
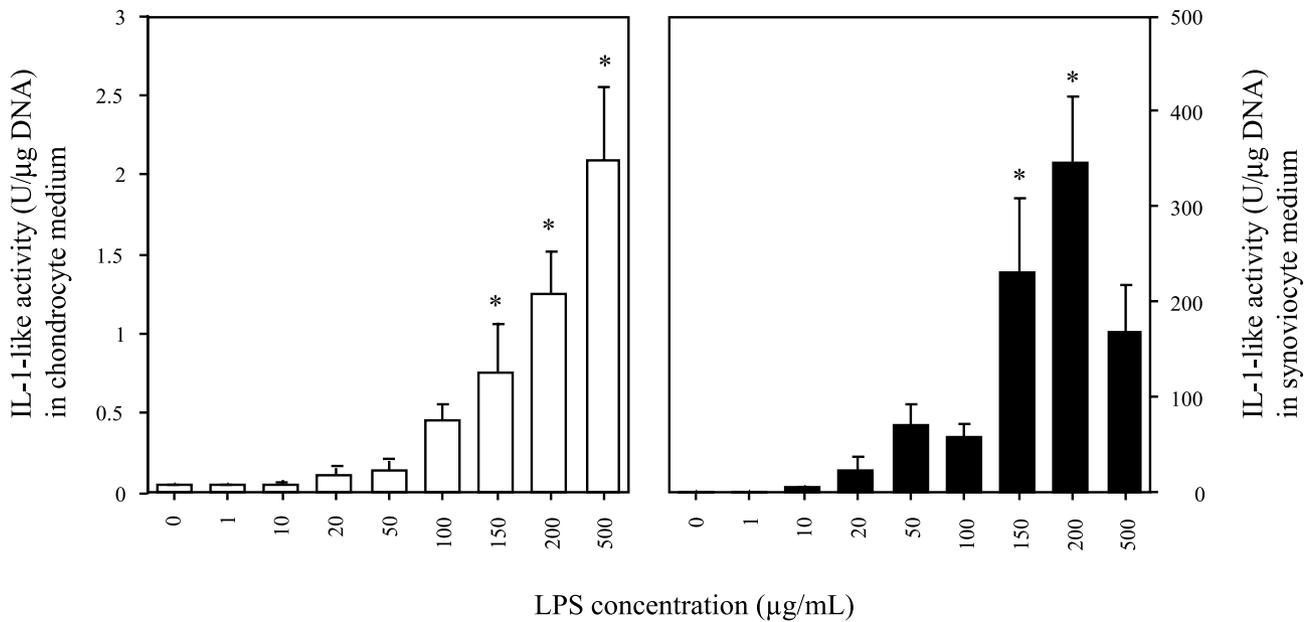
Fig. 1. Interleukin-1-like activity in medium from chondrocyte and synoviocyte cultures exposed to a range of concentrations of LPS for 4 h. *Significantly different from non-LPS treated cultures. Values are means of quadruplicate replicates with SEM error bars.

concentrations (46 and 183 μM) also reduced IL-6-like activity. Dexamethasone significantly reduced IL-6 production to baseline levels. Addition of carprofen (*R* and *S* enantiomers and racemate) to synoviocyte cultures produced significant or almost significant ($0.05 < P < 0.1$) reduction in IL-6 production at all concentrations tested (Fig. 8). Dexamethasone also significantly reduced IL-6 production.
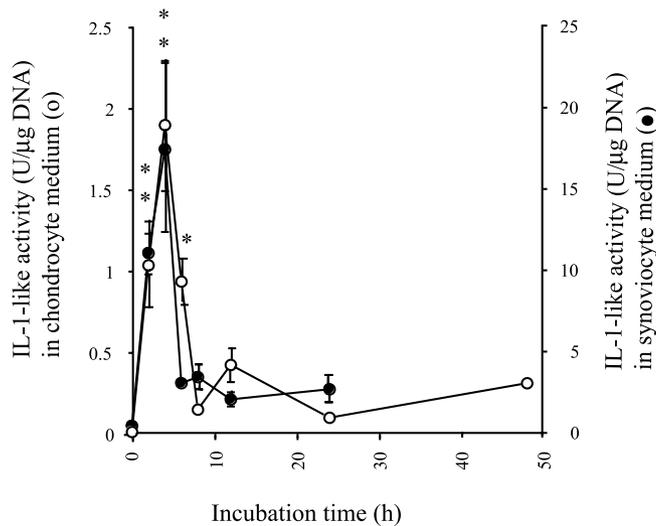

Fig. 2. Interleukin-1-like activity in medium from cultures exposed to 100 μg/mL (chondrocytes) or 200 μg/mL (synoviocytes) of LPS for a range of time periods. *Significantly different from time zero cultures. Values are means of quadruplicate replicates with SEM error bars.
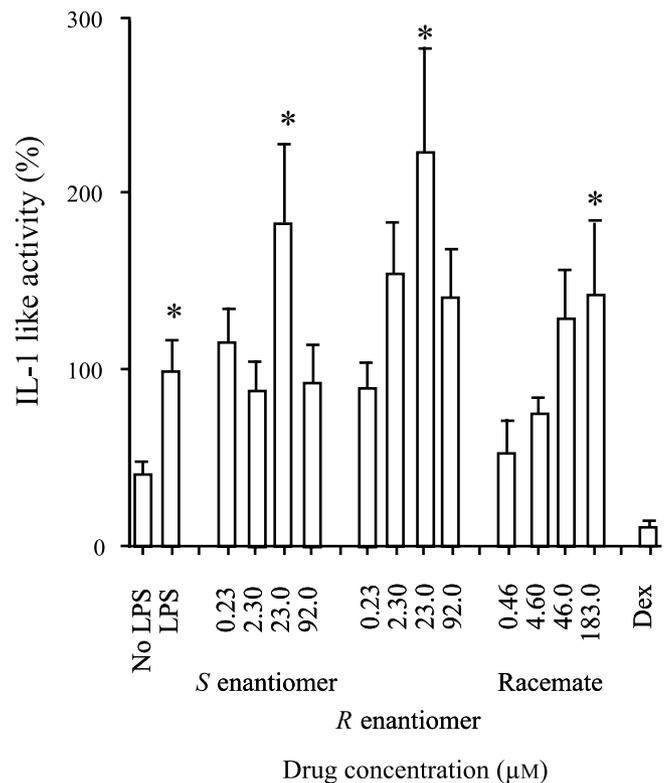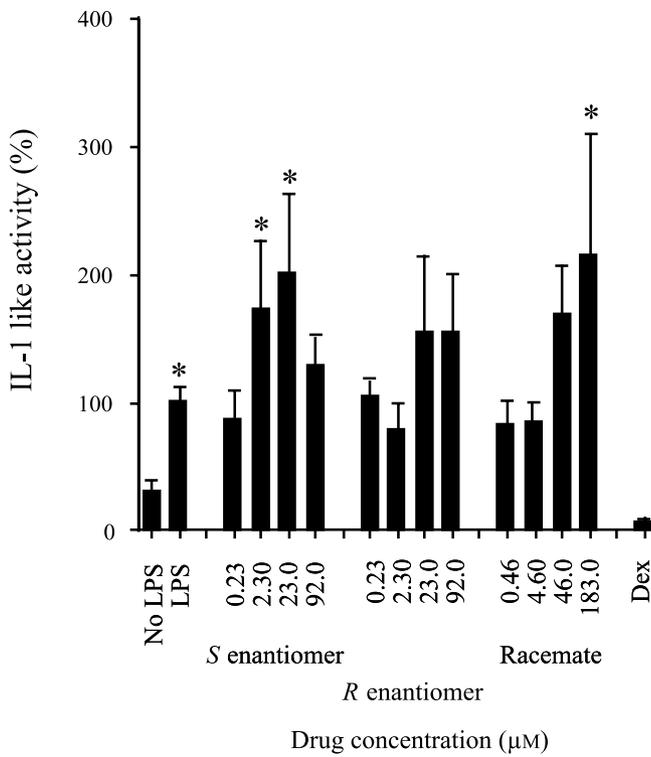

Fig. 3. Interleukin-1-like activity in medium from chondrocyte cultures exposed to 150 μg/mL LPS and a range of concentrations of *S*, *R* and racemic carprofen for 4 h. Results are expressed as a percentage of IL-1 like activity in cultures exposed to LPS alone. *Significantly greater than non-LPS treated cultures. Values are means of quadruplicate replicates with SEM error bars.

Fig. 4. Interleukin-1-like activity in medium from synoviocyte cultures exposed to 200 µg/mL LPS and a range of concentrations of *S, R* and racemic carprofen for 4 h. Results are expressed as a percentage of IL-1 like activity in cultures exposed to LPS alone. *Significantly greater than non-LPS treated cultures. Values are means of quadruplicate replicates with SEM error bars.

Tumour necrosis factor-α

Tumour necrosis factor-α-like activity was not detected in the medium of chondrocytes or synoviocytes.

DISCUSSION

The concentration of IL-1 in equine synovial fluid is elevated in OA (Alwan *et al.*, 1991). Interleukin-1 is a pro-inflammatory cytokine which also has specific degradative effects on articular cartilage matrix. Interleukin-1 stimulates proteoglycan breakdown, decreases proteoglycan synthesis and decreases collagen synthesis by chondrocytes (Arner & Pratta, 1989; Lefebvre *et al.*, 1990; MacDonnald *et al.*, 1992; Platt & Bayliss, 1994). Therefore, inhibition of IL-1 production is a potential mechanism by which osteoarthritic damage to articular cartilage could be minimized.

It has been reported that flunixin, tolfenamic acid and ketoprofen increase IL-1-like activity in medium from cultured equine synoviocytes (Landoni *et al.*, 1996). Also, indomethacin (100 µM) has been reported to increase IL-1 bioactivity in cultured human synovium, whereas meloxicam (0.1–10 µM) had no effect (Rainsford *et al.*, 1997). Piroxicam, however, has been shown to down-regulate IL-1 activity in human synovium from osteoarthritic patients, and this action appeared to be because of an increase in inhibitor production rather than a decrease in IL-1 synthesis (Herman *et al.*, 1991). The present study demonstrated that carprofen did not decrease LPS induced IL-1-like activity. In fact, there was an increase in IL-1-like activity in medium from equine chondrocytes cultured with 23 µM of either enantiomer or 183 µM racemate, and in synoviocytes cultured with 2.3 and 23 µM *S* enantiomer and
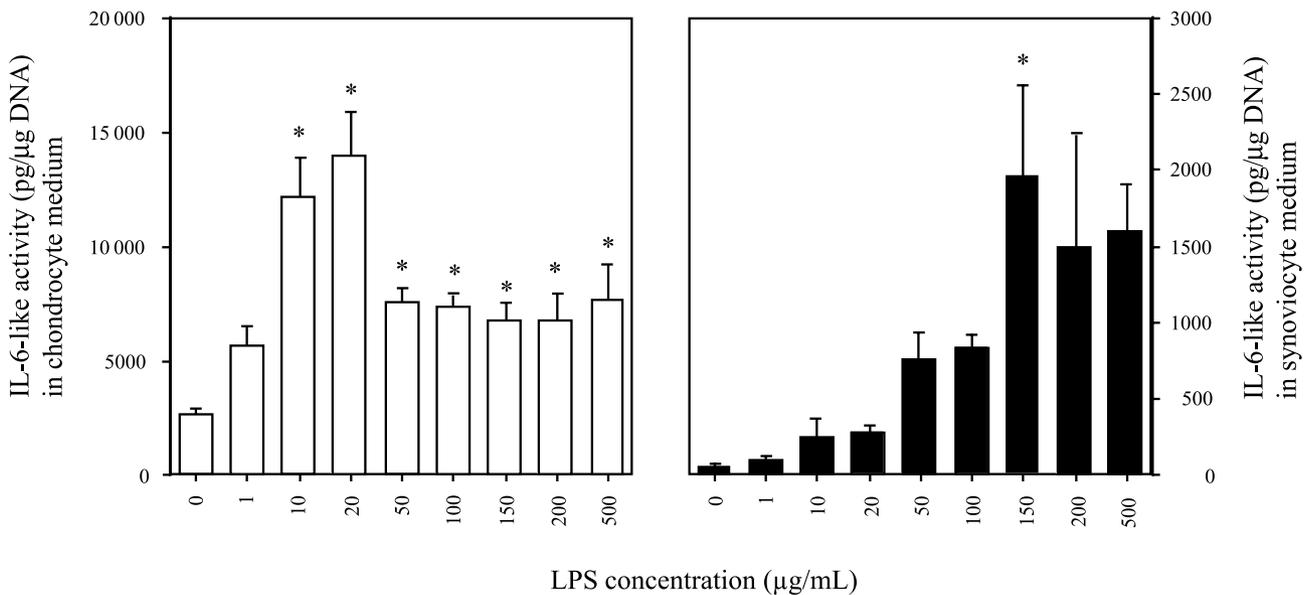
Fig. 5. Interleukin-6-like activity in medium from chondrocyte and synoviocyte cultures exposed to a range of concentrations of LPS for 4 h. *Significantly different from non-LPS treated cultures. Values are means of quadruplicate replicates with SEM error bars.
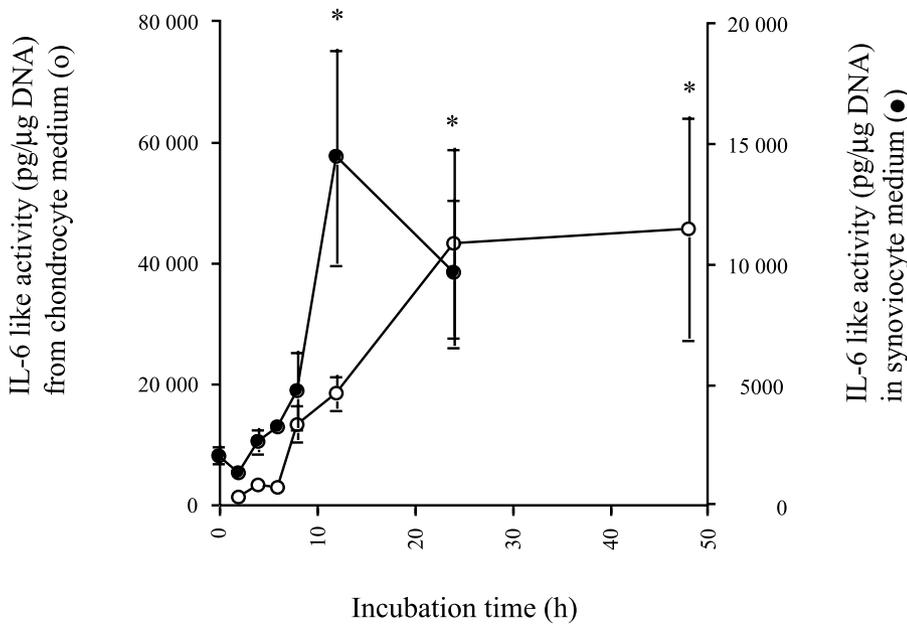
Fig. 6. Interleukin-6-like activity in medium from cultures exposed to 10 µg/mL (chondrocytes) or 200 µg/mL (synoviocytes) of LPS for a range of time periods. *Significantly different from time zero cultures. Values are means of quadruplicate replicates with SEM error bars.

183 µM racemate. It is worth noting, however, that the concentrations of NSAIDs used in the previously mentioned studies were not as high as 183 µM racemate carprofen used in the present study.

It has been proposed that stimulation of IL-1 production by NSAIDs may be a result of COX inhibition leading to decreased prostaglandin E2 (PGE2) concentrations which in turn removes PGE2 inhibition of IL-1 mRNA translation (Kunkel *et al.*, 1986; Landoni *et al.*, 1996). However, Bahl *et al.* (1994) proposed that, although this mechanism may account for increased IL-1 synthesis, a mechanism other than PGE2 inhibition accounts for the increase in IL-1 release from cells. The concentrations of carprofen required to stimulate IL-1 release from equine chondrocytes in this study were 10–1000 times greater than those which we have shown to significantly inhibit PGE2 production (0.23 µM *S* enantiomer, 2.3 µM *R* enantiomer, 0.46 µM racemate) (Armstrong & Lees, 1999), thus indicating that the effects of carprofen on IL-1 production may be mediated by mechanisms other than PGE2 inhibition. Chang *et al.* (1990) found that high concentrations of NSAIDs [e.g. 50 µg/mL (140 µM) indomethacin] inhibited IL-1 production by murine monocytes, although the NSAIDs that they examined produced no evidence for stimulation of IL-1 at lower concentrations.

Previously, we have shown that addition of carprofen enantiomers and racemate to equine chondrocyte cultures stimulates proteoglycan synthesis (Armstrong & Lees, 1999). The mechanism by which this effect was produced was not determined. The current results indicate that inhibition of IL-1 is an unlikely mechanism.

Synovial fluid concentrations of IL-6 are reported to increase in early OA (Venn *et al.*, 1993). The role of IL-6 in OA is not entirely clear. In general, it limits the catabolic effects of IL-1 and TNF-α, and hence protects the matrix from degradation induced by IL-1 (Shingu *et al.*, 1995). Thus, IL-6 has been shown to decrease IL-1 stimulated PGE2 production and proteoglycan loss,

increase tissue inhibitor of metalloproteinase production and possibly stimulate chondrocyte proliferation (Hauptmann *et al.*, 1991; Lotz & Guerne, 1991; Shinmei *et al.*, 1991; Venn *et al.*,
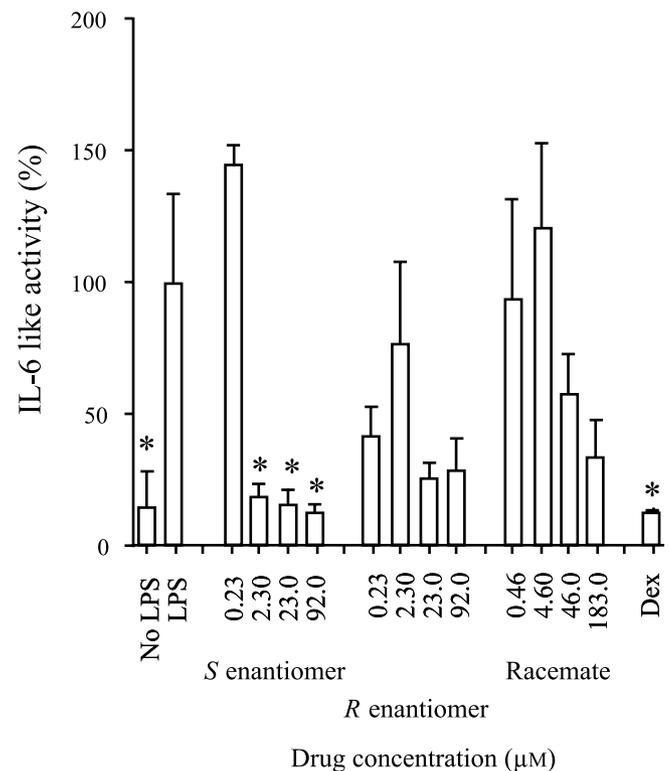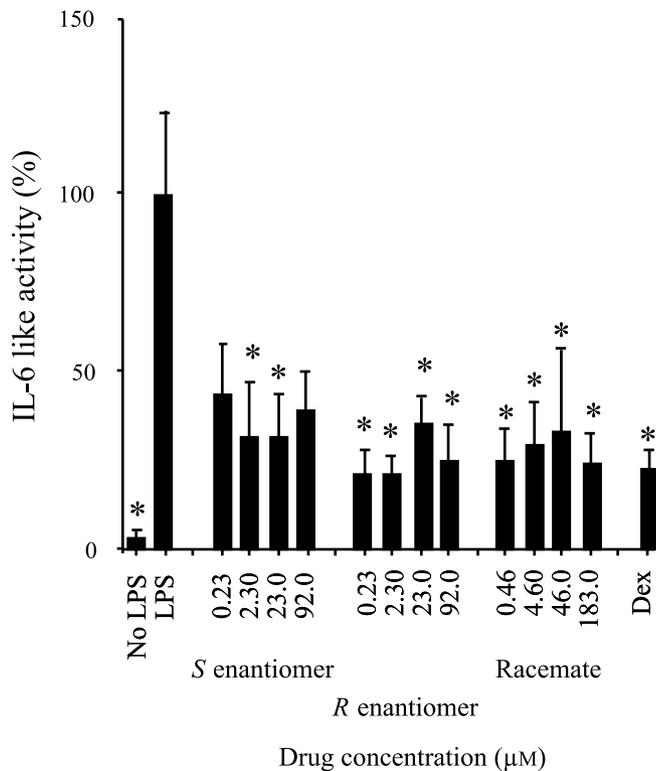

Fig. 7. Interleukin-6-like activity in medium from chondrocyte cultures exposed to 10 µg/mL LPS and a range of concentrations of *S*, *R* and racemic carprofen for 24 h. Results are expressed as a percentage of IL-6 like activity in cultures exposed to LPS alone. *Significantly lower than LPS treated cultures. Values are means of quadruplicate replicates with SEM error bars.

Fig. 8. Interleukin-6-like activity in medium from synoviocyte cultures exposed to 150 μg/mL LPS and a range of concentrations of *S*, *R* and racemic carprofen for 12 h. Results are expressed as a percentage of IL-6 like activity in cultures exposed to LPS alone. *Significantly lower than LPS treated cultures. Values are means of quadruplicate replicates with SEM error bars.

1993). However, IL-6 has also been shown to up-regulate TNF-α-receptor expression and this may result in increased TNF-α-induced glycosaminoglycan release (Van Bladel *et al.*, 1991; Venn *et al.*, 1993).

Interleukin-6 production by human chondrocytes is stimulated by IL-1 (Henrotin *et al.*, 1996). In the present study, the maximal production of IL-6-like activity occurred 8 and 20 h (in synoviocytes and chondrocytes, respectively) after the peak in IL-1-like activity, indicating that stimulation of IL-6 production by IL-1 may also occur in equine chondrocytes and synoviocytes.

Addition of carprofen (at intermediate concentrations of the enantiomers and higher concentrations of the racemate) tended to increase IL-1-like activity. However, instead of the expected subsequent stimulation of IL-6-like activity, there was significant inhibition of IL-6-like activity from synoviocytes (at all concentrations of enantiomer and racemate except 0.23 μM and 92 μM *S* enantiomer), and chondrocytes (at all concentrations of *S* enantiomer except 0.23 μM, and a similar trend was observed with the *R* enantiomer). Carprofen may therefore either have a direct effect on inhibition of IL-6 synthesis or release, or such an effect may be mediated via some indirect action of carprofen. Meloxicam (0.1 and 4.0 μM) and indomethacin (10 and 100 μM) increase IL-6 production by cultured osteoarthritic human synovial cells (Rainsford *et al.*, 1997) and flunixin, tolfenamic

acid and ketoprofen (all at concentrations between 0.1 and 1000 μM) are reported to have no significant effect on IL-6 production by equine synoviocytes (Landoni *et al.*, 1996). This suggests that carprofen may differ from many other NSAIDs in this respect. However, it has been reported that diclofenac (given to human OA patients at 50 mg t.i.d) reduces IL-6 production by subsequently cultured monocytes (Gonzalez *et al.*, 1994), so that this action is not unique to carprofen.

Tumour necrosis factor-α activity was not detected in any chondrocyte or synoviocyte cultures in this study. The lack of TNF-α production by equine chondrocytes was confirmed by polymerase chain reaction; primers detected no TNF-α mRNA production by LPS stimulated chondrocytes, whilst the same cells produced inducible nitric oxide synthase mRNA. Tumour necrosis factor-α mRNA was detected using the same primers in medium from LPS stimulated equine macrophages (unpublished data, this laboratory).

Tumour necrosis factor-α is detectable in equine synovial fluid and concentrations increase in OA (Billinghurst *et al.*, 1995). Also, chondrocytes do have TNF-α receptors (Kammerman *et al.*, 1996) and TNF-α has been shown to have deleterious effects on cartilage matrix integrity, such as reduced glycosaminoglycan synthesis and inhibition of tissue inhibitor of metalloproteinase production (Saklatvala, 1986; Yaron *et al.*, 1989; Shingu *et al.*, 1993). The source of synovial TNF-α may be inflammatory cells that infiltrate the synovium in OA, although leucocyte numbers are not greatly increased in OA. Alternatively, the cytokine may be transported into the joint from the systemic circulation. As we have demonstrated that carprofen affects the production of other cytokines, it is possible that it may alter synovial fluid TNF-α concentrations through effects at distant sites.

The literature reports a diverse range of effects of NSAIDs on cytokine production (Chang *et al.*, 1990; Gonzalez *et al.*, 1994; Landoni *et al.*, 1996; Rainsford *et al.*, 1997). Apart from differences between drugs, these variations may be because of differences in species and cell type and between *in vitro* and *in vivo* experiments. The range and sources of cytokines *in vivo* are far greater than *in vitro* and complex interactions occur between them. The observed ability of carprofen to modify production of IL-6 and possibly IL-1, is unlikely to be the mechanism by which it stimulates proteoglycan synthesis *in vitro*. However, the effects of carprofen on cytokine production described *in vitro* may play a role in the *in vivo* activity of this drug.

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