Evaluation of the ability of carprofen and flunixin meglumine to inhibit activation of nuclear factor kappa B

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Objective—To determine whether the nonsteroidal anti-inflammatory drugs (NSAIDs) carprofen, flunixin meglumine, and phenylbutazone have cyclooxygenase (COX)-independent effects that specifically inhibit activation of the proinflammatory transcription factor nuclear factor kappa B (NfκB).

Study Population—Purified ovine COX-1 and -2 and cultures of RAW 264.7 murine macrophages.

Procedure—The COX-1 and -2 inhibitory effects of the NSAIDs were tested in assays that used purified ovine COX-1 and -2. Prostaglandin production was analyzed by use of a radioimmunoassay. Inhibitory effects of these drugs on lipopolysaccharide (LPS)-induction of inducible nitric oxide synthase (iNOS) and LPS-stimulated translocation of NfκB were determined by use of RAW 264.7 murine macrophages.

Results—Flunixin meglumine and phenylbutazone were selective inhibitors of COX-1. Carprofen and flunixin meglumine, but not phenylbutazone, inhibited LPS-induction of iNOS. Carprofen and, to a lesser degree, flunixin meglumine had inhibitory effects on NFκB activation.

Conclusions and Clinical Relevance—The ability of drugs such as carprofen and flunixin meglumine to inhibit activation of NfκB-dependent genes such as iNOS, in addition to their effects on COX, suggests an additional mechanism for their anti-inflammatory effects and may explain the ability of flunixin meglumine to be an effective inhibitor of the effects of endotoxin in horses with endotoxemia. (*Am J Vet Res* 2003;64:211–215)

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for the treatment of animals with inflammatory disease and in pain management. Currently, phenylbutazone, flunixin meglumine, and carprofen are available and effective for use in horses.[1,2] Although phenylbutazone and flunixin meglumine are effective inhibitors of **cyclooxygenase (COX)**, carprofen is much less potent than those drugs at inhibiting COX and is considered to have a prostaglandin-sparing effect; hence, carprofen has reduced gastroenteric adverse effects when compared with other NSAIDs.[3-5] A possible explanation for this finding is that carprofen could be a selective COX-2 inhibitor. A number of studies[6-8] have investigated this hypothesis with varying conclusions, ranging from carprofen being a potent COX-2 inhibitor,[6] a weak inhibitor of COX-2 in canine cells,[7] and not having efficacy against COX-2 in equine blood.[8] This wide range of results probably reflects differences in the models used to study the COX-1 and -2 effects of the drugs. In contrast, from the limited data available from whole-blood assays, it can be determined that phenylbutazone and flunixin meglumine inhibit COX-1.[8]

In addition to inhibiting COX, it is now recognized that some NSAIDs, such as aspirin and salicylate, can elicit their anti-inflammatory effects by alternative COX-independent mechanisms. Aspirin and sodium salicylate inhibit activation of the proinflammatory transcription factor **nuclear factor kappa B (NfκB)** by tumour necrosis factor-α and **lipopolysaccharide (LPS)**.[9] Nuclear factor kappa B is a key transcription factor involved in the modulation of inflammatory and immune diseases.[10] It is retained in the cytoplasm by forming a complex with the inhibitory protein, **inhibitory kappa B (IκB)**. Translocation of NfκB to the nucleus is achieved by activation of a series of kinases that lead to phosphorylation of IκB by IκB kinase and subsequent release of NfκB.[10] Inhibition of NfκB by NSAIDs is mediated directly through inhibition of the kinases upstream of NFκB or indirectly through modulation of the activity of other signal transduction pathways that regulate the activity of this transcription factor (eg, p38 mitogen-activated protein kinase).[11] Additional mechanisms by which drugs such as aspirin may exert their anti-inflammatory effects include increasing concentrations of the anti-inflammatory mediator adenosine and inhibition of ribosomal S6 kinase to inhibit cAMP-dependent gene transcription.[11] Therefore, it is possible that carprofen and flunixin meglumine may exert their anti-inflammatory effects through COX-independent pathways.

Currently, mechanisms by which carprofen exerts its anti-inflammatory effects are far from clear. The objective of the study reported here was to test the hypothesis that carprofen mediates its anti-inflammatory effects by COX-independent mechanisms such as inhibition of the activation of NFκB and NFκB-dependent induction of **inducible nitric oxide synthase (iNOS)**. Effects of carprofen were compared with those of phenylbutazone and flunixin meglumine.

Materials and Methods

Assays of COX-1 and -2 activity—Each NSAID was dissolved in **dimethyl sulfoxide (DMSO)** to provide a solution

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with a final concentration of 10%. Solutions of each NSAID (10^{-2} to 10^{-8}M) were used to obtain dose-response curves, and the effects of each NSAID on COX-1 or -2 activities were compared with activity of 10% DMSO alone (control vehicle). Drug or control vehicle was placed in wells of a 96-well microassay plate. Then, ovine COX-1[a] or COX-2[b] enzyme (2 IU in Tris buffer at 21°C; pH, 7.5) was added to each well along with the cofactors hematin (1µM) and adrenaline (5mM). Each plate was covered with parafilm and incubated at 37°C for 30 minutes. The enzyme substrate arachidonic acid (1µM) was added to each well, and plates were incubated for an additional 15 minutes at 37°C. The enzyme reaction was terminated by addition of 1M HCl. Five minutes later, the HCl was neutralized by the addition of 1M NaOH. As an indication of COX-1 or -2 activity, concentrations of **prostaglandin E_2 (PGE_2)** were measured in the reaction mixtures by use of radioimmunoassay. For comparison, dose response curves for aspirin (as a nonselective COX inhibitor) and NS398 (as a selective COX-2 inhibitor) were prepared by use of the same assay conditions as those used for the reference NSAIDs.

PGE_2 radioimmunoassay—Concentrations of PGE_2 were determined by use of radioimmunoassay. Briefly, anti-PGE_2-antisera[c] and [^{3}H]-PGE_2[d] were added to each sample and a range of standards, and tubes were incubated for 18 to 24 hours at 0 to 4°C. After incubation, 200 µL of dextran-coated charcoal suspension (20 mg of charcoal/mL and 4 mg of dextran/mL) was added to each tube to adsorb free PGE_2, and the tubes were then centrifuged (2,000 × *g*, 4°C for 15 minutes) to separate the free and bound fractions. The supernatant (bound fraction) was decanted into scintillation vials, scintillant cocktail[e] was added, and radioactivity was determined by use of a beta counter. The effect of NSAIDs on COX-1 or -2 activity was determined by fitting PGE_2 concentration data obtained for a range of NSAID concentrations to sigmoid dose-response curves (data not shown) followed by calculation of **median inhibitory concentration (IC_{50})** values.[f] Ratios of COX-2 to COX-1 were calculated by dividing respective IC_{50} values. Data were reported as IC_{50} values with 95% confidence intervals (best fit of 4 dose response curves for COX-1 or -2 for each drug performed for 4 separate experiments).

Cell culture—Lipopolysaccharide-responsive RAW 264.7 macrophage-like cells were cloned and cultured in Dulbecco modified Eagle's medium containing 10% fetal calf serum supplemented with 2mM glutamine, 200 U of penicillin/mL, and 100 µg of streptomycin/mL. Prior to treatment with NSAIDs and LPS (*Escherichia coli*, serotype O157), fresh medium was added to confluent cells. Vehicle or an NSAID was added to the cells 1 hour prior to addition of LPS (1 µg/mL). For determination of the effects of NSAIDs on iNOS activity, medium was removed after 24 hours and frozen at –20°C for subsequent analysis of nitrite release. To assess the effects of NSAIDs on NFκB activity, cells were treated for 2 hours, and nuclear extracts were prepared. A crude assessment of cell death was made at the end of each experiment by counting the number of cells detached from the plate and comparing that to the total cell count to estimate the percentage of viable cells.

Nitrite release—After treatment with LPS for 24 hours, supernatant of the cultured RAW macrophages was removed and assayed for nitrite accumulation by use of the Griess reaction as an indication of iNOS activity.[12] Supernatant or sodium nitrite standard (0.1 to 200µM dissolved in Dulbecco modified Eagle's medium) was mixed with an equal volume of Griess reagent (4% sulphanilamide and 0.2% naphtylethylenediamine dihydrochloride in 10% phosphoric acid), and the colorimetric difference in optical density at 540 nm and 620 nm was determined immediately. A linear standard curve was plotted, and nitrite concentration in supernatant samples was obtained by comparing supernatant optical density against optical density of the standard curve. Nitrite data after the addition of each NSAID were expressed as a percentage of its respective value for control vehicle (ie, DMSO).

Electrophoretic mobility shift assay—Nuclear pellets were prepared from LPS-activated cells. Cells (3×10^6) were washed twice with ice-cold PBS solution and then pelleted by centrifugation (14,000 × *g* for 1 minute). Each pellet was resuspended in 400 µL of buffer 1 (10mM HEPES [pH, 7.9], 10mM KCl, 0.1mM EDTA, 1mM EGTA, 0.1mM dithiothreitol, 0.5mM **phenylmethanesulphonyl fluoride** [**PMSF**; dissolved in isopropanol], 10 µg of leupeptin/mL, 10 µg of pepstatin/mL, and 10 µg of aprotinin/mL) and put on ice to swell for 15 minutes. After addition of 25 µL of 10% (wt:vol) NP-40, samples were vortexed for 10 seconds and then centrifuged (14,000 × *g* for 20 seconds). Each pellet was resuspended in 50 µL of buffer 2 (20mM HEPES [pH, 7.9], 25% glycerol [wt:vol], 0.4M NaCl, 1mM EDTA, 1mM EGTA, 1mM dithiothreitol, 0.5mM PMSF, 10 µg of leupeptin/mL, 10 µg of pepstatin/mL, and 10 µg of aprotinin/mL). After being lightly vortexed, solutions were extracted on ice for 15 minutes. The extract was sonicated on ice for 30 seconds and then centrifuged (14,000 × *g* at 4°C for 15 minutes). Supernatant containing the nuclear fraction was stored at –70°C.

Oligonucleotide sequences[g] were the NFκB (ie, murine intronic k-chain κB site; 5'–AGT TGA GGG GAC TTT CCC AGC C–3') and AP1 (5'–TTC CGG CTG ACT CAT CAA GCG–3') consensus binding sites. Oligonucleotides were labeled with [γ-^{32}P]ATP by use of T4 polynucleotide kinase.[h] Electrophoretic mobility shift assays were performed in 10 µL of reaction mixture that contained 5 µg of nuclear extract, 5% glycerol, 1mM $MgCl_2$, 0.5mM EDTA, 0.5mM dithiothreitol, 50mM NaCl, 10mM Tris-HCl (pH, 7.5), 0.05 mg of poly(dI-dC).poly(dI-dC)/mL, and 0.2 ng of DNA probe. Tubes were incubated for 20 minutes at 21°C. After addition of 1 µL of gel-loading buffer (250mM Tris-HCl [pH, 7.5], 0.2% bromophenol blue, 40% glycerol), reaction products were analyzed on 4% acrylamide gels, and radioactive bands were visually examined by use of autoradiography. To evaluate for binding specificity to NFκB, 2 competitive reactions were conducted with 1.75 pmol of cold NFκB oligonucleotide or 1.75 pmol of cold AP1 oligonucleotide, respectively. Optical densities from autoradiographs from 4 separate experiments were measured and quantified by use of digital image analysis software.[i]

Statistical analysis—Nitrite release and optical density data from the electrophoretic mobility shift assays were analyzed by use of a paired Student *t*-test, with each set of data obtained for a dose of an NSAID being compared with data obtained for its appropriate paired control vehicle. Significance was designated at values of $P < 0.05$.

Results

Effects of NSAIDs on COX-1 activity—Effects of flunixin meglumine and phenylbutazone on COX-1 and -2 activities in the isolated enzyme assay were determined (**Table 1**). The effect of these drugs was compared with the effect of aspirin and the COX-2 selective inhibitor, NS398. In our assay, it was clear that flunixin meglumine and phenylbutazone were more effective at inhibiting COX-1 than COX-2. As predicted, NS398 selectively inhibited COX-2, and aspirin was a relatively nonselective inhibitor of COX.

We were unable to detect a significant inhibitory effect of carprofen on either isoform of COX in our assay.

Effects of NSAIDs on LPS-induced iNOS activity—Aspirin inhibits the activation of NfκB, and this is a key transcription factor involved in the induction of iNOS.[13-15] Inhibition of NfκB activation prevents induction of iNOS; thus, we tested the NSAIDs to determine whether they would inhibit the activation of this enzyme in RAW macrophages. As expected, only high concentrations of aspirin (1,000µM) significantly inhibited LPS-induced nitrite release (**Fig 1**). Similarly, high concentrations of carprofen (300 to 1,000µM) also significantly inhibited iNOS activation. Interestingly, flunixin meglumine significantly (P = 0.01) inhibited LPS-induced nitric oxide release at concentrations between 100 and 1,000µM. In contrast, phenylbutazone did not have inhibitory effects in this assay. At high concentrations of aspirin (1,000 to 10,000µM), approximately 5% of cells were detached and dead.

Table 1—Mean (95% confidence intervals) values for median inhibitory concentration (IC_{50}) and the ratio of cyclooxygenase (COX)-1 to COX-2 (COX-1:COX-2) for the nonsteroidal anti-inflammatory inhibitors NS398, aspirin, flunixin meglumine, and phenylbutazone as determined in an in vitro purified enzyme assay

Drug	IC_{50} COX-2 (µM)	IC_{50} COX-1 (µM)	COX-2:COX-1
NS398	131 (69–246)	3,766 (2,465–5,753)	0.03
Aspirin	100 (53–188)	92 (36–230)	1.09
Flunixin meglumine	3.24 (2.23–4.71)	0.55 (0.31–0.98)	5.84
Phenylbutazone	1,008 (724–1,404)	142 (112–179)	7.10

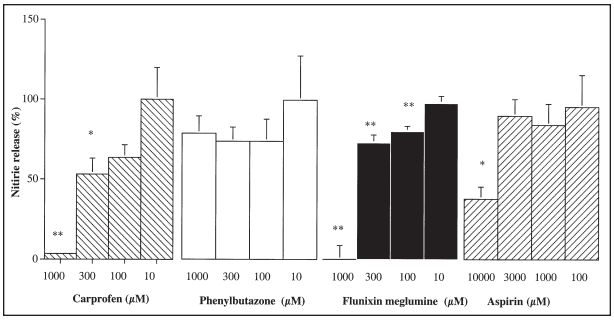

Figure 1—Effect of carprofen, phenylbutazone, flunixin meglumine, and aspirin on lipopolysaccharide (LPS)-induced activity of inducible nitric oxide synthase (iNOS) in RAW 264.7 murine macrophages. Macrophages were incubated with increasing concentrations of each nonsteroidal anti-inflammatory drug (NSAID) for 1 hour. Then, LPS (1 µg/mL) was added to the cells, and they were incubated at 37°C for 24 hours. Medium was removed, and nitrite concentrations were assayed by use of the Griess reaction. Data were analyzed by use of a paired Student *t*-test. Each value reported represents mean ± SD percentage of the value for the control vehicle (ie, dimethyl sulfoxide [DMSO]) for 4 experiments. *,**Within each NSAID, value differs significantly (*$P < 0.05$; **$P = 0.01$) from value for control vehicle.

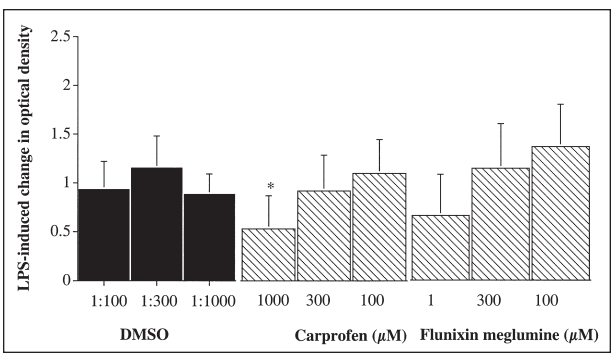

Figure 2—Effect of flunixin and carprofen on LPS-induced activation of nuclear factor kappa B (NfκB). Murine macrophages were incubated with increasing concentrations of DMSO (control vehicle), flunixin meglumine, or carprofen for 1 hour. Then, LPS (1 µg/mL) was added to the cells, and they were incubated at 37°C for 2 hours. Nuclear translocation of NfκB was determined by measuring gel retardation caused by nuclear protein binding to a radiolabeled oligonucleotide that contained an NfκB consensus binding site. Optical density of radiolabeled bands was determined, and data were analyzed by use of a paired Student *t*-test to compare values after addition of an NSAID to that of cells treated only with LPS. Values reported represent mean ± SD optical density for 4 experiments. *Within an NSAID, value differs significantly ($P < 0.05$) from value for control vehicle.

Effects of NSAIDs on NfκB activation—Because carprofen and flunixin meglumine inhibited LPS-induction of iNOS, we tested whether these NSAIDs could inhibit the activation of NFκB as determined by results of electrophoretic mobility shift assays. At high concentrations, aspirin inhibited the nuclear translocation of NFκB (data not shown). A similar effect was detected at the highest concentration of carprofen (**Fig 2**). Interestingly, flunixin meglumine, to a lesser degree, also inhibited NfκB activation. Because phenylbutazone did not have an effect on LPS-induced activation of iNOS, we did not test its effects on NFκB activation. Cell death was not detected in these experiments.

Discussion

In the study reported here, we provided evidence that carprofen and flunixin meglumine, but not phenylbutazone, inhibited activation of NFκB and induction of an NFκB-regulated gene iNOS. As expected, flunixin meglumine and phenylbutazone were potent COX inhibitors, both being relatively selective for COX-1. Although our IC_{50} values for inhibition of COX may differ from some values in the literature,[8,16] our rankings for COX-1 and -2 selectivity agree with data reported by other researchers.[8,16] Increasing amounts of evidence suggest that valid comparisons of the IC_{50} values for various COX inhibitors should be made within the same laboratory by use of the same assay system.[16] In contrast, comparisons of the ratios of COX-1 to COX-2 among laboratories and assay systems seem to be fairly consistent, and results obtained here with NS398 and aspirin compare favorably with result of other studies.[8,16] The only contentious area in this part of our study was the fact that we could not detect an inhibitory effect of carprofen on COX-1 or COX-2. It is possible that with additional optimization of our assay we may have been able to detect an effect of carprofen on COX in vitro. Analysis of data obtained in canine whole-blood assays reveals that carprofen is a potent COX-2 inhibitor (IC_{50}, 0.102µM).[6] Carprofen had limited effects on COX-2 in studies[7,8] that involved dogs and horses as models, and in a human whole-blood assay, carprofen was a potent COX-1 inhibitor.[16] These differences suggest that carprofen can inhibit COX in some assay systems and may reflect species dif-

ferences in selectivity for COX-1 and -2. Thus, the efficacy of carprofen as an inhibitor of COX-1 or -2 remains controversial, but it is likely that carprofen has most of its anti-inflammatory effects by mechanisms other than COX inhibition.

The potent anti-inflammatory therapeutic effects of carprofen in animals, in combination with minimal renal or enteric adverse effects, led us to investigate whether it may have alternative therapeutic mechanisms other than the inhibition of COX. High concentrations of several NSAIDs, including aspirin, salicylic acid, sulfasalazine, and ibuprofen, inhibit activation of the proinflammatory transcription factor NFκB.[9,15,17,18] Drugs such as aspirin and salicylate inhibit NFκB activation directly or indirectly (through activation of p38 mitogen-activated protein kinase) by preventing phosphorylation, and hence activation, of IκB kinase.[15,19] This has led us to speculate that part of the anti-inflammatory effects of carprofen could be mediated by inhibiting the activation of NFκB. First, we compared the ability of NSAIDs to prevent NFκB-dependent LPS-induction of iNOS activity. As expected, aspirin and carprofen, and more surprisingly, flunixin meglumine, significantly inhibited LPS-induction of iNOS at concentrations exceeding those required to inhibit COX. Analysis of these data suggests that carprofen and flunixin meglumine at concentrations comparable to those at which COX-independent effects of other NSAIDs have been detected may inhibit NFκB activity. Subsequently, in assays for detection of NFκB activation, we determined that carprofen and, to a lesser degree, flunixin meglumine but not phenylbutazone inhibited nuclear translocation of NFκB. In the study reported here, high concentrations of aspirin, comparable to those used in other studies,[9,15] also inhibited NFκB activation. It seems likely, therefore, that carprofen and, to a lesser degree, flunixin meglumine can inhibit the activation of NFκB, thus providing another COX-independent mechanism by which these NSAIDs can prevent or reduce the deleterious effects of endotoxin.

The high concentrations of NSAIDs required to inhibit NFκB activation make it difficult to assess the therapeutic importance of the effects of these drugs in vivo. However, because it is not easy to determine the local tissue concentrations of drugs after systemic administration, it is unclear whether the inhibition of NFκB activation will be an effect seen in vivo. There is a considerable body of evidence to support the fact that NSAIDs have anti-inflammatory effects independent of their ability to inhibit COX.[11] In addition, the COX-independent effects of NSAIDs such as salicylate or aspirin can also be mediated by NFκB-independent mechanisms, such as increasing the local concentrations of the anti-inflammatory mediator adenosine and inhibition of ribosomal S6 kinase to inhibit cAMP-dependent gene transcription.[20-22] The fact that carprofen is a relatively weak inhibitor of NfκB activation suggests that at least some of its anti-inflammatory effects may be through a NfκB-independent mechanism. Currently, we are not aware of evidence to suggest that carprofen or flunixin meglumine can affect any of these alternative anti-inflammatory mechanisms. It is also possible that these drugs may affect other as yet undetermined inflammatory mediators within cells.

Carprofen and, to a lesser degree, flunixin meglumine but not phenylbutazone inhibited the activation of NFκB. This observation could explain the reason that carprofen, with its relatively weak effects on COX activity, is an effective anti-inflammatory agent without many of the renal and enteric adverse effects associated with traditional COX inhibitors.

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[a]COX-1 (ovine), Cayman Chemical, Ann Arbor, Mich.
[b]COX-2 (ovine), Cayman Chemical, Ann Arbor, Mich.
[c]Anti-PGE2, Sigma-Aldrich Chemical Co, Dorset, UK.
[d]3H prostaglandin E_2, Amersham Biosciences UK Ltd, Little Chalfont, UK.
[e]Optiphase HiSafe II, PerkinElmer Life Sciences Ltd, Cambridge, UK.
[f]GraphPad Prism, version 3.0a for Macintosh, GraphPad Software, San Diego, Calif.
[g]5'–AGT TGA GGG GAC TTT CCC AGC C–3' and 5'–TTC CGG CTG ACT CAT CAA GCG–3', Promega Corp, Madison, Wis.
[h]T4 polynucleotide kinase, Promega Corp, Madison, Wis.
[i]Kodak digital science ID image analysis software, Kodak Ltd, Hemel Hempstead, UK.

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