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Enhanced anti-inflammatory potency of a nitric oxide-releasing prednisolone derivative in the rat

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1 Derivatization of nonsteroidal anti-inflammatory drugs, such that they release nitric oxide (NO) in small amounts, has been shown to significantly increase their anti-inflammatory activity and analgesic potency. In this study, we compared the anti-inflammatory potency of prednisolone to a nitric oxide-releasing derivative of prednisolone (NCX-1015).

2 Carrageenan-induced inflammation of an airpouch in the rat was used. The rats were pretreated with equimolar doses of prednisolone or NCX-1015 and the effects on leukocyte infiltration into the airpouch and exudates levels of prostaglandin E_2 (PGE_2), leukotriene B_4 (LTB_4) and nitrite (as an index of NO production) were measured 6 h later.

3 Injection of carrageenan into the airpouch resulted in a progressive increase in leukocyte infiltration, and accumulation of PGE_2, LTB_4 and nitrite. Carrageenan also induced elevated expression of cyclooxygenase-1 and -2, inducible nitric oxide synthase and 5-lipoxygenase in the inflammatory exudate.

4 Prednisolone dose dependently reduced the numbers of leukocytes within the airpouch exudates, as well as reducing PGE_2, LTB_4 and nitrite levels. NCX-1015 also reduced leukocyte numbers and inflammatory mediator levels. However, the doses of NCX-1015 required to produce a maximal reduction of each of these parameters was one-third to one-tenth the dose of prednisolone that produced a comparable effect.

5 The reduction of PGE_2 and NO production was likely to be at least in part due to reduced expression of the key enzymes responsible for their synthesis (cyclooxygenase-2, inducible NO synthase), with NCX-1015 producing greater suppression than prednisolone at an equimolar dose.

6 Coadministration of prednisolone with a nitric oxide donor (DETA-NONOate) resulted in a greater reduction of leukocyte infiltration and inflammatory mediator production than was observed with either drug alone.

7 These results support the notion that delivery of NO to a site of inflammation can markedly enhance the anti-inflammatory activity of a glucocorticoid.
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Keywords: Nitric oxide; glucocorticoid; inflammation; prostaglandin; leukotriene; leukocyte; cyclooxygenase

Abbreviations: COX, cyclooxygenase; DETA, diethylenetriamine; DMSO, dimethylsulfoxide; iNOS, inducible nitric oxide synthase; 5-LO, 5-lipoxygenase; LT, leukotriene; NO, nitric oxide; NSAID, nonsteroidal anti-inflammatory drug; PG, prostaglandin

Introduction

Glucocorticoids are the most effective treatment for a variety of allergic, inflammatory, autoimmune and rheumatic diseases. Glucocorticoids inhibit the expression of genes for a large number of chemokines, cytokines, adhesion molecules and arachidonic acid metabolites (Buckingham *et al*., 1994). While being very potent anti-inflammatory and immunomodulatory drugs, glucocorticoids can produce many adverse effects, including osteoporosis, hypertension and hyperglycemia. Such adverse effects can greatly limit the dose of a glucocorticoid that can be used and the duration of therapy.

Nonsteroidal anti-inflammatory drugs (NSAIDs) represent another class of anti-inflammatory drugs that are greatly limited in their utility by significant adverse effects. Most notably, NSAIDs cause ulceration and bleeding in the gastrointestinal tract (Wallace, 1997). NSAID-induced gastrointestinal toxicity is directly related to the ability of these drugs to inhibit the enzyme cyclooxygenase (COX), which is the rate-limiting step in the synthesis of prostaglandins (Vane, 1971). As prostaglandins (PG) and nitric oxide (NO) both play an important role in gastric mucosal defense (Elliott & Wallace, 1998), the coupling of an NO-releasing moiety to NSAIDs (NO-NSAIDs) was proposed as a novel approach to decrease the adverse effects of NSAIDs (Wallace *et al*., 1994; Wallace *et al*., 1995). NO-NSAIDs have been shown not only to have greatly reduced toxicity relative to conventional NSAIDs, but also to exhibit increased potency as analgesics and to exhibit a

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broader range of anti-inflammatory effects (Wallace *et al*., 1994; Davies *et al*., 1997; Fiorucci *et al*., 2000). This raised the possibility that other anti-inflammatory drugs might be modified in the same manner in order to reduce toxicity or to increase efficacy and potency. Indeed, an NO-releasing derivative of paracetamol has been shown to be considerably more potent than the parent drug in terms of both analgesic and anti-inflammatory activity (Al-Swayeh *et al*., 2000). An NO-releasing derivative of prednisolone (NCX-1015) has been shown to be more potent than the parent drug in reducing neutrophil extravasation, expression of inducible NO synthase (iNOS) and cytokine and chemokine production in a mouse model of acute inflammation (Paul-Clark *et al*., 2000). In addition, NCX-1015 suppressed the clinical signs of collagen-induced arthritis in rats to a greater degree than prednisolone, with milder side effects on bone (Paul-Clark *et al*., 2002), and showed enhanced activity over prednisolone in a mouse model of colitis (Fiorucci *et al*., 2002a).

In the present study, we have further explored the possibility that an NO-releasing prednisolone derivative would exert more potent anti-inflammatory effects than prednisolone itself, using the carrageenan-airpouch model in the rat. We have also explored the role of NO in augmenting the anti-inflammatory potency of prednisolone.

Methods

Animals

Male, Wistar rats (175–200 g) were obtained from Charles River Breeding Farms (Montreal, Canada). Rats were housed in the Animal Care Service of the University of Calgary and were fed standard laboratory chow and water *ad libitum*. In all experiments described herein, the minimum sample size per group was five. All experimental procedures were approved by the Animal Care Committee of the University of Calgary and were in accordance with the guidelines of the Canadian Council on Animal Care.

Carrageenan-airpouch model

An airpouch was produced in each rat by the subcutaneous injection of 20 ml of air on the back of the rats on the first day (Edwards *et al*., 1981; Wallace *et al*., 1999). On the third day, an additional 10 ml of air was injected into the airpouch. At 5 days after the first injection, another 10 ml of air was injected at the same site. On the sixth day, 2 ml of either saline or a 1% $w\,v^{-1}$ solution of carrageenan was injected into the pouch. All injections were performed under 5% ($v\,v^{-1}$) halothane anesthesia. At 1 h before the carrageenan injection, one of the test drugs (prednisolone or NCX-1015) at doses ranging from 0.8 to 28 μmol kg^{-1}, or the vehicle (dimethylsulfoxide, DMSO), was injected directly into the airpouch (1 ml kg^{-1}). The direct injection of the test drugs into the airpouch was selected as the route of administration in order to eliminate the possibility of different bioavailability within the airpouch if delivered via another route, which would confound direct comparisons of drug potency. Samples were collected at 1, 2, 4 and 6 h after the carrageenan injection. Rats were euthanized with an overdose of sodium pentobarbital (MTC Pharmaceuticals, Cambridge, ON, Canada). A small incision was performed on the airpouch and samples of the exudate were collected into sterile tubes. The tissue was frozen immediately on dry ice and kept at -80°C until used for Western blot analysis.

The volume of the exudate was measured. An aliquot of the exudate was used to quantify the number of leukocytes using a Coulter Particle Counter (Coulter Electronics, Beds., U.K.). Differential cell counts were also performed by light microscopy, using Wright's stain for characterization of the leukocytes. Finally, the rest of the exudate was centrifuged at $1000 \times g$ for 10 min. Cell pellets were frozen on dry ice and kept at -80°C for Western blot analysis. The supernatants were also collected and stored at -80°C for subsequent measurement of prostaglandin E_2 (PGE_2), leukotriene B_4 (LTB_4) and nitrite concentrations. PGE_2 and LTB_4 concentrations were determined using specific ELISAs, while nitrite concentrations were determined using the Griess reaction.

Pilot studies were performed to determine if the vehicle in which the test drugs were administered (DMSO) would significantly affect the inflammatory response to carrageenan administration. Groups of five rats each were treated with 1 ml kg^{-1} of DMSO or 0.9% saline 1 h prior to carrageenan administration (as described above). Samples of the exudate were collected 6 h later. There were no significant differences between these two groups in terms of the numbers of infiltrating leukocytes or the production of inflammatory mediators.

A series of experiments were performed to determine if administration of an NO donor together with prednisolone would produce anti-inflammatory effects in the carrageenan-airpouch model greater than would be achieved with either drug alone. Rats were treated with either diethylenetriamine NONOate (DETA-NONOate) or prednisolone at doses of 1.4 and 2.8 μmol kg^{-1}, respectively, or with a combination of both drugs. DETA-NONOate spontaneously releases NO in aqueous solution (Keefer *et al*., 1996). The dose of DETA-NONOate was selected such that it would have an equal number of NO-releasing moieties, on a molar basis, to that of a 2.8 μmol kg^{-1} dose of NCX-1015. As in the other studies, the vehicle was DMSO (1 ml kg^{-1}).

Nitrite measurements

NO production was quantified using the Griess reaction (Gilliam *et al*., 1993). Briefly, nitrate in the supernatant was reduced to nitrite by incubation with 50 μl of 1 U ml^{-1} nitrate reductase and 35 μl of 110 μM β-NADPH at room temperature for 30 min. A volume of 100 μl of 1% sulfanilamide in 5% H_3PO_4 and 100 μl of 0.1% *N*-(1-naphtyl)-ethylenediamine dihydrochloride were incubated with 100 μl of the reduced sample for 10 min at room temperature, and the absorbance was measured at 540 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.). Nitrite concentrations were calculated by comparison with standard solutions of sodium dinitrite (1–100 μM), prepared in phosphate-buffered saline (0.01 M, pH 7.4).

In an attempt to determine the amount of NO released by NCX-1015 and DETA-NONOate in the absence of inflammation (where the generation of high amounts of NO confounds detection of NO released from these drugs), these drugs were administered without subsequent administration of carrageenan. NCX-1015 was tested at a dose of 28 μmol kg^{-1}, while

DETA-NONOate was administered at a dose of 1.4 μmol kg^{-1}. After 7 h, fluid was collected from the airpouch for more measurement of nitrate/nitrite concentrations as described above.

Western blot analysis

Samples were homogenized in 1 ml of lysis buffer containing a cocktail of protease inhibitors (0.1% Triton X-100, 500 mM NaCl, 50 mM HEPES, 0.1 mg ml^{-1} leupeptin and 10 mg ml^{-1} phenylmethyl sulfonyl fluoride) and centrifuged at 10 000 $\times$ *g* for 5 min at 4°C. Protein concentrations of the supernatants were determined with a protein assay kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Samples (50 μg protein per lane) and prestained SDS – polyacrylamide gel (PAGE) molecular weight control markers (Bio-Rad Laboratories, Hercules, CA, U.S.A.) were resolved by gel electrophoresis on 10% SDS – PAGE and transferred electrophoretically to a nitrocellulose membrane (Pall Corporation, Ann Arbor, MI, U.S.A.). After the transfer, the membrane was blocked for an hour in 5% milk in Tris-buffered saline (20 mM Tris, 100 mM NaCl) with 0.05% Tween 20 and then incubated overnight with a primary antibody. Finally, the primary antibody was detected by a horseradish peroxidase-conjugated IgG and visualized using a Hyperfilm ECL (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, U.K.). The X-ray film was analyzed with a GS-710 calibrated imaging densitometer and Quantity One software (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Western blotting studies were performed for endothelial NOS (eNOS), iNOS, COX-1, COX-2 and 5-lipoxygenase (5-LO) at 1, 2, 4 and 6 h after carrageenan administration. Samples of both the cell infiltrate and the pouch lining were analysed.

Materials

DMSO was obtained from Merck (Darmstadt, Germany). NCX-1015 (prednisolone 21-[(4′-nitrooxymethyl) benzoate]) was synthesized by NicOx S.A. (Sophia Antipolis, France). Prednisolone and lambda carrageenan were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). DETA NONOate, the ELISA kit for LTB_4, the antibody to 5-LO (1 : 2000; rabbit-anti-human polyclonal) and the COX-2 antibody (1 : 200; rabbit-anti-human polyclonal) were obtained from Cayman Chemical (Ann Arbor, MI, U.S.A.). The ELISA kit for PGE_2 was obtained from Neogen (Medicorp, PQ, Canada). The COX-1 antibody (1 : 200; rabbit-anti-human polyclonal) and eNOS antibody (1 : 200; rabbit-anti-human polyclonal) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The iNOS antibody (1 : 10,000; rabbit-anti-mouse polyclonal) was obtained from BD Pharmingen (Mississauga, ON, Canada). The secondary antibody (1 : 10,000; donkey-anti-rabbit IgG, horseradish peroxidase-conjugated) was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, U.S.A.). Lambda carrageenan was obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.).

Statistical analysis

Data are expressed as the mean $\pm$ s.e.m. Comparisons among groups were made using a one-way analysis of variance followed by the Student – Newman – Keuls test. A *P*-value of less than 5% was considered as significant.

Results

Pilot studies were performed to establish the time course of peak increases in leukocyte infiltration and inflammatory mediator content of the airpouch exudates. Leukocyte infiltration into the airpouch was evident within the first hour after carrageenan administration, and gradually increased throughout the 6 h experiment. Differential staining indicated that the majority of the cells were neutrophils (>90%) at all time points. Carrageenan administration also resulted in a progressive increase in nitrate/nitrite, LTB_4 and PGE_2 levels in the airpouch. Thus, the studies of prednisolone and NCX-1015 were performed at the 6 h post-carrageenan time point.

The increase in nitrate/nitrite accumulation was likely due to increased production via iNOS, as the expression of this

Table 1 Time course of expression of 5-lipoxygenase, cyclooxygenase and nitric oxide synthase following injection of carrageenan into the airpouch

Time (h):	*0*	*1*	*2*	*4*	*6*
Cellular infiltrate					
COX-1	13.8 $\pm$ 4.3	13.3 $\pm$ 3.5	19.4 $\pm$ 4.9	45.9 $\pm$ 6.7*	75.9 $\pm$ 6.4*
COX-2	ND	ND	ND	2.2 $\pm$ 0.9	24.6 $\pm$ 7.2
eNOS	ND	ND	ND	ND	ND
iNOS	ND	ND	ND	9.5 $\pm$ 4.5	43.6 $\pm$ 11.2
5-LO	ND	ND	ND	17.5 $\pm$ 5.2	20.7 $\pm$ 3.9
Airpouch lining					
COX-1	4.9 $\pm$ 1.0	3.6 $\pm$ 1.3	4.1 $\pm$ 2.5	2.8 $\pm$ 0.9	3.3 $\pm$ 0.7
COX-2	ND	ND	1.0 $\pm$ 0.3	12.3 $\pm$ 1.4	7.9 $\pm$ 2.9
eNOS	7.2 $\pm$ 2.5	7.2 $\pm$ 1.9	6.6 $\pm$ 2.3	7.0 $\pm$ 2.7	7.1 $\pm$ 2.2
iNOS	3.9 $\pm$ 1.0	2.6 $\pm$ 0.5	3.4 $\pm$ 1.4	3.1 $\pm$ 1.6	4.8 $\pm$ 0.3
5-LO	4.3 $\pm$ 0.4	3.2 $\pm$ 1.0	3.2 $\pm$ 1.1	3.1 $\pm$ 2.3	6.0 $\pm$ 1.8

All data are presented in arbitrary densitometry units (mean $\pm$ s.e.m. of five to six rats per group). Statistical analysis was performed only in those cases where there was detectable enzyme in the vehicle-treated (time zero) group (*$P<0.05$ *versus* the vehicle-treated group). ND = not detectable.

isozyme was substantially increased in the infiltrate 4–6 h after carrageenan administration (Table 1), while there was no detectable eNOS expression. Expression of both COX-1 and COX-2 was markedly increased in the infiltrating cells at 6 h post-carrageenan, but in the lining of the pouch only COX-2 was upregulated. Previously, studies utilizing selective COX inhibitors in this model demonstrated that COX-2 is the principal source of PGE_2 generation in response to carrageenan administration (Wallace *et al.*, 1999). There was also marked induction of 5-LO in the infiltrating cells in response to carrageenan injection, but no change in the airpouch lining (Table 1).

Effects of prednisolone and NCX-1015

Prednisolone dose dependently reduced leukocyte infiltration into the airpouch in response to injection of carrageenan, with the 8.3 and 28 μmol kg^{-1} doses producing significant reductions relative to the vehicle-treated group (Figure 1). NCX-1015 also produced reductions in leukocyte infiltration, but this drug was more potent than prednisolone. Thus, a dose of NCX-1015 of 2.8 μmol kg^{-1} produced a significant reduction of leukocyte infiltration, and the 8.3 μmol kg^{-1} dose produced a reduction comparable to that achieved with the 28 μmol kg^{-1} dose of prednisolone (Figure 1).

A difference in the potency of prednisolone and NCX-1015 was also observed with respect to effects on accumulation of nitrate/nitrite, PGE_2 and LTB_4 in the airpouch. Exudate levels of nitrate/nitrite levels 6 h after carrageenan administration were significantly reduced by NCX-1015 at doses $\geqslant$2.8 μmol kg^{-1}, whereas prednisolone was only effective at doses $\geqslant$8.3 μmol kg^{-1} (Figure 1). NCX-1015 at a dose of 2.8 μmol kg^{-1} produced a similar reduction in nitrate/nitrite levels as was seen with prednisolone at a dose of 28 μmol kg^{-1}. In an attempt to determine the contribution of NCX-1015 to the nitrate/nitrite that was recovered from the exudate, we determined the concentration of nitrate/nitrite in the airpouch of rats given NCX-1015 (28 μmol kg^{-1}) but not given carrageenan. At 7 h after administering NCX-1015 into the airpouch, the concentration of nitrate/nitrite in the airpouch was 1.3 ± 0.2 μM ($n = 5$). In the experiments in which rats were treated with vehicle and carrageenan, nitrate/nitrite concentrations in the airpouch reached $\sim$750 μM by 6 h postcarrageenan administration. Thus, the contribution of NCX-1015 to the total nitrate/nitrite generated in the airpouch following carrageenan administration was relatively small.

To test the possibility that differential effects of prednisolone and NCX-1015 on inflammatory mediator production might be related to suppression of expression of the key enzymes for production of those mediators, we performed Western blotting analyses of samples taken from rats treated with the two test drugs at a dose of 2.8 μmol kg^{-1}. As shown in Figure 2 and in Table 2, both drugs reduced the expression of COX-1, COX-2 and iNOS, but a greater effect was seen with NCX-1015 than with prednisolone. Neither of the drugs, at the dose tested, affected expression of 5-LO.

Co-administration of prednisolone and an NO donor

As in the studies described above, prednisolone at a dose of 2.8 mol kg^{-1} did not significantly affect carrageenan-induced leukocyte infiltration into the airpouch (Figure 3). Treatment

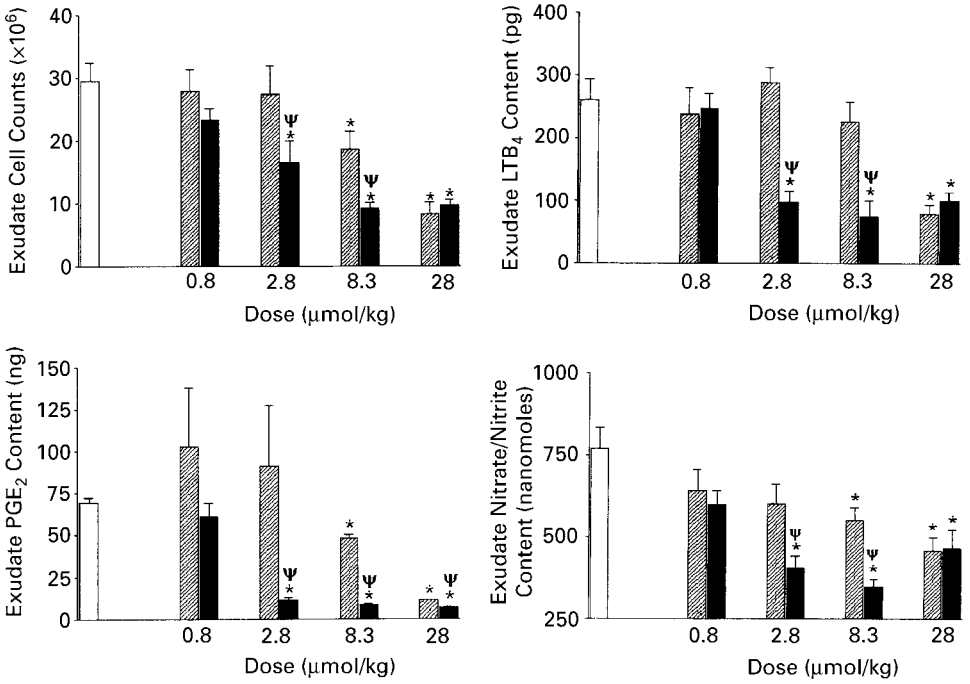

Figure 1 Effects of predisolone (hatched bars) and NCX-1015 (solid bars) on inflammation induced by injection of carrageenan into an airpouch in rats. The groups treated only with saline prior to carrageenan administration are represented by the open bars. The groups treated with the vehicle (DMSO) are represented by the stippled bars. The top panels show the effects of the test drugs on number of infiltrating leukocytes (left) and the exudate LTB_4 content (right). The bottom panels show the effects of the test drugs on exudate levels of PGE_2 (left) and nitrate/nitrite (right). Each bar represents the mean $\pm$s.e.m. of six to eight rats. *$P > 0.05$ *versus* the vehicle-treated group. $^{\Psi}P < 0.01$ *versus* the equivalent dose of prednisolone.

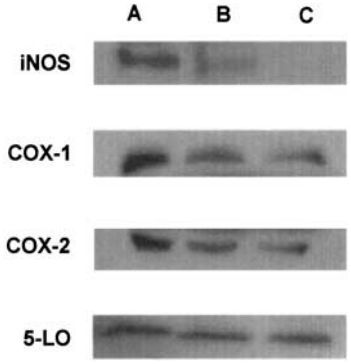

Figure 2 The effects of drug treatment on iNOS (130 kDa), COX-1 (70 kDa), COX-2 (72 kDa) and 5-LO (78 kDa) expression in the cellular infiltrate, 6 h after carrageenan administration. Lanes A–C show representative Western blots of cellular infiltrates recovered from rats treated with vehicle, prednisolone (2.8 μmol kg^{-1}) and NCX-1015 (2.8 μmol kg^{-1}), respectively. Each lane was loaded with 35 μg of protein.

Table 2 Effects of prednisolone and NCX-1015 on cyclooxygenase, nitric oxide synthase and 5-lipoxygenase expression in cellular infiltrates

	Vehicle	*Prednisolone*	*NCX-1015*
COX-1	88.3±7.4	40.6±4.5*	33.8±3.9*
COX-2	27.9±5.8	16.1±3.1	9.6±2.4*
iNOS	38.2±4.2	19.8±4.1*	3.3±1.1*,**
5-LO	25.8±5.6	22.5±4.8	30.4±4.0

Western blotting was performed on samples collected 6 h after carrageenan administration into the airpouch. The rats were treated 1 h before carrageenan administration with vehicle (DMSO), prednisolone (2.8 μmol kg^{-1}) or NCX-1015 (2.8 μmol kg^{-1}). Each lane was loaded with 35 μg of protein. Results are expressed (in arbitrary densitometry units) as the mean ±s.e.m. of four rats per group. *$P<0.05$ *versus* the corresponding vehicle-treated group. **$P<0.05$ *versus* the corresponding prednisolone-treated group.

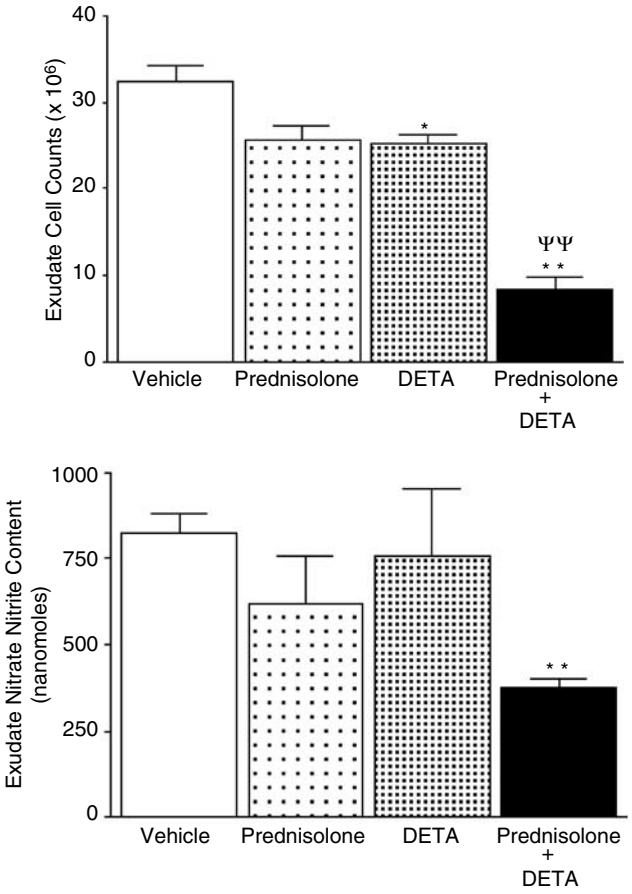

Figure 3 Effects of prednisolone (2.8 μmol kg^{-1}) or DETA-NONOate (1.4 μmol kg^{-1}) on carrageenan-induced leukocyte infiltration (top panel) and nitrate/nitrate accumulation (bottom panel). The test drugs were given alone or in combination. *$P<0.05$, **$P<0.01$ *versus* the vehicle-treated group. Each bar represents the mean ±s.e.m. of five rats. $^{\Psi\Psi}P<0.01$ *versus* the groups treated with prednisolone or DETA-NONOate alone.

with DETA-NONOate reduced leukocyte infiltration into the airpouch by approximately 20% ($P<0.05$). The combination of prednisolone and DETA-NONOate, on the other hand, reduced carrageenan-induced leukocyte infiltration by >70% ($P<0.01$ *versus* prednisolone alone or DETA-NONOate alone) (Figure 3).

The combination of prednisolone and DETA-NONOate also produced greater reductions in exudate levels of nitrate/nitrite than produced by either drug alone (Figure 3). While the combination of drugs reduced nitrate/nitrite levels by ~60%, neither drug alone exerted significant effects. Generation of nitrate/nitrite from DETA-NONOate (in the absence of stimulation with carrageenan) was negligible (concentrations of 1.4±0.2 μM in the airpouch fluid; $n=5$) relative to the levels of nitrate/nitrite generated in response to carrageenan (~750 μM). Thus, such release did not significantly mask any inhibitory effects on nitrate/nitrite production.

Discussion

The results of this study in rats confirm previous observations of a marked increase in anti-inflammatory potency of an NO-releasing glucocorticoid in comparison to the native glucocorticoid in mice (Paul-Clark *et al*, 2000). Irrespective of which index of inflammation was examined (leukocyte infiltration, PGE_2, LTB_4 or nitrate/nitrite), there was a three- to 10-fold greater potency of NCX-1015 *versus* prednisolone. These differences cannot be attributed to differential absorption or penetration into the airpouch, as we administered the test drugs directly into the lumen of the pouch. It should be noted, however, that NCX-1015 does exhibit significant anti-inflammatory activity, and increased potency compared to prednisolone, when administered orally or intraperitoneally in mice (Paul-Clark *et al*., 2000). That a marked increase in anti-inflammatory effect could also be seen by co-administration of prednisolone and an NO donor (DETA-NONOate) strongly suggests that NO release from NCX-1015 was responsible for the increase in anti-inflammatory potency. NO release from NCX-1015 has previously been demonstrated (Paul-Clark *et al*., 2000).

The enhancement of activity of a glucocorticoid through incorporation of an NO-releasing moiety is consistent with similar enhancement of activity of other types of drugs. NSAIDs, acetaminophen and ursodeoxycholic acid have all been shown to exhibit enhanced activities and reduced toxicity

relative to the parent drugs (Davies *et al*., 1997; Al-Swayeh *et al*., 2000; Fiorucci *et al*., 2001). Whether or not the mechanism underlying the enhancement of activity and reduced toxicity is the same for all of these types of derivatives is not clear, but certainly it would appear likely that NO mediates a substantial component of the improvements in therapeutic activity.

There are several possible mechanisms through which NO could enhance the anti-inflammatory effects of a glucocorticoid. NO itself has potent inhibitory effects on leukocyte adherence to the vascular endothelium (Gaboury *et al*., 1993) and can suppress the release of inflammatory mediators from mast cells (Hogaboam *et al*., 1993), lymphocytes (Fiorucci *et al*., 2002a) and platelets (Radomski & Moncada, 1993). Thus, reduced production of inflammatory mediators in the airpouch could be in part due to these inhibitory effects of NO on leukocyte recruitment and/or inflammatory mediator release. These are rapid-onset effects, as opposed to the slower-onset effects of glucocorticoids that require ongoing protein synthesis. The Western blot studies suggest that NCX-1015 also has enhanced activity in terms of suppressing expression of some of the key enzymes in producing prostaglandins and NO (COX-2 and iNOS, respectively), but did not significantly affect the expression of one of the key enzymes in LTB_4 synthesis, 5-LO. Thus, at least in the case of LTB_4, NCX-1015 must have produced inhibitory effects on synthesis through other mechanisms than just suppression of expression of 5-LO. NO has been shown to interfere with 5-LO activity by interfering with substrate availability and by post-translational modification of the enzyme, such as through *S*-nitrosylation (Coffey *et al*., 2001; 2002). We can not exclude the possibility that NCX-1015 modified the expression or activity of another enzyme in the LTB_4 biosynthetic or degradative pathways.

It is also possible that the delivery of NO together with a glucocorticoid results in enhanced activation of the glucocorticoid receptor. Paul-Clark *et al*. (2000) reported that NCX-1015 exhibited increased potency in inducing CD163 expression on human mononuclear cells. CD163 expression is an index of glucocorticoid receptor activation (Morganelli & Guyre, 1988; Hogger *et al*., 1998). They also showed increased potency of NCX-1015 *versus* prednisolone in suppressing endotoxin-induced IL-1β production from human peripheral blood mononuclear cells (Paul-Clark *et al*., 2000). These results are consistent with the notion that there is enhanced activation of the glucocorticoid receptor with NCX-1015. However, it is also possible that the enhanced inhibition of IL-1β production by NCX-1015 was related to inactivation of caspase-1 (also known as interleukin-1 converting enzyme), as has been demonstrated to occur in rat splenocytes (Fiorucci *et al*., 2000).

NFκB activation is a critical step in the induction of expression of several proinflammatory enzymes, including COX-2 and iNOS. NO can decrease NFκB activation through inhibition of IκB phosphorylation and degradation (Katsuyama *et al*., 1998). NO can also block NF-κB binding to DNA (Parks *et al*., 1996). It is possible, therefore, that the enhanced potency of NCX-1015, including the suppression of iNOS and COX-2 induction, could be related to effects on NFκB activation. The ability of NO-releasing derivatives of aspirin and other NSAIDs to suppress expression of COX-2 and iNOS has been observed previously, but the mechanism of action was not delineated (Cirino *et al*., 1996; Fiorucci *et al*., 2002b).

The present study was focused on examining potential differences in anti-inflammatory potency between NCX-1015 and prednisolone. An increase in anti-inflammatory potency, if not accompanied by a parallel increase in adverse effects, could mean that lower, and therefore safer, doses of the drug could be employed. It is noteworthy that NCX-1015 has been shown to exhibit reduced adverse effects on bone than are seen with prednisolone. Unlike prednisolone, NCX-1015 did not elevate the bone-resorbing activity of rat primary osteoblasts. Moreover, collagen-induced arthritis in rats was associated with elevated levels of serum pyridinoline, consistent with bone and cartilage erosion. This was prevented by treatment with NCX-1015, but not prednisolone (Paul-Clark *et al*., 2002).

In summary, the NO-releasing prednisolone derivative, NCX-1015, exhibits enhanced anti-inflammatory potency in a rat model of acute inflammation relative to preqdnisolone. NCX-1015 was three to 10 times more potent in suppressing leukocyte adherence and inflammatory mediator production. NCX-1015 may offer an attractive alternative to older glucocorticoids for the treatment of inflammatory conditions.

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