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SPECIAL REPORT

Effect of a new non-steroidal anti-inflammatory drug, nitroflurbiprofen, on the expression of inducible nitric oxide synthase in rat neutrophils

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The effects of a non-steroidal anti-inflammatory drug, flurbiprofen, and its nitro-derivative, nitroflurbiprofen, on inducible nitric oxide synthase in rat neutrophils were examined. Nitroflurbiprofen was shown to inhibit nitric oxide synthase induction caused by lipopolysaccharide administration, while flurbiprofen had no effect on nitric oxide synthase induction. This inhibitory action may be ascribed to nitric oxide released from nitroflurbiprofen.

Keywords: Nitroflurbiprofen; nitric oxide; nitric oxide synthase; neutrophil

Introduction Inflammation is a pathological event to which nitric oxide (NO), released by NO synthase (NOS) induced in activated macrophages, neutrophils and hepatocytes at the injured site, seems to contribute. Some steroid hormones such as dexamethasone were reported to be inhibitory agents of the expression of the inducible form of NOS (Boughton-Smith *et al.*, 1993): this seems to account in part for the anti-inflammatory effects of dexamethasone.

Non-steroidal anti-inflammatory drugs are widely used in the treatment of inflammatory conditions, but gastrointestinal lesions have often limited their clinical utilization (Carson & Strom, 1992). Flurbiprofen (FP) is a well-established anti-inflammatory agent having a potent pharmacological action mostly due to its ability to inhibit cyclo-oxygenase activity, causing a concomitant deficiency of cellular prostacyclin content and tissue damage. Nitroflurbiprofen (NFP) is a new anti-inflammatory drug obtained by the incorporation of a nitroxybutyl moiety through an ester linkage to the carboxylic group of FP; previous experiments have shown its anti-inflammatory efficacy and good gastrointestinal tolerability (Wallace *et al.*, 1994).

In the present work, we investigated the possible effects of the two non-steroidal anti-inflammatory drugs on NOS induction in the neutrophils of lipopolysaccharide (LPS)-treated rats.

Methods *Animal treatment* Female Sprague-Dawley rats (Charles River, Italy), weighing 220-240 g, were treated with FP or NFP (10 mg kg^{-1} body weight) suspended in carboxymethylcellulose 0.5% and administered orally in a volume of 1 ml 100 g^{-1} body weight; control rats received an equal volume of the vehicle. In rats co-treated with LPS (5 mg kg^{-1} body weight), it was given via the tail vein 1 h after the drug administration. Four hours later, the rats were killed by injection of sodium pentobarbitone (60 mg kg^{-1} body weight) i.p.

Neutrophil preparation Neutrophils were separated by Ficoll-Paque density gradient centrifugation as described previously (Mariotto *et al.*, 1995).

Quantitation of nitrite/nitrate in the plasma Nitrite/nitrate concentrations in the plasma were measured according to a modification of a method described previously (Bartholomew, 1984): nitrate reductase prepared from *E. coli* ATCC 25922 (Difco) was used to convert nitrate to nitrite. Nitrite was quantitated colorimetrically after reaction with the Griess reagent (Green *et al.*, 1982).

Assay of NOS activity in the neutrophils NOS activity was estimated by measurement of the conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline as described by Bredt & Snyder (1990) with a slight modification.

Chemicals L-[2,3,4,5-^{3}H]-arginine monohydrochloride (specific activity: 60 Ci $mmol^{-1}$; 1 Ci = 27 GBq) was from Amersham Life Science. (6**R**)-5,6,7,8-tetrahydro-l-biopterin was from Dr B Schircks Laboratories (Jona, Switzerland). Nitroflurbiprofen was synthesized and kindly supplied by Pharmaceutical Discovery Service (Milano, Italy).

Statistical analysis Statistical analysis of the data was performed by one-way analysis of variance followed by Student's *t* test. $P < 0.05$ or less was considered as indicative of a significant difference.

Results and Discussion NOS activity in the neutrophils of control rats was undetectable under our assay conditions. Neither FP nor NFP had any effects on the induction of NOS activity in the neutrophils. On the other hand, NFP treatment caused a 3 fold increase in nitrite/nitrate plasma concentrations compared to the basal value: this indicates an exogenous NO release from NFP and confirms recent data on the elevation of plasma nitrite levels *in vivo* following NFP administration (Wallace *et al.*, 1994). After LPS-treatment, NOS activity became detectable, with a 8 fold increase in nitrite/nitrate plasma levels. When FP was co-administered, NOS activity did not change significantly, while the plasma nitrite/nitrate level halved compared to LPS-treated rats. Since FP alone did not change the basal plasma nitrite/nitrate concentration and had no effects on the induction of neutrophil NOS, the decreased nitrite/nitrate levels observed in rats co-treated with LPS and FP could be partially due to the cyclo-oxygenases inhibition with production of free radicals reacting with NO and/or to an inhibition of NOS present in other tissues (Table 1).

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Table 1 Plasma NO_2^-/NO_3^- levels and neutrophil NOS activity

Group	*Plasma NO_2^-/NO_3^- (μM)*	*NOS activity (pmol/10^6 cells)*
Controls	32.80 ± 18.03	0
Flurbiprofen	36.08 ± 15.67	0
Nitroflurbiprofen	83.20 ± 26.25(*)	0
LPS	230.00 ± 88.36(**)	10.25 ± 6.28
LPS + flurbiprofen	121.20 ± 41.25(•)	11.94 ± 6.28
LPS + nitroflurbiprofen	252.80 ± 74.59	6.13 ± 4.97

The values are expressed as mean ± s.d. of 5–7 experiments for each treatment. Student's *t* test: nitroflurbiprofen vs controls, $*P<0.01$; LPS vs controls, $**P<0.001$; LPS + flurbiprofen vs LPS, $•P<0.05$.

NFP and LPS co-administration caused a marked decrease (40%) in neutrophil NOS activity, while no change in nitrite/nitrate plasma levels was observed; these data raise the question of the inhibitory effect of NFP on iNOS activity.

Since NFP is hydrolyzed *in vivo* to FP and NO as reported recently (Wallace *et al.*, 1994) and as indicated also in this work, and FP alone does not exert any inhibitory action on neutrophil NOS induction, it seems reasonable to postulate that the inhibitory action of NFP should be ascribed to NO released from NFP. Our recent report on a possible inhibition of neutrophil NOS induction by exogenous NO derived from sodium nitroprusside (Mariotto *et al.*, 1995) seems to be consistent with the present data. Furthermore, similar results were reported recently on an inhibitory action of NFP on iNOS in J774 cells (Cirino *et al.*, 1994). Recent work has pointed out a possible feedback inhibition of the induction of iNOS expression by endogenously produced NO (Park *et al.*, 1994); our data do not exclude this possibility.

Another aspect should be considered concerning the inhibition of neutrophil NOS by NFP. Recently Assreuy *et al.* (1993) demonstrated a direct inhibition of iNOS by NO; on the other hand, Cirino *et al.* (1994) reported that NFP did not inhibit J774 iNOS activity. This does not exclude a possible direct inhibition of neutrophil NOS activity by NFP during the course of experiments.

The present data indicate the possibility that exogenous NO derived from NFP, as endogenous NO (Park *et al.*, 1994), exerts an inhibitory action on iNOS expression, probably at the transcriptional level. The nature of exogenous NO remains to be elucidated.

According to the above description, plasma nitrite/nitrate levels in rats receiving both NFP and LPS should result from the balance between the following two distinct phenomena: (1) increase in plasma nitrite/nitrate concentration after oxidation of NO released from NFP and (2) decrease in nitrite/nitrate concentrations caused by inhibition of NOS induction due to NO released from NFP.

In conclusion, a novel non-steroidal anti-inflammatory drug, NFP, seems to act as an inhibitory agent on the induction of NOS expression in neutrophils, while its analogue, FP, does not.

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