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RESEARCH PAPER

Effect of a nitric oxide releasing derivative of paracetamol in a rat model of endotoxaemia

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Background and purpose: Nitroparacetamol is a nitric oxide-releasing paracetamol with novel anti-inflammatory properties compared to the parent compound. This study has investigated the anti-inflammatory activity of nitroparacetamol in a model of endotoxaemia in rats to probe the mechanisms underlying this effect.
Experimental approach: Nitroparacetamol (92 mg kg^{-1}), paracetamol (50 mg kg^{-1}) or vehicle were administered to male, Wistar rats 15 min prior to or 3 h after lipopolysaccharide (0.5 mg kg^{-1}, serotype 0127:B8). Mean arterial pressure and heart rate were measured for 5 h and plasma and organs were then obtained to determine organ dysfunction, inducible nitric oxide synthase and cyclooxygenase-2 expression (lung, liver and kidney tissue) and plasma nitrate/nitrite. In separate experiments, nitroparacetamol, paracetamol or vehicle was administered 1 h before acetylcholine (0.1 μg kg^{-1}) or sodium nitroprusside (0.25 μg kg^{-1}) to determine if nitroparacetamol desensitizes responses to exogenous/endogenous nitric oxide.
Key results: Nitroparacetamol prevented but did not reverse the lipopolysaccharide-induced hypotension. There was no effect on heart rate or plasma markers of organ dysfunction. Nitroparacetamol prevented the increased plasma nitrate/nitrite and expression of COX-2 and iNOS, whereas paracetamol exerted partial inhibition of COX-2 in lung alone. Nitroparacetamol also reduced responses to acetylcholine and sodium nitroprusside.
Conclusions and implications: NO is the active component of nitroparacetamol in this model of endotoxaemia. Pro-inflammatory processes targeted by nitroparacetamol have been shown to include iNOS/COX-2 induction and possibly vascular soluble guanylyl cyclase. Precise mechanisms underlying the NO effect are unclear but inhbition of cytokine formation may be important.
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Keywords: nitroparacetamol; paracetamol; lipopolysaccharide; sodium nitroprusside; acetylcholine; cyclooxygenase-2; inducible nitric oxide synthase

Abbreviations: AST, aspartate aminotransferase; COX-2, cyclooxygenase-2; iNOS, inducuble nitric oxide synthase; LPS, lipopolysaccharide; MAP, mean arterial blood pressure; NO, nitric oxide; NOPARA, nitroparacetamol; PARA, paracetamol; SNP, sodium nitroprusside; TNF-α, tumour necrosis factor-alpha

Introduction

Nitroparacetamol (NOPARA) is a nitric oxide (NO)-releasing derivative of paracetamol (PARA) that has an enhanced pharmacological profile compared to the parent compound. It is chemically related to a novel family of NO-releasing adduct drugs, for example the nitro-nonsteroidal anti-inflammatory drugs and nitroprednisolone (for review, see Moore and Marshall, 2003). NOPARA is a more effective analgesic agent compared to PARA, as shown in the acetic acid-induced abdominal constriction model in the mouse (Al-Swayeh *et al*., 2000) and by its effect on spinal 'wind-up' following the injection of hyperalgesic agents in the rat hindpaw (Romero-Sandoval *et al*., 2002). Moreover, we have shown in our laboratory that NOPARA, but not PARA, is anti-inflammatory in the carrageenan-induced hindpaw oedema model in the rat (Al-Swayeh *et al*., 2000). We consider NOPARA to be of particular interest as an anti-inflammatory drug considering it does not have the gastrointestinal and cardiovascular side effects of conventional NSAIDs (for review, see Moore and Marshall, 2003). Furthermore, NOPARA does not cause the hepatic damage associated with PARA (Futter *et al*., 2001: Fiorucci *et al*., 2002; for review see Wallace, 2004). We have therefore extended our investigation of the anti-inflammatory effects of NOPARA into models of endotoxaemia.

Endotoxaemia and sepsis involve a major systemic inflammatory response, frequently associated with hypotension, hypoperfusion and subsequent tissue injury and organ

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failure. The activation of monocytes/macrophages and neutrophils, with the consecutive release of pro-inflammatory mediators (e.g. cytokines) and activation of the coagulation cascade play a major role in the pathogenesis of endotoxaemia (for review see Jacobi, 2002). Thus, we consider this to be a particulary relevant disease process for an investigation of the anti-inflammatory effect of NOPARA. We have previously investigated the effect of NOPARA in an *in vitro* model of endotoxaemia where we have demonstrated an inhibition of lipopolysaccharide (LPS)-induced cytokine production in human whole blood by NOPARA but not PARA (Marshall and Moore, 2004). We further showed that the NO moiety of NOPARA was responsible for the anti-inflammatory activity by reversing the effect with an NO scavenger, 2-4-(carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO). In contrast, 3-2-hydroxy-1-(1-methylethyl)-2-nitrosohydrazino-1-propanamine (NOC)-5 (a slow-releasing NO donor) was unable to mimic the effect of NOPARA (Marshall and Moore, 2004), indicating that the particularly slow release of NO from NOPARA is essential for its anti-inflammatory properties (for review see Keeble and Moore, 2002).

We have now extended our studies into an *in vivo* model of LPS-induced endotoxaemia in the rat where we have determined the effect of NOPARA and PARA as regards hypotension, organ dysfunction and induction of inducuble nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). Both the preventative (pretreatment) and therapeutic (post-treatment) effect of NOPARA have been investigated to give further insight into the anti-inflammatory activity of NOPARA. Overall, the present study demonstrates an anti-inflammatory effect of NO-PARA in endotoxaemia that depends on the time of administration and which has a variety of underlying mechanisms.

Methods

Animals

Male, Wistar rats (200–260 g, Charles River Ltd, UK) were housed in the biological services unit of King's College London and allowed food and water *ad libitum* throughout this period. All procedures were in accordance with the UK Home Office Scientific Procedures Act (1986).

Surgical procedure

Male, Wistar rats (Harlan, UK; 250–300 g) were anaesthetized with either urethane (1.75 g kg^{-1}, intraperitaneously (i.p.)) or sodium pentobarbitone (60 mg kg^{-1}, i.p.) and placed on a heating blanket to maintain body temperature. Level of anaesthesia was assessed by the paw pinch and corneal reflex tests and anaesthetic administered as necessary. The trachea was cannulated to facilitate respiration. The jugular vein and carotid artery were also cannulated for the administration of drugs and measurement of blood pressure, respectively. The carotid artery cannula was connected via a transducer to a BP amplifier (AD Instruments, Oxford, UK) to a Powerlab (AD Instruments, UK) which, in turn, was connected to a Dell Inspiron 4000 computer. Mean arterial blood pressure (MAP), mm Hg and heart rate were routinely recorded for 10–15 min before the administration of drugs in order to establish stable baseline readings and thereafter for the remainder of the experiment.

Effect of NOPARA or PARA on consequences of LPS injection

Initial experiments were performed in sodium-pentobarbitone-anaesthetized rats to determine if NOPARA (92 mg kg^{-1}), an equimolar dose of PARA 50 mg kg^{-1}, i.p.) or vehicle (0.5 w v^{-1} carboxymethylcellulose (CMC) containing 5% v v^{-1} dimethylsulphoxide (DMSO), 2 ml kg^{-1}, i.p.) would affect MAP and heart rate (HR) in control animals over a 1 h period. Subsequently, NOPARA (92 mg kg^{-1}, i.p.), PARA (50 mg kg^{-1}, i.p.) or vehicle (0.5 w v^{-1} CMC containing 5% v v^{-1} DMSO, 2 ml kg^{-1}, i.p.) were administered to urethane-anaesthetized rats 15 min before a bolus injection of *Escherichia coli* LPS (0.5 mg kg^{-1}, 0.5 ml, i.v., serotype 0127: B8) or vehicle (saline, 0.5 ml, i.v.). In separate experiments, the NOPARA (92 mg kg^{-1}) or PARA (50 mg kg^{-1}, i.p.) were administered 3 h after LPS injection. In all cases, MAP and HR were continuously recorded for a total period of 5 h after LPS injection. At the end of the experiment, blood (3 ml, heparinized 100 units ml^{-1}) was collected via the carotid artery cannula. The lung, liver and kidneys were immediately removed and snap frozen in liquid nitrogen for subsequent assay of iNOS/COX-2 expression, as described below.

Measurement of organ failure

Plasma was separated from blood by centrifugation (5 min, 10 000 *g*, MSE Centaur, Sanyo Ltd, UK) and analysed by a contract veterinary laboratory (VETLAB Services Horsham, West Sussex, UK) for aspartate aminotransferase (AST) and creatinine as markers of liver and kidney failure, respectively.

Preparation of organ homogenates

Lung, liver and kidney removed from animals injected with LPS-treated animals were removed from storage at −70 °C, placed in liquid nitrogen and ground to a fine powder using a pestle and mortar before being transferred to centrifuge tubes. Lysis buffer (1.5 w/v; composition; 10% glycerol, 2% sodiumdodecyl sulphate (SDS), 76.5 mM Tris pH 6.8) was then added and samples mixed thoroughly. Samples were then centrifuged (5000 *g*, 5 min, MSE Microcentaur, Sanyo Ltd) and sonicated (3 × 15 s Ultrasons, LAG-Plant Ltd, UK) to break down any remaining solid material. Samples were then centrifuged (5000 *g* for 10 min, MSE Microcentaur, Sanyo Ltd) and supernatant removed and incubated on ice for 5 min. A protein assay (bicinchoninic acid assay kit, Sigma Chemical Co., UK) was then performed to determine the protein content of each of the lysates. For Western blot assays, bromophenol blue (2 μl of 2% solution) and β-mercaptoethanol (5 μl) were added to 100 μl aliquots of each sample before heating (95 °C 5 min) and centrifugation (5000 *g*, 5 min, MSE Microcentaur, Sanyo Ltd).

Western blot analysis of iNOS and COX-2

A resolving gel (6.96 ml ultra pure water, 30% acrylamide/bisacrylamide mixture, 10% SDS, 10% ammonium persulphate in 1.5 M Tris-HCl, pH 8.8) was set in a 3 mm gap between glass plates and polymerized with tetramethylethylenediamine (TEMED, 0.007 ml to 15 ml resolving gel solution). The top of the gel was evened out by the addition of butan-1-ol, saturated with H_2O, which was then removed and the gel left to set for 20 min at room temperature (RT). After which time the stacking gel (2.44 ml ultra pure water, 30% acrylamide/bisacrylamide mixture, 10% SDS, 10% ammonium persulphate in 0.5 M Tris-HCl, pH 6.8, and polymerized with 0.004 ml TEMED) was added on top of the resolving gel and left to set (45 min at room temp) with a plastic comb set into the gel to provide the wells for the addition of sample.

Protein matched samples (20 μg of protein per well) were then loaded onto the gel and subjected to electrophoresis (Protean II, Bio-Rad Laboratories Ltd, UK) at 200 V for 45 min. The resolved proteins were then transferred onto PVDF membranes at 140 mA for 2 h using a transfer buffer (39 mM glycine, 48 mM Tris-base, 0.0375 SDS, 20% methanol).

After transfer was complete, blots were washed in buffer (10 mM Tris-base, 100 mM NaCl, 0.1% Tween 20) and incubated at RT for 1 h in blocking buffer (5% milk, 10 mM Tris-base, 100 mM NaCl, 0.1% Tween 20). Membranes were then washed with buffer tris-buffered saline containing 0.5% Tween-20 (TBST) (10 mM Tris-base, 100 mM NaCl, 0.1% Tween 20), before incubation (RT, 1 h) with primary goat anti-mouse antibody (0.25 mg/ml stock in 50% glycerol, 20 mM sodium phosphate pH 7.5, 150 mM NaCl, 1.5 ml NaN_3, 1 mg/ml BSA, diluted 1:2500 in blocking buffer). Membranes were then washed with buffer (10 mM Tris-base, 100 mM NaCl, 0.1% and Tween 20) for 30 min with the wash buffer changed every 5 min. The enzyme conjugate, anti-mouse immunoglobulin (Ig)G:horseradish peroxidase (HRP) was then diluted 1:10 000 in blocking buffer and incubated with membranes for 1 h at RT. After the residual anti-serum was removed with a 3×10 min washes with TBST, immunoreactive bands were detected using enhanced chemiluminescence (ECL plus kit, Amersham, UK).

ELISA analysis of iNOS and COX-2

The levels of iNOS and COX-2 in organ homogenate were quantified by enzyme-linked immunosorbent assay (ELISA), using polyclonal anti-human iNOS and COX-2 antibodies (Biogenesis, Poole, UK).

Organ homogenates were diluted in sodium bicarbonate coating buffer (0.05 M, pH 9.6; Sigma, Dorset, UK), and 50 μl added to wells of a 96-well Nunc Maxisorb ELISA plate (Life Technologies, Paisley, UK). After incubation for 24 h at 4 °C, the plates were washed three times with phosphate-buffered saline (PBS) containing 0.05% v/v Tween-20 (PBST). The wells were then blocked with 2% w v^{-1} ELISA-grade bovine serum albumin (BSA) in PBS for 1 h at RT. After washing with PBST, 50 μl of polyclonal anti-iNOS or anti-COX-2 antibody was added, and plates incubated for 1 h at RT. After washing, anti-rabbit IgG-conjugated HRP 100 μl diluted 1/200 was added for 1 h at RT, and colour was developed by the addition of freshly prepared *O*-phenylenediamine 100 μl (Sigma, Dorset, UK). The absorbance was thereafter measured at 490 nm (Anthus Labtec spectrophotometric plate reader, model Ht 111). Human recombinant iNOS and COX-2 (Biogenesis, Poole, UK) were used as standard, and the sensitivity of the assay was 0.3 ng/ml. Levels of iNOS and COX-2 were normalized to the total protein concentration.

Measurement of plasma nitrite

Plasma nitrate plus nitrite (NO_x) concentration was assayed by the Griess reaction (Green *et al*., 1982). Aliquots (80 μl) were incubated (30 min, 37°C) with nitrate reductase (10 mU) and nicotinamide adenine dinucleotide phosphohydrogenase (100 μM) to convert all nitrate to nitrite. Griess reagent (100 μl; 5% v/v H_3PO_4 containing 1% w/v sulphanilic acid and 0.1% w/v *N*-1-napthylethylenediamine) was then added and samples incubated for a further 15 min (37°C). The absorbance at 550 nm was measured (Anthus Labtec spectrophotometric plate reader, model Ht 111). The concentration of NO_x in each sample was determined by comparison with a standard curve of sodium nitrite ($NaNO_2$).

Effect of NOPARA and PARA pretreatment on vasodepressor responses to SNP and ACh

In preliminary experiments, sodium pentobarbitone-anaesthetized animals were injected with increasing doses of either sodium nitroprusside (SNP) (2.5–10 μg kg^{-1}, 0.5 ml kg^{-1}, i.v.) or acetylcholine (ACh) (0.01–1.0 μg kg^{-1}, 0.5 ml kg^{-1}, i.v.) at 5 min intervals and MAP and HR determined before, and at the peak of each resulting response. Thereafter, approximate ED_{70} doses (SNP; 2.5 μg kg^{-1} and ACh; 0.25 μg kg^{-1}) were chosen. In separate experiments, animals were injected with NOPARA (92 mg kg^{-1}, i.p), PARA (50 mg kg^{-1}, i.p.) or the equivalent volume of vehicle (0.5 w v^{-1} CMC containing 5% v v^{-1} DMSO, 2 ml kg^{-1}, i.p.). MAP and HR were monitored for a further 60 min, at which point either SNP (2.5 μg kg^{-1}, i.v.) or ACh (0.25 μg kg^{-1}, i.v.) was administered.

Statistical analysis

Results are given as means ± s.e.m. Statistical significance between groups was performed using analysis of variance (ANOVA) followed by Dunnett's test. In all cases, a probability (*P*)-value of 0.05 or less was taken to denote statistical significance.

Results

Effect of NOPARA and PARA on LPS-induced hypotension

Treatment of animals with LPS (0.5 mg kg^{-1}, i.v.) resulted in a time-dependent, profound fall in MAP (Figure 1) which was associated with an increase in HR in these animals (Figure 2). Changes in MAP and HR were significant within 3 h of LPS administration (Figures 1 and 2). Pretreatment of animals with NOPARA, but not PARA, significantly attenuated the

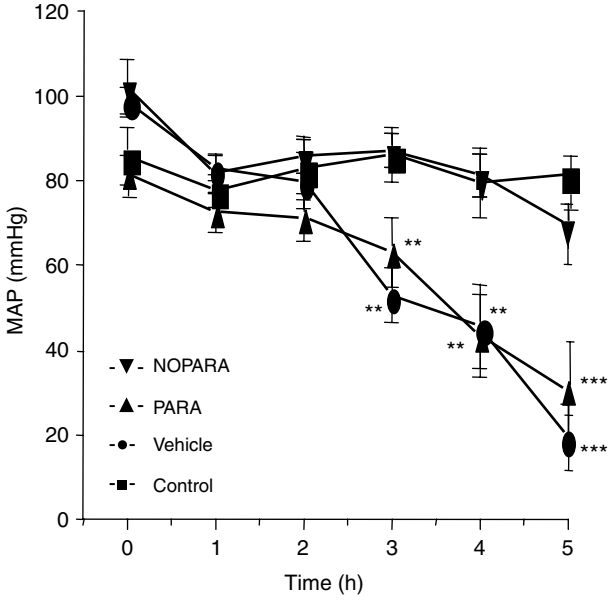

Figure 1 Effect of pretreatment (5 min before LPS) with NOPARA, PARA or vehicle on MAP in the LPS-injected rat. The MAP of control animals that were administered saline and drug vehicle is also shown. Results show MAP in mm Hg and are mean ± s.e.m., $n = 5$–12, $^{*}P < 0.01$, $^{**}P < 0.005$, c.f. LPS and drug vehicle control at corresponding times (ANOVA plus *post hoc* student's *t*-test).

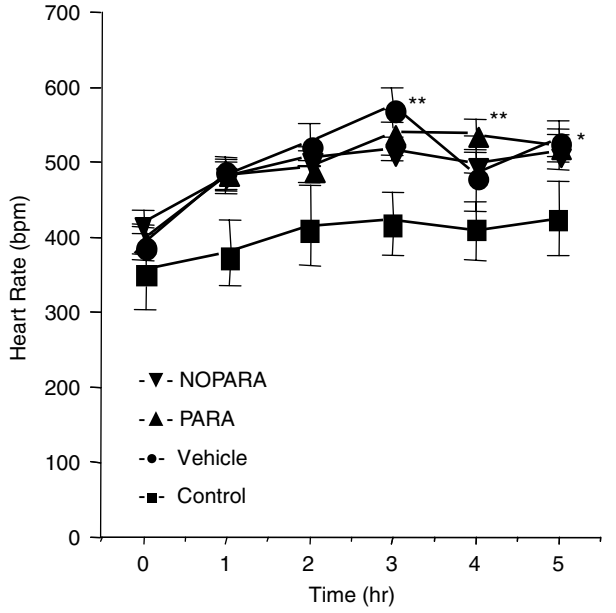

Figure 2 Effect of pretreatment (5 min before LPS) with NOPARA, PARA or vehicle on HR in the LPS-injected rat. The HR of control animals that were administered saline and drug vehicle is also shown. Results show HR in b.p.m and are mean ± s.e.m., $n = 5$–12, $^{*}P < 0.01$, $^{**}P < 0.005$, c.f. LPS and drug vehicle control at corresponding times (ANOVA plus *post hoc* student's *t*-test).

LPS-induced fall in MAP (Figure 1). However, when NOPARA or PARA were administered 3 h after LPS administration, they did not affect the existing LPS-induced fall in MAP (Figure 3), that is, no reversal of MAP was apparent at any time point up

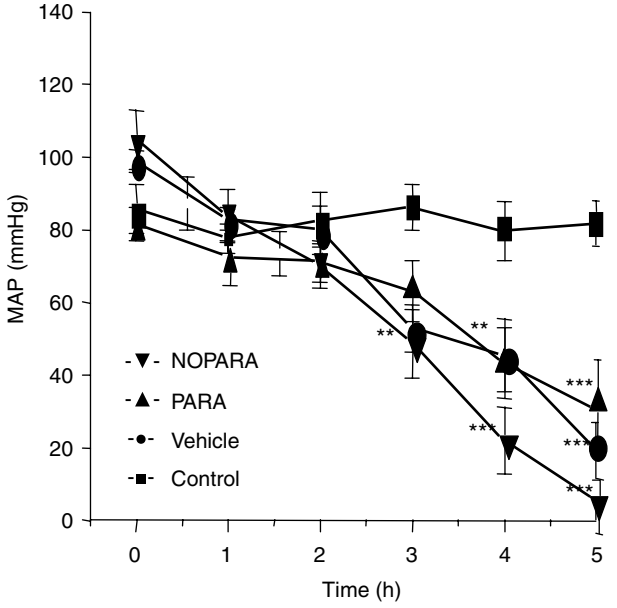

Figure 3 Effect of post-treatment (3 h after LPS) with NOPARA, PARA or vehicle on MAP in the LPS-injected rat. The MAP of control animals that were administered saline and drug vehicle is also shown. Results show MAP in mm Hg in mean ± s.e.m., $n = 5$–12, $^{**}P < 0.005$, $^{***}P < 0.001$, c.f. LPS and drug vehicle control at corresponding time points (ANOVA plus *post hoc* student's *t*-test).

Table 1 The effect of NOPARA, PARA and vehicle on MAP and HR 1 h after administration in control rats

Drug treatment	*MAP (mm Hg)*		*HR (b.p.m.)*	
	T = *0*	T = *1 h*	T = *0*	T = *1 h*
Vehicle	112 ± 6	107 ± 12	408 ± 8	388 ± 14
NOPARA	110 ± 16	112 ± 15	423 ± 29	406 ± 10
PARA	108 ± 16	96 ± 10	419 ± 17	404 ± 18

Abbreviations: b.p.m; beats per minute; HR, heart rate; MAP, mean arterial blood pressure; NOPARA, nitroparacetamol; PARA, paracetamol; T, time.
Results are mean ± s.e.m., $n = 5$. No significant differences ($P > 0.05$) were noted (ANOVA plus *post hoc* Dunnett's test).

to 5 h. In contrast to MAP, pretreatment with NOPARA or PARA did not significantly affect the LPS-mediated rise in HR (Figure 2). Furthermore, neither NOPARA nor PARA significantly altered MAP or HR in control animals, not receiving LPS (Table 1).

Effect of NOPARA and PARA on organ failure

Plasma obtained from control, vehicle-treated rats (5 h) exhibited AST and creatinine levels all within the normal range (Table 2). Administration of LPS resulted in a significant increase in both plasma AST enzyme activity and creatinine concentration. These data suggest significant liver and kidney damage in these animals over this time period. Neither pretreatment with NOPARA nor PARA affected the LPS-induced rise in plasma markers of organ damage in these animals.

Table 2 The effect of NOPARA and PARA on plasma markers of organ dysfunction in rats 5 h after injection of LPS

Drug treatment	*AST (units ml^{-1})*	*Creatinine (μM)*
Vehicle	258.8±34.0	61.25±5.9
LPS	542.9±101.3[a]	197.1±12.7[a]
LPS + NOPARA	718.0±89.2[a]	106.6±16.6[a]
LPS + PARA	415.3±161.8[a]	129.0±19.0[a]

Abbreviations: Ast, aspartate aminotransferase; LPS, lipopolysaccharide; NOPARA, nitroparacetamol; PARA, paracetamol.
Results show the AST activity or creatinine concentration in plasma and are mean±s.e.m., $n=8$. Injection of LPS raised both plasma AST and plasma creatinine but pretreatment with NOPARA or PARA did not affect the LPS-induced increase in these plasma markers.
[a]Significantly different from values after vehicle ($P>0.05$; ANOVA plus *post hoc* Dunnett's test).

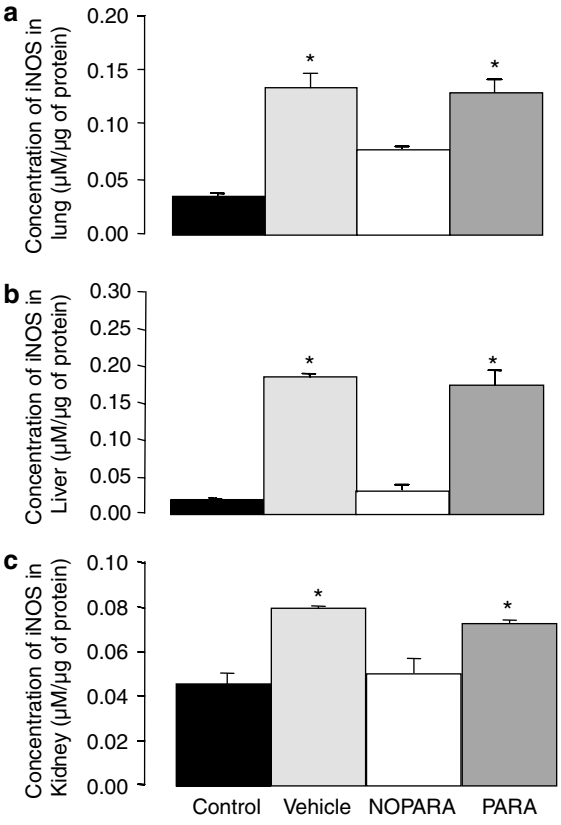

Figure 4 Effect of pretreatment with NOPARA, PARA and vehicle on levels of iNOS in (**a**) lung, (**b**) liver and (**c**) kidney removed from LPS-injected rats at 5 h. The level of iNOS found in animals that received saline and drug vehicle is also presented. Results are concentration (μM) and are mean±s.e.m., $n=4$, *$P<0.01$, **$P<0.005$ (ANOVA plus *post hoc* Dunnett's test compared with saline control).

Effect of NOPARA and PARA on organ expression of iNOS and COX-2

Treatment of rats with LPS resulted in significant increases in iNOS expression in lung, liver and kidney (Figure 4). COX-2 expression increased in lung and liver, but not in kidney (Figure 5). Pretreatment of rats with NOPARA, but not PARA, significantly reduced iNOS and COX-2 expression in all affected organs from LPS-treated rats (Figures 4 and 5).

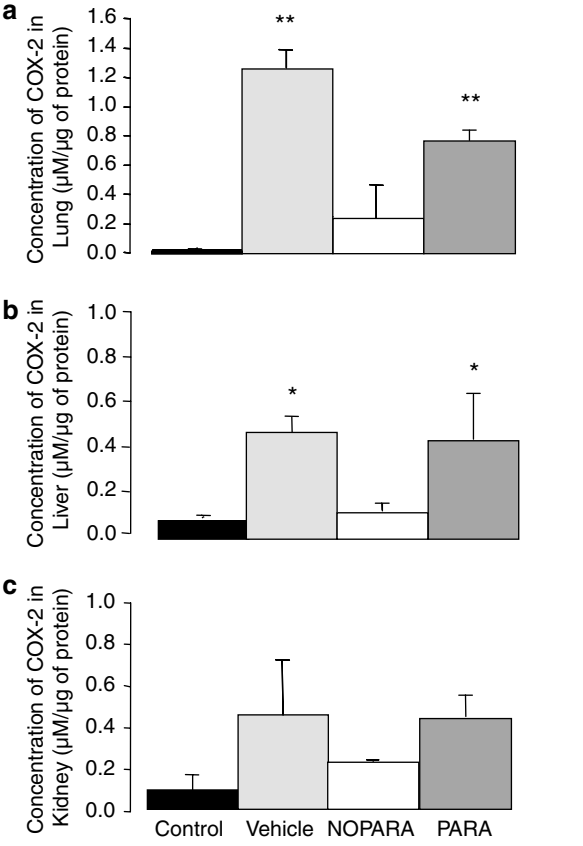

Figure 5 Effect of pretreatment with NOPARA, PARA and vehicle on levels of COX-2 in (**a**) lung, (**b**) liver and (**c**) kidney removed from LPS-injected rats at 5 h. The level of COX-2 found in animals that received saline and drug vehicle is also presented. Results are concentration (μM) and are mean±s.e.m., $n=4$, **$P<0.005$, ***$P<0.001$ (ANOVA plus *post hoc* Dunnett's test compared with saline control).

Interestingly, PARA did partially reverse the increased COX-2 expression in lung following LPS administration (Figure 5).

Effect of NOPARA and PARA on plasma levels of NO_x

LPS caused a significant increase in NO_x levels that was prevented by pretreatment with NOPARA, but not PARA (Figure 6). To account for NO released from NOPARA itself, the mean concentration of plasma NO_x in rats pretreated with NOPARA but not LPS, was subtracted from the value obtained for animals treated with LPS and NOPARA.

Effect of NOPARA and PARA on vascular responsiveness to SNP and ACh in vivo

Approximate EC_{70} doses of SNP and ACh elicited decreases in MAP that were rapid in onset and transient with MAP returning to basal ('pre-injection') values within approximately 5 min (SNP) or 1 min (ACh). In animals pretreated with NOPARA before SNP or ACh administration, a significant reduction in the vasodepressor effect of both SNP and ACh was observed compared to responses in vehicle-

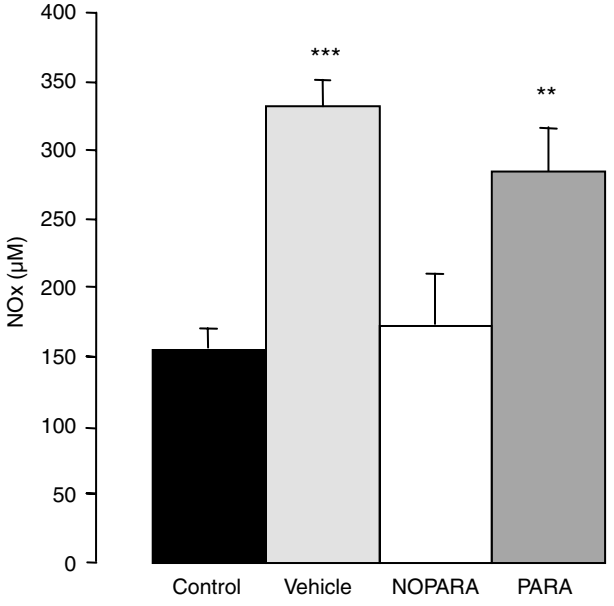

Figure 6 Effect of pretreatment with NOPARA, PARA and vehicle on plasma levels of NO_x in the LPS-injected, rat at 5 h. The plasma NO_x concentration of control animals that were administered saline plus drug vehicle is also shown. Results show NO_x in μM and are mean $\pm$ s.e.m., $n=6$, $^*P<0.01$ (ANOVA plus *post hoc* student's *t*-test compared with saline controls).

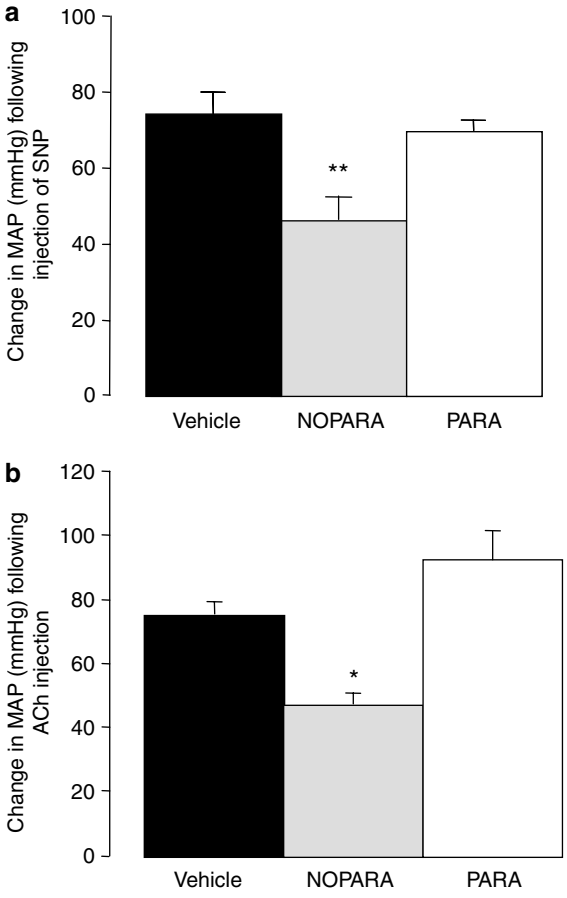

Figure 7 Effect of pretreatment (1 h before SNP or ACh) of rats with NOPARA, PARA, or vehicle on the (**a**) SNP and (**b**) ACh-induced decrease in MAP. Results show decrease in MAP in mm Hg and are mean $\pm$ s.e.m., $n=7$–8, $^*P<0.01$, $^{**}P<0.005$ (ANOVA plus *post hoc* student's *t*-test compared with vehicle-treated animals).

pretreated rats (Figure 7). PARA had no significant effect on responses to ACh or SNP (Figure 7).

Discussion

The present study was carried out in order to investigate the anti-inflammatory properties of NOPARA in a systemic model of inflammation. We report here, for the first time, that pretreatment with NOPARA, but not PARA, had a protective effect in a rat model of endotoxaemia. NOPARA attenuated LPS-induced hypotension, iNOS/COX-2 induction and increased plasma nitrite. However, it had no effect on the LPS-induced increase in HR or multiple organ dysfunction. Furthermore, if given 3 h after LPS injection, the protective effect of NOPARA was lost. These findings give us interesting information regarding the anti-inflammatory mechanism of NOPARA.

Firstly, the present study confirmed previous work showing the NO moiety of NOPARA is responsible for its anti-inflammatory effect. PARA failed to affect any of the parameters of endotoxaemia measured and it may thereby be deduced that the NO moiety was the active component. Furthermore, the results were confirmation that PARA lacks anti-inflammatory activity.

Secondly, the paradox that NOPARA prevented the LPS-induced decrease in MAP without affecting HR is suggestive that the beneficial effect of the drug is, at least in part, vascular in origin, that is, that the decrease in total peripheral resistance is prevented without a concomitant effect on cardiac output. Increased iNOS synthesis is considered to be a major contributor to the hypotension observed in endotoxaemia, causing decreased peripheral resistance owing to NO-induced vasodilation. NOPARA has been shown in the present study to decrease iNOS induction and, furthermore, to prevent the increase in the level of NO_x in the plasma of LPS-treated rats (NO_x concentration being a measure of NO production).

We also investigated the possibility that exposure of vascular smooth muscle cells *in vivo* to the relatively low concentrations of NO released from NOPARA over a sustained period could reduce the sensitivity of the vasculature to NO. Other authors have previously shown that exposure of vascular smooth muscle cells in culture to SNP causes soluble guanylyl cyclase (sGC) to become tolerant to activation by NO-releasing drugs (Papapetropoulos *et al.*, 1996). In the present experiments, we observed that approximate ED_{70} doses of both SNP (NO donor) and ACh (releaser of endogenous endothelium-derived NO) elicited significantly less vasodepressor activity when animals were pretreated with NOPARA. In contrast, vascular responsiveness to both agents was unaltered in animals administered an equimolar dose of PARA. Based on these studies, it may be suggested that the slow delivery into the bloodstream of low concentrations of NO from NOPARA is sufficient to reduce

vascular responsiveness to both exogenous and endogenous NO.

The finding that pretreatment, but not post-treatment (3 h after LPS) with NOPARA is protective against LPS-induced hypotension gives us futher information regarding the mechanism of NO. We did not observe a significant decrease in MAP and increase in HR until 3 h after LPS administration. Although these pathophysiological parameters are not apparent until 3 h there is a significant inflammatory response that precedes it, for example activation of monocytes/macrophages and neutrophils, release of pro-inflammatory mediators (e.g. cytokines) and activation of the coagulation cascade (for review, see Jacobi, 2002). We have previously shown *in vitro* that NOPARA inhibits the LPS-induced synthesis of tumour necrosis factor-alpha(TNF)-α and interleukin-1beta in human whole blood (Marshall and Moore, 2004). These cytokines are key mediators of iNOS and COX-2 induction (Maier *et al*., 1994; Akarasereenont *et al*., 1995). Treatment with NOPARA 3 h after LPS administration may have failed to have targeted this critical stage in the inflammatory response, possibly contributing to the lack of effect of NOPARA in these conditions.

An alternative reason for the failure of NOPARA to reverse the endotoxaemia concerns the pharmacokinetics of NO release from this compound. NOPARA and related NO-releasing adduct drugs are particularly interesting NO donors because their administration to animals (Aldini *et al*., 2002) or man (Zacharowski *et al*., 2004) results in esteratic cleavage leading to a slow release of the NO and parent molecule such that their concentrations peak at 2–4 h after administration. The time course of LPS-induced endotoxaemia in rats is fairly rapid compared to the situation in humans and administration of NOPARA 3 h after LPS may not have allowed sufficient levels of the cleaved drug to accumulate before the end of the 5 h time course. The particular kinetics of NO release from NOPARA is critical for its anti-inflammatory effect as its inhibitory effect on cytokine production in LPS-treated human whole blood cannot be mimicked by other slow-releasing NO donors (Marshall and Moore, 2004).

To conclude, endotoxaemia and sepsis in humans have an extremely high mortality rate with patients often not receiving treatment until the latter stages of the disease. With this in mind, the anti-inflammatory mechanism of NOPARA may not appropriate to the treatment of this disease unless treatment can be given in the early stages. Furthermore, NOPARA had no effect of organ dysfunction, which is a critical symptom of endotoxaemia. Nevertheless, the present study has given an interesting insight into the novel anti-inflammatory activity of NOPARA. Firstly, the NO moiety of NOPARA appears to be the active component in this model of endotoxaemia. Furthermore, pro-inflammatory processes targeted by NO-PARA have been shown to include induction of iNOS and COX-2, in addition to a possible effect on vascular sGC. The precise mechanisms underlying the NO effect are unclear but our previous studies suggest that inhibition of cytokine formation (particularly TNF-α) may be an important component (Marshall and Moore, 2004).

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Conflict of interest

The authors state no conflict of interest.

References

Akarasereenont P, Bakhle YS, Thiemermann C, Vane JR (1995). Cytokine-mediated induction of cyclo-oxygenase-2 by activation of tyrosine kinase in bovine endothelial cells stimulated by bacterial lipopolysaccharide. *Br J Pharmacol* **115**: 401–408.

Aldini G, Carini M, Orioli M, Facino RM, Wenk GL (2002). Metabolic profile of NO-flurbiprofen (HCT1026) in rat brain and plasma: a LC-MS study. *Life Sci* **71**: 1487–1500.

Al-Swayeh OA, Futter LE, Clifford RH, Moore PK (2000). Nitroparacetamol exhibits anti-inflammatory and anti-nociceptive activity. *Br J Pharmacol* **130**: 1453–1456.

Fiorucci S, Antonelli E, Mencarelli A, Palazzetti B, Alvarez-Miller L, Muscara M *et al*. (2002). A NO-releasing derivative of acetaminophen spares the liver by acting at several checkpoints in the Fas pathway. *Br J Pharmacol* **135**: 589–599.

Futter L.E., Al Swayeh O.A., Moore P.K. (2001). A comparison of the effect of nitroparacetamol on liver injury. *Br J Pharmacol* **132**: 10–12.

Green L.C., Wagner D.A., Glogowski J., Skipper P.L., Wishnok J.S., Tannenbaum S.R. (1982). Analysis of nitrate, nitrite and [15N]nitrate in biological fluids. *Anal Biochem* **126**: 131–138.

Jacobi J. (2002). Pathophysiology of sepsis. *Am J Health Syst Pharm* **59**: S3–S8.

Keeble JE, Moore PK (2002). Pharmacology and potential therapeutic applications of nitric oxide-releasing non-steroidal anti-inflammatory and related nitric oxide-donating drugs. *Br J Pharmacol* **137**: 295–310.

Maier R, Bilbe G, Rediske J, Lotz M (1994). Inducible nitric oxide synthase from human articular chondrocytes: cDNA cloning and analysis of mRNA expression. *Biochem Biophys Acta* **1208**: 145–150.

Marshall M., Moore PK (2004). Effect of nitric oxide releasing paracetamol and flurbiprofen on cytokine production in human blood. *Eur J Pharmacol* **483**: 317–322.

Moore PK, Marshall M (2003). Nitric oxide releasing acetaminophen (nitroacetaminophen). *Digestive Liver Dis* **35** S2, 49–60.

Papapetropoulos A, Abou-Mohamed G, Marczin N, Murad F, Caldwell RW, Catravas JD (1996). Downregulation of nitrovasodilator-induced cyclic GMP accumulation in cells exposed to endotoxin or interleukin-1beta. *Br J Pharmacol* **118**: 1359–1366.

Romero-Sandoval R, Mazario J, Howat D, Heereiro JF (2002). NCX-701 (nitroparacetamol) is an effective anti-nociceptive agent in rat withdrawal reflexes and wind up. *Br J Pharmacol* **135**: 1556–1562.

Wallace JL (2004). Acetaminophen hepatotoxicity: NO to the rescue. *Br J Pharmacol* **143**: 1–2.

Zacharowski P, Zacharowski K, Donnellan C, Johnston A, Vojnovic I, Forte P *et al*. (2004). The effects and metabolic fate of nitroflurbiprofen in healthy volunteers. *Clin Pharmacol Ther* **76**: 350–358.