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Effect of nitric oxide releasing paracetamol and flurbiprofen on cytokine production in human blood

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Abstract

Exposure of anti-coagulated human blood to *Escherichia coli* lipopolysaccharide (50 ng/ml) resulted in the time-dependent (maximum at 5 h) biosynthesis of interleukin-1β and tumour necrosis factor-α (TNF-α). Preincubation with nitroparacetamol or nitroflurbiprofen (but not paracetamol or flurbiprofen) caused dose-related inhibition of the formation of interleukin 1β (IC_{50}s, 44.5 and 362 μM, $n=12$) and tumour necrosis factor-α (IC_{50}s, 9.0 and 0.0009 μM, $n=12$). The inhibitory effect of nitroparacetamol was completely reversed by (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazol-1-yloxy-3-oxide potassium (carboxy-PTIO, 100 μM; NO scavenging agent). Neither the nuclear factor-κB transduction inhibitor, pyrrolidinedithiocarbamate (10–1000 μM) nor the nitric oxide donor, 1-hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene (NOC-5, 10–1000 μM), affected cytokine formation in these experiments.

Keywords: Nitroparacetamol, Nitroflurbiprofen; Interleukin-1β; TNF-α (tumour necrosis factor-α); Lipopolysaccharide; Blood

1. Introduction

Nitric oxide releasing non-steroidal anti-inflammatory drugs such as nitroaspirin, nitroflurbiprofen and nitronaproxen, as well as other nitric oxide releasing donor drugs, such as nitroprednisolone and nitroparacetamol, have recently been described (for reviews, see Del Soldato et al., 1999; Keeble and Moore, 2002). These compounds were originally devised in an attempt to develop aspirin-like agents with fewer gastrointestinal side effects. Indeed, they exhibit negligible gastrotoxicity both in animals and man (Davies et al., 1997; Hawkey et al., 2003). Perhaps more surprisingly is the observation that such nitric oxide releasing derivatives exhibit more pronounced anti-inflammatory activity than the parent drug (e.g. nitroaspirin vis-à-vis aspirin, Al-Swayeh et al., 2000a and nitroprednisolone vis-à-vis prednisolone; Paul-Clark et al., 2000). Perhaps the best example is nitroparacetamol, which exhibits potent anti-inflammatory activity in the carrageenan-induced hind-paw oedema (rat, Al-Swayeh et al., 2000b) and zymosan-induced air pouch (mouse, Paul-Clark et al., 2001) models whilst paracetamol itself has little or no anti-inflammatory activity in these tests. Furthermore, both nitroaspirin and nitroparacetamol exhibit markedly greater anti-nociceptive potency than their respective parent compounds (Al-Swayeh et al., 2000a,b; Romero-Sandovai et al., 2002). The mechanism(s) underlying the enhanced anti-inflammatory activity of nitroparacetamol and related compounds are not yet known but blockade of transcription of pro-inflammatory molecules via the intracellular nuclear factor κB pathway (Chen et al., 1999) as well as inhibition of caspase-1 (interleukin converting enzyme; Fiorucci, 2001) which forms pro-inflammatory interleukin-1β have both been put forward as possibilities.

In the present study, we have examined the effect of nitroparacetamol and nitroflurbiprofen (plus their corresponding parent compounds, paracetamol and flurbiprofen) on lipopolysaccharide-induced production of interleukin-1β and tumour necrosis factor-α (TNF-α) in incubated human whole blood. Some of these data have been pub-

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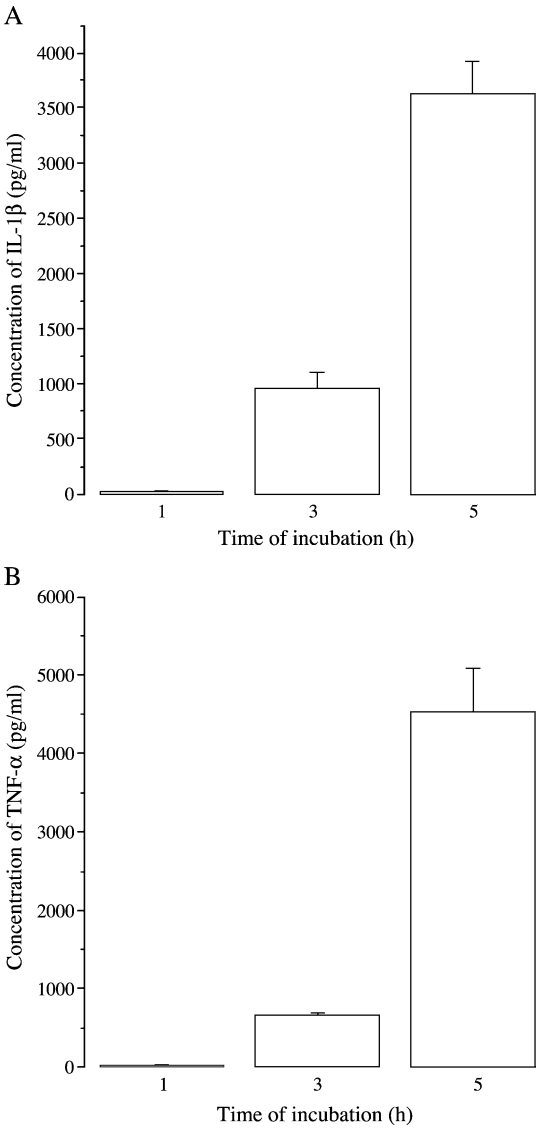

Fig. 1. Time course of interleukin-1β (A) and tumour necrosis factor-α (B) production in lipopolysaccharide (50 ng/ml) incubated (37 °C, up to 5 h), anticoagulated human blood. Plasma samples were analysed by ELISA. Plasma interleukin-1β and tumour necrosis factor-α concentration in blood prior to incubation with lipopolysaccharide were 8.7 ± 1.0 and 5.3 ± 2.1 pg/ml, respectively ($n = 12$). No change in cytokine concentration was detected in blood samples incubated in the absence of added lipopolysaccharide. Results show concentration of each cytokine (pg/ml) and are mean ± S.E.M., $n = 12$.

lished previously in preliminary form (Marshall and Moore, 2003).

2. Material and methods

Heparinised (12.5 U/ml) blood was obtained from healthy volunteers and diluted 1:1 v/v in RPMI-1640 medium (Sigma, Poole, UK). Aliquots (1.5 ml) of blood/medium were incubated (37 °C) with *Escherichia coli* lipopolysaccharide (50 ng/ml, serotype 0127: B8). In preliminary experiments, the time course of LPS-evoked interleukin-1β and tumour necrosis factor-α generation was determined following incubation of blood for 1, 3 or 5 h at 37 °C. All subsequent experiments with inhibitory drugs were carried out using a 5 h incubation period.

To assess the effect of test drugs, aliquots of human blood (prepared as above) were incubated with *E. coli* LPS in the presence of nitroparacetamol (10–1000 μM), paracetamol (10–1000 μM), nitroflurbiprofen (0.0001–10 000 μM) or flurbiprofen (10–1000 μM) or an appropriate volume (15 μl) of vehicle (dimethylsuphoxide; 1% v/v). All samples were gently agitated for the entire duration of the incubation. At the end of the incubation period, samples

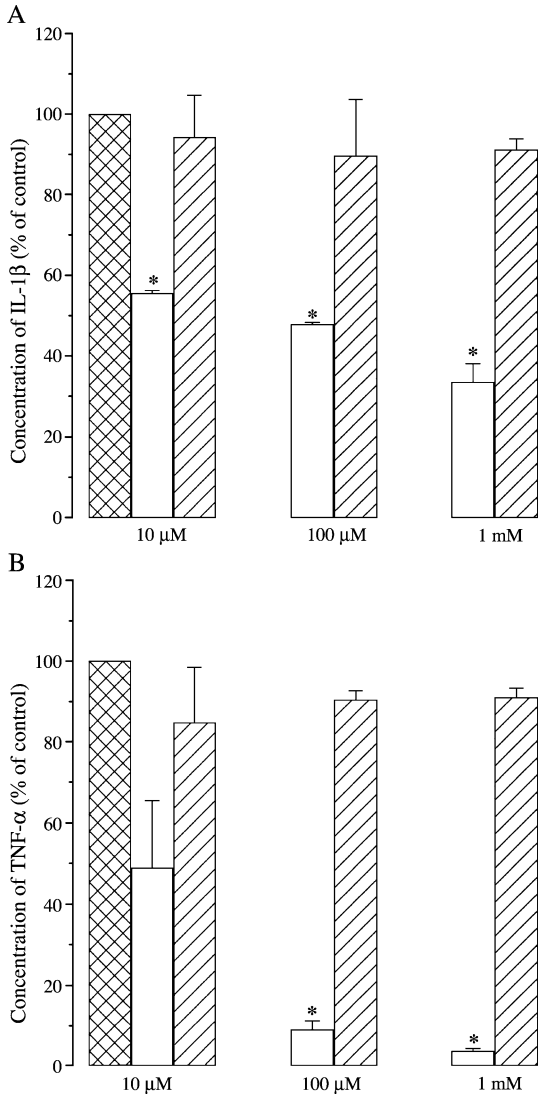

Fig. 2. Effect of nitroparacetamol (open columns) and paracetamol (striped columns) on lipopolysaccharide (50 ng/ml) induced interleukin-1β (A) and tumour necrosis factor-α (B) production. Hatched columns represent lipopolysaccharide-induced cytokine formation in the presence of vehicle (15 μl dimethylsulphoxide). Results indicate production of cytokine as a percentage of control formation (i.e. in presence of vehicle) and are mean ± S.E.M., $n = 12$, $^*P < 0.01$ (ANOVA plus post-hoc Dunnett's test).

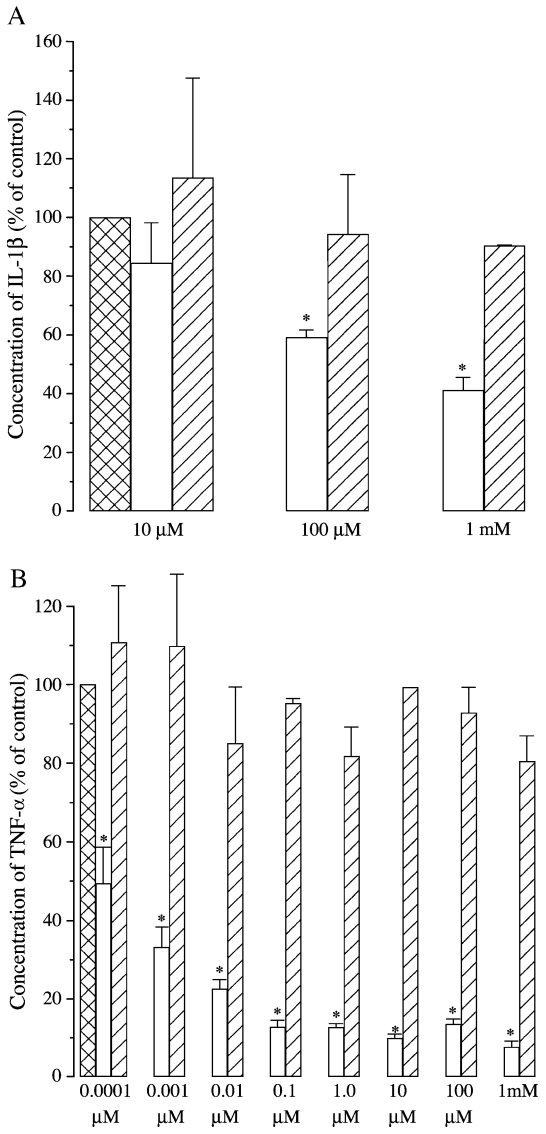

Fig. 3. Effect of nitroflurbiprofen (open columns) and flurbiprofen (striped columns) on lipopolysaccharide (50 ng/ml) induced interleukin-1β (A) and tumour necrosis factor-α (B) production. Hatched columns represent lipopolysaccharide-induced cytokine formation in the presence of vehicle (15 μl dimethylsulphoxide). Results indicate production of cytokine as a percentage of control formation (i.e. in presence of vehicle) and are mean ± S.E.M., $n = 12$, $^*P < 0.01$.

were centrifuged (6000 × *g*, 5 min) and the resulting plasma supernatant aspirated prior to storage at − 20 °C until assay.

In order to examine the part played by released nitric oxide in the effect of nitroparacetamol on cytokine production, further experiments were conducted in which aliquots of lipopolysaccharide-treated blood were incubated with nitroparacetamol (10–1000 μM) in the presence of the nitric oxide scavenging agent, carboxy-PTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazol-1-yloxy-3-oxide potassium, 100 μM; Akaike et al., 1993). In separate experiments, the effect of the NF-κB inhibitor, pyrrolodinedithiocarbamate (PDTC, 10–1000 μM; Chiang et al., 2003) and the slow releasing nitric oxide donor, NOC-5 (10–1000 μM; 1-hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene; Shibuta et al., 1998) on lipopolysaccharide-induced interleukin 1-β and tumour necrosis factor-α formation was also assessed.

At the end of the incubation period, aliquots of plasma (10–100 μl) were assayed for interleukin-1β and tumour necrosis factor-α using commercially available ELISA kits.

Results indicate mean ± standard error of the mean (S.E.M.). Statistical significance between groups was

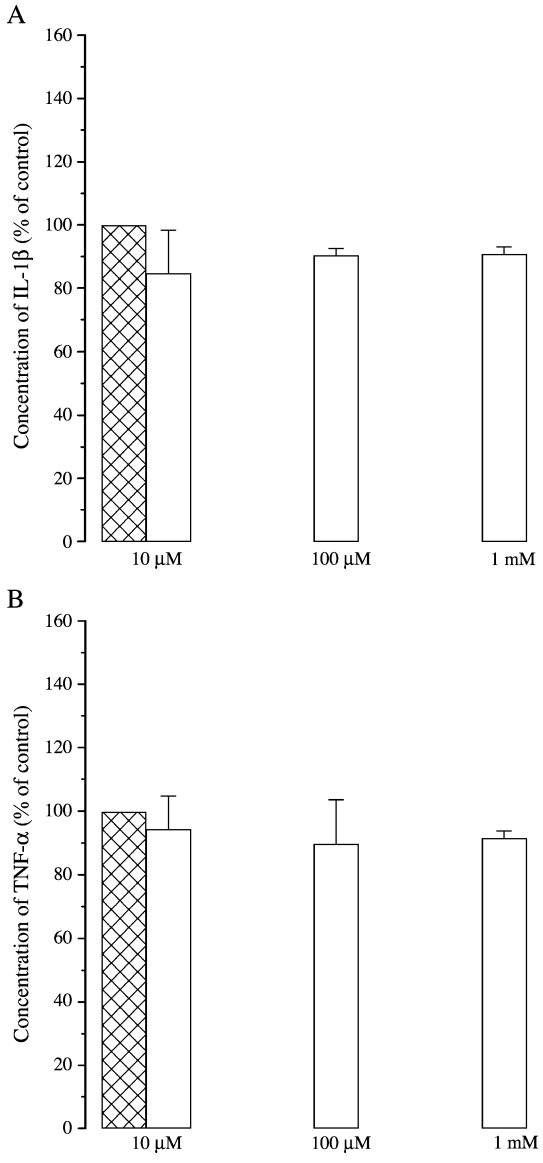

Fig. 4. Effect of incubation with (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazol-1-yloxy-3-oxide potassium (carboxy-PTIO; 100 μM) on the inhibitory action of nitroparacetamol (10–1000 μM; grey columns) on lipopolysaccharide (50 ng/ml) induced interleukin-1β (A) and tumour necrosis factor-α (B) production. Hatched columns represent cytokine formation in the presence of vehicle (15 μl dimethylsulphoxide). Results indicate production of cytokine as a percentage of control formation (in presence of vehicle) and are mean ± S.E.M., $n = 12$, $P < 0.05$.

assessed 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.

Carboxy-PTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide 2-(4-arboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazol-1-yloxy-3-oxide potassium, carboxy-PTIO), dimethylsulphoxide, paracetamol, flurbiprofen, *E. coli* lipopolysaccharide (serotype 0127:B8), pyrrolodinedithiocarbamate (PDTC) and RPMI-1640 medium were obtained from Sigma. Human interleukin-1β and tumour necrosis factor-α ('Quantikine') sandwich ELISA kits were obtained from Research and Development Systems, Oxfordshire, UK, NOC-5 (1-hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene) was purchased from Calbiochem. Nitroparacetamol and nitroflurbiprofen were the generous gifts of Dr. P. Del Soldato, NicOx, Sophia-Antipolis, France.

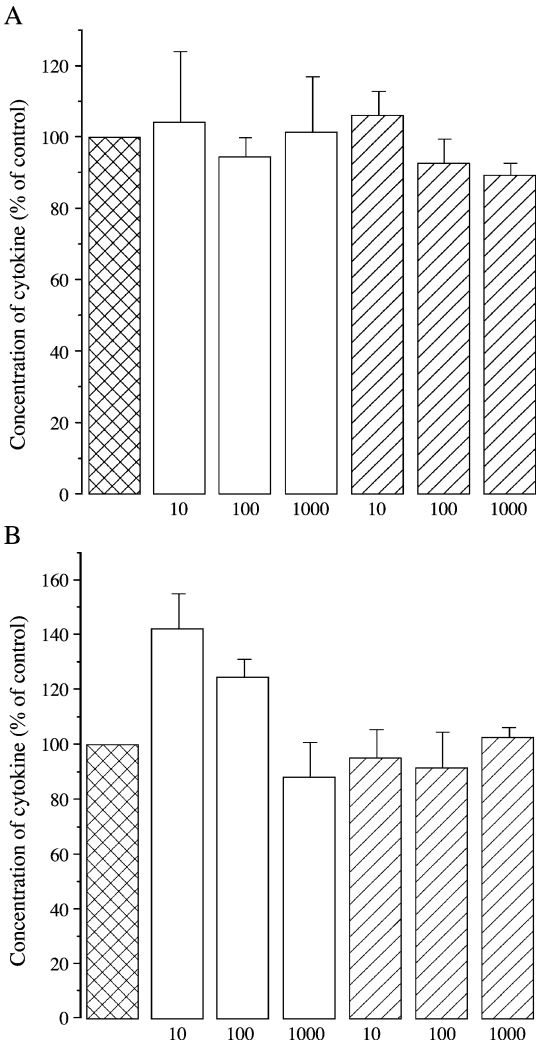

Fig. 5. Effect of (A) pyrrolodinedithiocarbamate (PDTC; 10–1000 μM) and (B) 1-hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene (NOC-5; 10–1000 μM) on lipopolysaccharide (50 ng/ml) induced interleukin-1β (open columns) and tumour necrosis factor-α (striped columns) production. Hatched columns represent cytokine formation in the presence of vehicle (15 μl dimethylsulphoxide). Numbers indicate concentration of drug in μM. Results indicate production of cytokine as a percentage of control formation (in presence of vehicle) and are mean ± S.E.M., $n=12$, $P<0.05$.

3. Results

Basal levels of interleukin-1β and tumour necrosis factor-α in human blood plasma incubated (5 h) in the absence of lipopolysaccharide were very low (<30 pg/ml). In contrast, incubation of human blood with lipopolysaccharide (50 ng/ml) over a 5-h period resulted in a marked (approx. 100 fold) and highly significant increase in the concentration of both interleukin-1β (3815.5 ± 336.3 pg/ml, $n=6$, $P<0.005$) and tumour necrosis factor-α (3160 ± 880 pg/ml, $n=12$, $P<0.001$). Production of both cytokines was undetectable at 1 h and intermediate at 3 h of incubation (Fig. 1).

Neither paracetamol (10–1000 μM, Fig. 2) nor flurbiprofen (10–1000 μM, Fig. 3) affected lipopolysaccharide-induced interleukin-1β or tumour necrosis factor-α production in human whole blood. In contrast, incubation of blood with either nitroparacetamol (10–1000 μM, Fig. 2) or nitroflurbiprofen (0.01–1000 μM, Fig. 3) resulted in a significant ($P<0.01$) inhibition of the formation of both cytokines.

The inhibitory effect of nitroparacetamol was concentration-dependent (IC_{50}, 44.5 μM, $n=12$ and IC_{50}, 9.0 μM, $n=12$) for inhibition of interleukin-1β and tumour necrosis factor-α production, respectively. Nitroflurbiprofen also inhibited lipopolysaccharide-induced interleukin-1β biosynthesis in a concentration-dependent manner (IC_{50}, 362.0 μM, $n=12$). Intriguingly, compared with nitroparacetamol, nitroflurbiprofen proved to be a very much more effective inhibitor of the biosynthesis of tumour necrosis factor-α with an IC_{50} of 0.0009 μM ($n=12$). Maximal (about 90%) inhibition of tumour necrosis factor-α production by nitroflurbiprofen under these experimental conditions occurred at concentrations greater than 1 μM.

Incorporation into incubated blood of carboxy-PTIO (100 μM) abolished the ability of nitroparacetamol to inhibit lipopolysaccharide-induced cytokine formation (Fig. 4). In contrast, neither the slow releasing nitric oxide donor, NOC donor (10–1000 μM), nor the nuclear transcription factor κB blocker, pyrrolidinedithiocarbamate (10–1000 μM), affected the formation of either cytokine (Fig. 5).

4. Discussion

Nitric oxide releasing non-steroidal anti-inflammatory drugs have previously been reported to inhibit caspase-1 enzyme activity with a resulting decline in the synthesis of interleukin-1β. Since interleukin-1β exhibits pro-inflammatory activity (Badolato and Oppenheim, 1996; Dinarello,

2000) it has been suggested that such an action underlies the enhanced (cf. the parent compound) anti-inflammatory effect of these nitric oxide releasing derivatives (reviewed by Fiorucci, 2001) as well as their reduced gastric toxicity (Zhang et al., 2000a). At the molecular level, these compounds most probably nitrosylate strategically important sulphydryl groups in caspase-1, thereby inhibiting enzyme activity.

In the present experiments, we report that nitroparacetamol (but not paracetamol) inhibits lipopolysaccharide-induced cytokine production in incubated human blood. Cytokine production under these conditions most probably takes place in activated cells of the monocyte-phagocytic system. Whole blood was used in these experiments (cf. isolated monocytes) in a deliberate attempt to mimic more closely the situation in vivo and to avoid the introduction of possible artifacts following cell separation and washing.

The finding that carboxy-PTIO, an efficient scavenger of nitric oxide (Akaike et al., 1993), abolished the cytokine-inhibitory effect of nitroparacetamol suggests that nitric oxide, released from nitroparacetamol most probably by the action of esterase enzymes in plasma and/or cellular blood components (for review, see Keeble and Moore, 2002), is the active moiety. This conclusion is supported by the observation that lipopolysaccharide-induced cytokine production is not restricted to nitroparacetamol, since nitroflurbiprofen (but not flurbiprofen) was also active. However, this conclusion is at odds with the inability of the slow releasing NO donor, NOC-5, to mimic the ability of nitroparacetamol or nitroflurbiprofen to inhibit lipopolysaccharide-induced interleukin 1β or tumour necrosis factor-α production in human blood. Clearly, further experiments are required to investigate the precise role of NO in the control of cytokine production under these circumstances. It is conceivable that nitric oxide release from NOC-5 is insufficient to inhibit cytokine formation in these experimental conditions. However, this seems unlikely in that the reported half-life of NOC-5 breakdown to nitric oxide is 1.5 h (Hrabie et al., 1993) which is less than the incubation period used in the present work. One possibility is that nitroparacetamol and nitroflurbiprofen act as 'carrier' molecules allowing nitric oxide to gain access to intracellular sites at sufficient concentration to inhibit cytokine production. The lack of effect of NOC-5 may therefore be explained by rapid extracellular binding to proteins such as haemoglobin.

A particularly intriguing finding in the present study is that nitric oxide, released from nitroparacetamol and nitroflurbiprofen following incubation in blood, inhibited the production, not only of interleukin 1-β (presumably by caspase-1 inhibition), but also of tumour necrosis factor-α which is formed from a membrane precursor by the activity of a specific metalloprotease (TNF converting enzyme). To the best of our knowledge there have been no reports in the literature suggesting an inhibitory effect of nitric oxide on this enzyme. Indeed, in contrast, Zhang et al. (2000b) reported that nitric oxide activates (not inhibits) purified TNF converting enzyme in vitro by a mechanism which involves nitrosation of the inhibitory motif of the enzymes prodomain.

The precise manner by which nitroparacetamol and nitroflurbiprofen inhibit the formation of interleukin-1β and tumour necrosis factor-α is therefore unclear. One possibility is that nitric oxide prevents the mobilization of a range of pro-inflammatory molecules (e.g. vascular cell adhesion molecule 1, E-selectin, cytokines, cyclooxygenase-2, superoxide dismutase) by blocking transduction through the intracellular nuclear factor-κB pathway. A growing body of evidence in the literature testifies to the ability of nitric oxide to block this pathway either by inhibiting proteasome enzyme activity (e.g. Chen et al., 1999) and thereby stabilizing nuclear factor-κB in the cytoplasm or, once translocated into the nucleus, by preventing nuclear factor-κB binding to DNA (Matthews and Hay, 1995). In either case, reduced cytokine (both interleukin-1β and tumour necrosis factor-α) production would be expected. However, we note here that incubation of human blood with pyrrolidinedithiocarbamate at a concentration previously reported to inhibit nuclear factor-κB transduction in other cells (e.g. Chiang et al., 2003; Kim et al., 2003) did not affect the inhibitory action of nitroparacetamol on cytokine generation in lipopolysaccharide-exposed human blood. The possibility that pyrrolidinedithiocarbamate is unstable in, or perhaps becomes bound to formed elements of the blood, and therefore not available to inhibit NF-κB transduction, seems unlikely since this compound is active in vivo after parenteral administration (Hong et al., 2000) and in cell culture experiments in the presence of human serum (Morigi et al., 1998). In consequence, it seems unlikely that inhibition of transduction via the nuclear factor κB pathway underlies the cytokine inhibitory effect of nitroparacetamol in human blood.

In conclusion, we provide evidence here that two nitric oxide releasing donor drugs (nitroparacetamol and nitroflurbiprofen) inhibit production of interleukin-1β and tumour necrosis factor-α in lipopolysaccharide-challenged human blood. Both compounds are considerably more potent inhibitors of tumour necrosis factor-α formation. Indeed, nitroflurbiprofen is active at submicromolar concentrations and this action may contribute to the very pronounced anti-inflammatory effect of this agent in vivo (Al-Swayeh et al., 1999). We also show that release of nitric oxide from combination with nitroparacetamol or nitroflurbiprofen accounts for the cytokine synthesis inhibitory effect of these compounds. The precise molecular mechanism remains unclear but does not appear to involve inhibition of nuclear factor κB transduction. Inhibition of cytokine formation (particularly tumour necrosis-α) may be an important mechanism underlying the potent anti-inflammatory activity of these drugs.

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