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Neutrophil-associated inflammatory responses in rats are inhibited by phenylarsine oxide

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Abstract

NADPH oxidase is a phagocyte-specific enzyme which produces O_2^- and so initiates a cascade of reactive oxygen species formation. Inflammatory diseases involve overproduction of reactive oxygen species which induce tissue damage. Phenylarsine oxide has been described previously as a complete and direct inhibitor of NADPH oxidase in vitro that acts by covalently binding to vicinal thiol groups of a membrane-associated component of the enzyme. In the present work, the potential anti-inflammatory effect of phenylarsine oxide was tested on two experimental models in rats, carrageenan-induced paw oedema and lipopolysaccharide-mediated lung inflammation. Intraperitoneal injection of phenylarsine oxide reduced (i) reactive oxygen species production by rat phagocytes, (ii) neutrophil infiltration into the lung after inhalation of lipopolysaccharide and (iii) neutrophil-dependent oedema induced by carrageenan in hindpaws. We conclude that phenylarsine oxide has anti-inflammatory properties which are probably exerted by its ability to inhibit neutrophil NADPH oxidase-dependent reactive oxygen species production. The present work provides the basis for the development of new anti-inflammatory, arsenic-free agents reacting at the phenylarsine oxide site, which seems to be the Achilles' heel of NADPH oxidase.

Keywords: Neutrophil; NADPH oxidase; Phenylarsine oxide; Inflammation; NADPH oxidase inhibitor; Anti-inflammatory drug

1. Introduction

Neutrophils and macrophages play a crucial role in host defence by performing effective killing and digestion of pathogens (Smith, 1994). For this purpose they are equipped for chemotaxis, phagocytosis, exocytosis of bactericidal enzymes contained in granules and generation of toxic oxygen metabolites (Smith, 1994). Phagocytes can be stimulated by a variety of inflammatory agents in addition to pathogens. Inappropriate tissue infiltration and activation of neutrophils can result in tissue damage (Smith, 1994; Halliwel, 1987). Neutrophil-derived reactive oxygen species have been implicated in tissue injury associated with inflammation, organ ischemia and reperfusion, adult respiratory distress syndrome, rhumatoid arthritis and asthma (Smith, 1994; Halliwel, 1987). Reactive oxygen species are formed subsequent to the assembly and activation of the phagocyte-specific enzyme, NADPH oxidase (Chanock et al., 1994). This enzyme produces superoxide anions (O_2^-) which initiate the formation of other toxic oxygen metabolites such as H_2O_2, $OH^{\cdot}$ and HOCl (Ward et al., 1986). Scavenging of O_2^- or inhibition of NADPH oxidase are therefore two pharmacological strategies for the treatment of inflammatory diseases: the use of superoxide dismutase which dismutates O_2^- into H_2O_2, has a protective effect in different inflammatory models (Jadot et al., 1986; Hardy et al., 1994; Johansson et al., 1990; Suzuki et al., 1992b) and diphenylene iodonium which inhibits the production of O_2^- by binding to NADPH (O'Donnell et al., 1993), has anti-inflammatory effects in peroxochromate-induced arthritis in mice (Miesel et al., 1995) but blocks mitochondrial respiratory chain enzymes (Majander et al., 1994). Direct inhibition of the phagocyte-specific NADPH oxidase should therefore be the most efficient pharmacological approach, and it may limit undesired side effects. We have recently reported that phenylarsine oxide produces complete inhibition of O_2^- generation in isolated human neutrophils (Le Cabec and

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Maridonneau-Parini, 1995). This arsenical derivative reacts specifically with the NADPH oxidase and does not affect other neutrophil functions such as phagocytosis or degranulation, at a concentration which completely blocks the production of O_2^- (Le Cabec and Maridonneau-Parini, 1995).

In the present study, we investigated in vivo the potential anti-inflammatory properties of phenylarsine oxide, using two models: carrageenan-induced hindpaw oedema and lipopolysaccharide-induced lung inflammation in rats. We report that, under both experimental conditions, phenylarsine oxide is a potent anti-inflammatory agent.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (Charles River, France) received a standard laboratory diet and water ad libitum in an air conditioned room at 21°C and were kept under a 12/12 h light cycle. The experimental protocols followed were approved by the Comité Régional d'Ethique Biomédicale (Toulouse, France).

2.2. Bronchoalveolar lavage

The animals were killed with an overdose of sodium pentobarbital and bronchoalveolar lavages were performed via a cannula in the trachea with 5-times 7 ml of saline at 25°C. Bronchoalveolar lavages obtained from each animal were pooled and centrifuged at $150 \times g$ for 10 min at 4°C. The cell pellet was resuspended in saline and total cell counts were performed using a Coulter counter (Coultronics, France). Cell viability was measured by Trypan blue exclusion. To estimate the proportion of neutrophils and mononuclear cells, cells were centrifuged at $200 \times g$ for 10 min, stained with May-Grünwald Giemsa dye (Le Cabec and Maridonneau-Parini, 1994) and 200 cells were examined by light microscopy.

2.3. Measurement of reactive oxygen species

Alveolar macrophages (purity > 95%) or neutrophils (purity > 90%) were isolated from bronchoalveolar lavages performed on rats after inhalation of saline or lipopolysaccharide, respectively, as described below. Cells (5×10^4/ml), resuspended in Hanks buffer saline, were stimulated with 0.8 mg/ml opsonized zymosan (Sigma, St. Louis, MO, USA) prepared in rat serum as previously described (Halstensen et al., 1986). In other experiments, the production of reactive oxygen species elicited by opsonized zymosan was measured in the whole blood collected on 2 mg/ml EDTA. The generation of reactive oxygen species was determined by chemiluminescence (0.2 mM luminol, LKB luminometer) and the light emission was recorded continuously for 5–10 min under agitation at 37°C (Halstensen et al., 1986).

2.4. Inflammatory models

Carrageenan-induced hindpaw oedema: inflammation was produced by injection in the left hind foot pad of 50 μl of carrageenan suspension (Sigma) (1% in saline) as described (Winter et al., 1962). Plethysmometric measurements of the volume of both hindpaws were made after 1 h and measurement of the left hindpaw was then performed 2, 3 and 4 h after the carrageenan injection. Oedema volume (in ml) and the inflammation percentage (oedema volume/control paw volume × 100) were calculated for each animal.

Lipopolysaccharide-induced pulmonary inflammation: the animals were housed in a 10 l chamber where they received an aerosolized solution of 0.9% saline or 500 μg/ml lipopolysaccharide (from *E. coli* 026:B6, Difco, Detroit, MI, USA) in saline delivered by a compressed air nebulizer at 11 l/min for 30 min. Bronchoalveolar lavages were performed 4 h after lipopolysaccharide inhalation.

2.5. Phenylarsine oxide treatments

Phenylarsine oxide (Aldrich, Steinheim, Germany) was solubilized in polyethylene glycol 200 (Hoechst France) at 0.4 mg/ml and subsequent dilutions were made in polyethylene glycol to inject the same ratio (v/w, 2.5 ml/kg) to each animals. Phenylarsine oxide was injected i.p. 30 min before carrageenan or 10 min before lipopolysaccharide. In some experiments, chronic treatments with 0.5 mg/kg phenylarsine oxide were performed either as two i.p. injections made at 48 h interval or as nine injections (three injections per week).

2.6. Statistical analyses

In carrageenan experiments, comparisons between treated and non-treated groups were made using the unpaired Student's *t*-test. To compare the dose effect with the control values in Fig. 2, one-way analysis of variance (ANOVA) test was used followed by the Dunnett post hoc test. A P value of less than 0.05 was used as the criterion of statistical significance.

3. Results

3.1. Effect of phenylarsine oxide on reactive oxygen species production by isolated rat phagocytes

First we studied whether phenylarsine oxide is able to inhibit the production of reactive oxygen species in rat phagocytes. To obtain cell populations enriched either in macrophages or neutrophils, bronchoalveolar lavages were performed in rats that had received saline or lipopolysaccharide, respectively. Macrophages, which normally constitute the major alveolar cell population, were obtained by performing bronchoalveolar lavages in control rats,

Table 1
Phenylarsine oxide inhibits reactive oxygen species production by isolated alveolar macrophages and neutrophils

	Saline	Lipopolysaccharide
Total cells ($\times 10^6$)	1.8 ± 0.2	13.1 ± 2.2
Cell type (%) [a]		
Macrophages	98.6 ± 0.3	10.0 ± 0.3
Neutrophils	0.3 ± 0.1	89.0 ± 0.2
Phenylarsine oxide inhibition of reactive oxygen species production (IC_{50}, $\times 10^{-7}$ M) [b]	0.82 (3×10^{-8}–2.6×10^{-7} M)	2.0 (8×10^{-8}–5.4×10^{-7} M)

Rats received aerolized saline or lipopolysaccharide and bronchoalveolar lavages were performed 4 h later. [a] Cells were counted differentially as described in Section 2. [b] Cells exposed in vitro to different concentrations of phenylarsine oxide were stimulated by opsonized zymosan, reactive oxygen species production was measured and IC_{50} were calculated by linear regression analysis; in parentheses are 95% confidence limits ($n = 3$).

whereas neutrophils were obtained in bronchoalveolar lavage from rats exposed to lipopolysaccharide aerosols. After lipopolysaccharide inhalation, the total number of cells increased significantly ($P < 0.01$) as the result of the recruitment of blood neutrophils (Table 1).

Isolated phagocytes were exposed to different concentrations of phenylarsine oxide and stimulated with opsonized zymosan, a potent activator of the respiratory burst enzyme, NADPH oxidase (Maridonneau-Parini et al., 1986), and the production of reactive oxygen species was measured. We observed that phenylarsine oxide inhibited the generation of reactive oxygen species in alveolar macrophages and neutrophils (Table 1) without modification of cell viability.

3.2. Effect of phenylarsine oxide on reactive oxygen species production in blood

Next, to study whether phenylarsine oxide is also able to inhibit the production of reactive oxygen species by phagocytes in vivo, rats received an i.p. injection of phenylarsine oxide (1 mg/kg) and blood was collected 1 h later. The production of reactive oxygen species by blood phagocytes (mostly neutrophils) elicited by opsonized zymosan was inhibited by $37.7 \pm 2.6\%$ ($P < 0.05$, $n = 3$) in the blood of phenylarsine oxide-treated rats compared to controls.

3.3. In vivo anti-inflammatory effects of phenylarsine oxide

The anti-inflammatory action of phenylarsine oxide was first studied by measuring its effects on hindpaw oedema induced by carrageenan, a model frequently used to test anti-inflammatory agents (Winter, 1965; Vinegar et al., 1976). After carrageenan injection, the oedema volume increased steadily during the experiments. When compared to that in control animals, the inflammatory response was significantly reduced 3 and 4 h after carrageenan injection in rats that received 0.5 or 1 mg/kg phenylarsine oxide (Fig. 1A), while 0.1 mg/kg did not modify the oedema (data not shown).

Next, we studied whether two injections of phenylarsine oxide would produce an increased anti-inflammatory effect compared to a single administration. Two i.p. injections of phenylarsine oxide (0.5 mg/kg) were made at a 48-h interval. We observed that the anti-inflammatory effect was not increased when compared to the effect obtained

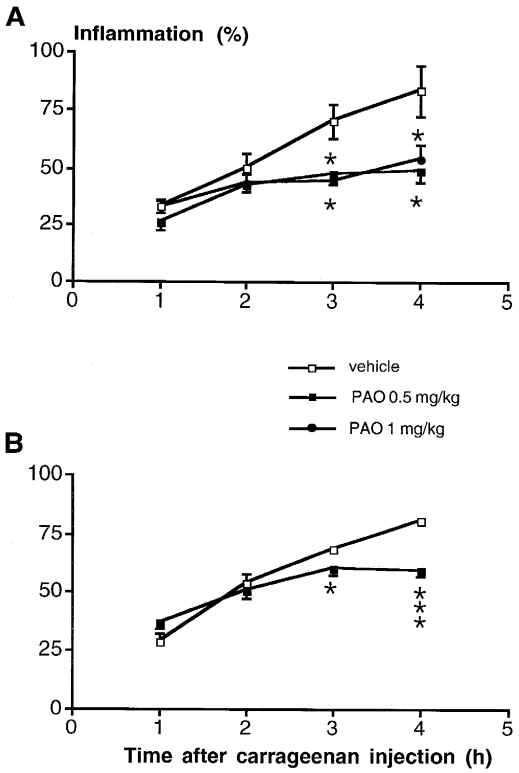

Fig. 1. Anti-inflammatory effect of phenylarsine oxide in carrageenan-induced hindpaw oedema in rats. (A) Three groups of 5 rats received an i.p. injection of poly(ethylene glycol) (vehicle), phenylarsine oxide 0.5 or 1 mg/kg 30 min before carrageenan (1%). (B) Rats received two injections of phenylarsine oxide at a two-day interval, the last injection being performed 30 min before carrageenan administration. In parallel, a group of control animals received poly(ethylene glycol). The oedema volume was measured 1–4 h after carrageenan injection in the left paw. The percentage of inflammation was determined by comparing the volume of the right paw to the volume of the left paw in each animal. Results are expressed as means ± S.E. The differences between control and phenylarsine-oxide-treated rats was analyzed by Student's *t*-test ($n = 5$) (* $P < 0.05$ and *** $P < 0.001$).

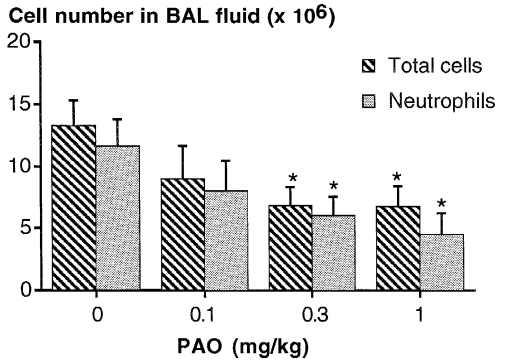

Fig. 2. Effect of phenylarsine oxide treatment on cell recruitment in bronchoalveolar lavage fluid after lipopolysaccharide inhalation. Groups of 6 rats that received an i.p. injection of phenylarsine oxide at the concentrations shown were exposed to lipopolysaccharide aerosols for 30 min (0.5 mg/ml) and bronchoalveolar lavages were performed 4 h later. Total cells (hatched bars) and neutrophils (grey bars) were counted. Results are expressed as means ± S.E. (* $P < 0.05$ as compared to control).

after a single injection of phenylarsine oxide (Fig. 1B). A longer treatment involving nine injections of 0.5 mg/kg phenylarsine oxide (three injections per week) also did not produce a greater effect (data not shown).

Arsenical derivatives can be toxic (Webb, 1966). The effect of phenylarsine oxide on the rat body weight (used as an index of toxicity) was measured. Although the experiments involved a single or two injections of phenylarsine oxide (see Fig. 1), the potential toxicity of this arsenical derivative was studied as a longer treatment (nine i.p. injections of 0.5 mg/kg phenylarsine oxide at 48 h interval). However, there was no significant difference between control and chronically treated rats, indicating that phenylarsine oxide did not detectably poison the animals (data not shown).

A second inflammatory model was tested to confirm the anti-inflammatory action of phenylarsine oxide and to better characterize its mechanism of action in vivo. In this model, the inflammatory reaction was elicited by lipopolysaccharide inhalation and invasion of cells into rat lungs was measured as an index of the inflammatory response.

I.p. injection of phenylarsine oxide at 0.3 or 1 mg/kg significantly reduced the number of cells present in bronchoalveolar lavage fluids from lipopolysaccharide-treated rats, indicating that phenylarsine oxide diminished the migration of neutrophils to the inflammation site (Fig. 2).

4. Discussion

Activation of neutrophils has been shown to result in tissue damage in a variety of chronic inflammatory diseases (Smith, 1994; Halliwel, 1987). Hydrogen peroxides and hydroxyl radicals, formed as the consequence of the massive generation of O_2^- by neutrophil NADPH oxidase, are implicated as the oxidants responsible for tissue injury (Smith, 1994; Halliwel, 1987). Therefore, selective inhibitors of the O_2^--generating enzyme should have a potent anti-inflammatory action. We have previously described that phenylarsine oxide is a complete and selective inhibitor of NADPH oxidase in isolated human neutrophils and monocytes (Le Cabec and Maridonneau-Parini, 1995). We report herein that phenylarsine oxide has an anti-inflammatory effect in two rat models of inflammation.

First, we showed that, in rat neutrophils and macrophages, NADPH oxidase is inhibited in vitro at the same range of phenylarsine oxide concentrations. Furthermore, the production of reactive oxygen species by human or rat neutrophils was similarly inhibited with an IC_{50} of 1.8×10^{-7} M (Le Cabec and Maridonneau-Parini, 1995) or 2.0×10^{-7} M, respectively. When systemic treatment was performed with phenylarsine oxide, the production of reactive oxygen species by blood phagocytes was also inhibited, indicating that phenylarsine oxide can exert its inhibitory action on the NADPH oxidase in vivo as well as in vitro.

Second, we report that the inflammatory responses to carrageenan in paws or lipopolysaccharide in lungs were significantly reduced by phenylarsine oxide. It has been shown that carrageenan-induced paw oedema has a first phase involving kinins, prostaglandins and serotonin (30–90 min), and a second phase consisting of massive neutrophil migration to the inflammatory site about 2–4 h after carrageenan injection (Vinegar et al., 1987). Administration of phenylarsine oxide reduced paw oedema 3 h after injection of the pro-inflammatory agent, suggesting that it inhibits the inflammatory phase mediated by neutrophils.

In the lipopolysaccharide inflammatory model, a large number of neutrophils migrate to the lung and produce reactive oxygen species which are implicated in lung damage (Heflin and Brigham, 1981; Snella and Rylander, 1982; Suzuki et al., 1992b). It was consistent with this that, in the present study, a marked increase in neutrophil number was observed in bronchoalveolar lavage fluid after lipopolysaccharide inhalation. In rats treated with phenylarsine oxide, the migration of neutrophils to the lung was reduced.

These findings suggest that phenylarsine oxide exerts its anti-inflammatory effects via neutrophils: (i) in the carrageenan model, the anti-inflammatory effect of phenylarsine oxide occurred during the neutrophil-dependent oedematous response, (ii) in the lipopolysaccharide model, the number of neutrophils in bronchoalveolar lavage was reduced and (iii) chronic treatment with phenylarsine oxide prior to injection of carrageenan did not improve the anti-inflammatory effect of phenylarsine oxide. Blood neutrophils have a very short life of about 10 h (Bainton, 1992) which can explain why multiple injections of phenylarsine oxide did not produce an additive anti-inflammatory effect as the drug cannot accumulate in these short-lived cells.

The mechanism of the anti-inflammatory action of phenylarsine oxide can, at least in part, result from NADPH oxidase inhibition. First, the reduced production of reactive oxygen species by phagocytes in rat blood collected after i.p. injection of phenylarsine oxide indicates that NADPH oxidase can also be inhibited in vivo. Second, migration of neutrophils to the inflammatory site requires adhesion to endothelial cells, followed by diapedesis through the vascular barrier and this step is, to some extent, reactive oxygen species-dependent. Indeed, neutrophil adherence to endothelium is partially mediated by leukocyte adhesion molecule-1 or GMP-140 on endothelial cells whose expression is activated by reactive oxygen species (Suzuki et al., 1992a; Patel et al., 1991). Therefore, inhibition of NADPH oxidase by phenylarsine oxide might reduce neutrophil diapedesis. Accordingly, apocynin, an inhibitor of reactive oxygen species generation by neutrophils, and superoxide dismutase, has been shown to reduce neutrophil migration to the lungs of septic guinea pigs (Wang et al., 1994; Suzuki et al., 1992b). Third, in the carrageenan model, reactive oxygen species are directly implicated in the formation of the paw oedema (Vinegar et al., 1976). Although, in this model, we cannot distinguish between inhibition of the recruitment and inhibition of the activation of neutrophils at the inflammatory site, reduction of the NADPH oxidase activity can explain the anti-inflammatory effect of phenylarsine oxide in both cases.

To conclude, we propose that neutrophil NADPH oxidase is targetted by phenylarsine oxide in vivo as well as in vitro, thus explaining, at least in part, its anti-inflammatory effect.

NADPH oxidase is a multicomponent enzyme comprising cytosolic and membrane-associated proteins (Chanock et al., 1994). Upon activation of phagocytic cells, several of the oxidase components assemble in the plasma membrane, and the O_2^--producing enzyme becomes functional. In previous work, we have shown that phenylarsine oxide does not interfere with the assembly of the enzymatic complex and exerts its inhibitory action by covalently binding vicinal thiol groups of a membrane-associated component of the NADPH oxidase (Le Cabec and Maridonneau-Parini, 1995). We now established that phenylarsine oxide is a potent anti-inflammatory agent but, being an arsenical derivative, it cannot be used as a therapeutic agent. Precise identification of the two amino acids bearing the thiol groups reacting with phenylarsine oxide, which we found to be the Achilles' heel of NADPH oxidase, should offer possibilities for the future development of new anti-inflammatory molecules.

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