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Parasiticidal effects of peroxynitrite on ovine liver flukes *in vitro*

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Abstract

Peroxynitrite ($ONOO^-$) is a cytotoxic anion, produced by interaction between nitric oxide and superoxide *in vivo* in some inflammatory cells. This study investigated its effects on *Fasciola hepatica* and *Dicrocoelium dendriticum* isolated from ovine livers and kept in bile at room temperature. Peroxynitrite was synthesized using a quenched flow reactor and assayed spectrophotometrically. It was applied at different concentrations ($10^{-3.5}$ to $10^{-2.3}$ M) to the flukes kept in bile. The viability of the peroxynitrite-treated flukes was compared with a control group (n = 5–7 per group). Control *F. hepatica* and *D. dendriticum* lived for 226 ± 11 and 208 ± 14 min, respectively. Life times were decreased by peroxynitrite at all concentrations used ($P < 0.001$). At the highest concentration of peroxynitrite, *F. hepatica* and *D. dendriticum* lived only for 6.1 ± 0.4 and 4.1 ± 4.1 ± 0.2 min, respectively. Correlation between peroxynitrite concentration and parasite viability was significant in the case of *F. hepatica* ($r = -0.842$; $P = 0.0035$). A single application of peroxynitrite can decrease the life span of ovine liver flukes. A failure in the activation of hepatic macrophages in infected animals may lead to a decreased production of free radicals and, thus, peroxynitrite. Such a failure is likely to deprive the body of a defence tool against multicellular parasites.

Introduction

The liver fluke, *Fasciola hepatica*, is infective to a wide variety of mammals. Flukes that pass through the gut wall are attacked and, perhaps, irreversibly damaged by host cells in the peritoneal cavity including eosinophils, neutrophils, macrophages and mast cells (Hughes, 1987). A critical role for the eosinophil in the expression of immunity to *F. hepatica* is suggested by the presence of large numbers of eosinophils in the peritoneal cavities after challenge of resistant rats and the attachment of these cells to juvenile flukes prior to the destruction of the parasite (Davies & Goose, 1981; Burden *et al.*, 1983).

Peroxynitrite ($ONOO^-$) is a cytotoxic anion which is produced from a rapid reaction between two free radicals, nitric oxide ($^{\cdot}NO$) and superoxide anion ($O_2^{\cdot -}$). The *in vivo* production of peroxynitrite has been shown in some inflammatory cells, including macrophages. This substance has not only serious damaging effects on the body cells, but can also kill some microorganisms. The latter is considered to be a mechanism involved in non-specific immune responses against infection and, therefore, can be beneficial (Muijsers *et al.*, 1997; Sadeghi-Hashjin *et al.*, 1998). Peroxynitrite has been suggested to be the reactive intermediate responsible for some of the pathologies associated with an overproduction of nitric oxide (Giorgio *et al.*, 1996).

Murine macrophages can be activated to produce nitric oxide, superoxide and, hence, peroxynitrite, a powerful oxidant which may be involved in parasite killing (Assreuy *et al.*, 1994). For instance, peroxynitrite released from activated macrophages has been shown to be highly cytotoxic against *Trypanosoma cruzi* epimastigotes (Rubbo *et al.*, 1994). To the knowledge of the authors, there is no report dealing with the effect of this substance on the multicellular parasites. For this reason, this study was designed to evaluate its effect of a group of trematodes, i.e. *Fasciola hepatica* and *Dicrocoelium dendriticum*.

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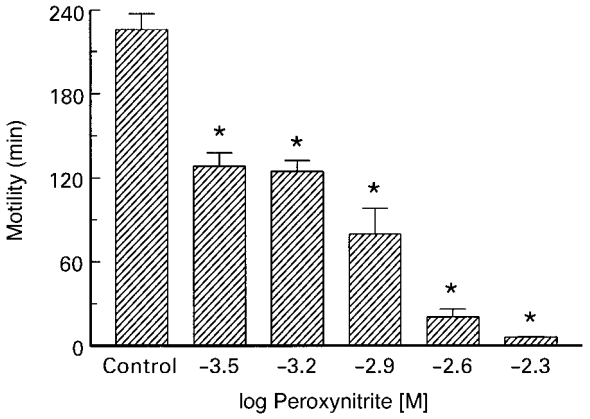

Fig. 1. Effect of peroxynitrite on the life span of *Fasciola hepatica* in vitro. Peroxynitrite was added to ovine bile which contained the parasite and was kept at room temperature. It decreased the life span of the worm in a concentration-dependent fashion. *$P < 0.001$, as tested by Bonferroni's t test after one-way ANOVA.

Materials and methods

Chemicals

Chemicals used in this study, including $NaNO_2$, HCl, H_2O_2 and NaOH all were purchased from Merck (Darmstadt, Germany).

Peroxynitrite synthesis

Peroxynitrite was synthesized in a quenched flow reactor as described previously (Sadeghi-Hashjin *et al.*, 1996). Briefly, solutions of (i) 0.6 M $NaNO_2$ and (ii) 0.6 M HCl plus 0.7 M H_2O_2 were pumped into a T junction and mixed in a glass tube. The acid-catalysed reaction of nitrous acid with H_2O_2 to form peroxynitrous acid was quenched by pumping 1.5 M NaOH at the same rate into a second T junction at the end of the glass tubing. The solution was frozen for a week at −20°C. As a result of freeze fractionation, peroxynitrite formed a yellow top layer which was retained for further analysis and application. The concentration of peroxynitrite in this layer was determined by absorbance at 302 nm ($\varepsilon_{302} = 1.67 \times 10^3$/M/cm). Stock solutions of peroxynitrite were kept at −80°C at alkaline pH, and the concentration was determined each time before use. At room temperature and a concentration of 10 mM in Krebs buffer, peroxynitrite decomposed approximately by 50%, 92% and 100% after 15, 30 and 60 min, respectively.

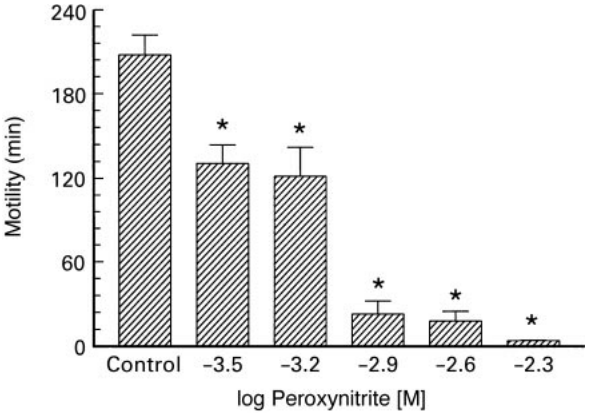

Fig. 2. Effect of peroxynitrite on the life span of *Dicrocoelium dendriticum* in vitro. Peroxynitrite was added to ovine bile which contained the parasite and was kept at room temperature. It decreased the life span of the worm in a concentration-dependent fashion. *$P < 0.001$, as tested by Bonferroni's t test after one-way ANOVA.

Tissue preparation and parasite isolation

Bile and infected livers from slaughtered sheep of either sex were obtained from a local abattoir and were immediately transported to the laboratory, where *F. hepatica* and *D. dendriticum* were isolated. The parasites were kept in the bile at room temperature. Peroxynitrite was applied at different concentrations ($10^{-3.5}$, $10^{-3.2}$, $10^{-2.9}$, $10^{-2.6}$ and $10^{-2.3}$ M) to the flukes kept in bile. The viability of the peroxynitrite-treated flukes was compared with a control group (n = 5–7 per group).

Statistical analysis

The time courses between addition of peroxynitrite to the bile and the absence of fluke motility were averaged for the various experimental groups. Values are given as mean ± SEM. The data were analysed first for normal distribution by one-way analysis of variance (ANOVA) and then for statistical significance by *post-hoc* Bonferroni's t test. In both cases the 0.05 significance level was adopted. In addition, the relationship between the concentrations of peroxynitrite and the life span of the parasites were evaluated by correlation and regression analyses.

Results

Control *F. hepatica* and *D. dendriticum* survived for 225.8 ± 11.4 and 207.5 ± 14.4 min, respectively. Life times were decreased by peroxynitrite at all concentrations used ($P < 0.001$; Bonferroni's t test) (figs 1 and 2). At the highest concentration of peroxynitrite, *F. hepatica* and *D. dendriticum* lived for only 6.1 ± 0.4 and 4.1 ± 4.1 ± 0.2 min, respectively. Correlation between peroxynitrite concentration and parasite viability was significant in the case of *F. hepatica* ($r = -0.842$; $P = 0.0035$) but not *D. dendriticum* ($r = -0.776$; $P = 0.070$).

Discussion

The present study showed that a single application of peroxynitrite can decrease the life span of two ovine liver flukes, *F. hepatica* and *D. dendriticum*, *in vitro*.

By utilizing a force and displacement transducer, *F. hepatica* preserved its movements for up to 4 days (Fairweather *et al.*, 1983). The method used in the

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present study was based on a visual assessment of the worm using a loop. When no movement was observed, the fluke was considered to be dead. At room temperature and in the bile taken from sheep, the parasite lived for less than 4 h. The experiments were performed at room temperature in order to prevent peroxynitrite from faster degradation. Indeed, its half life has been reported to be only 1 s at pH 7.4 and 37°C (Marla *et al.*, 1997).

In the rat, primary infected and challenged with *F. hepatica*, the number of eosinophils are increased in the peritoneal cavity (Smith *et al.*, 1992). Bovine eosinophils were shown to attach the tegument of juvenile *F. hepatica*, leading to ultrastructural changes in some parts and to death of the parasite (Glauert *et al.*, 1985). Many inflammatory cells, such as macrophages, produce and release superoxide anions and nitric oxide as part of their microbicidal effector molecules. The simultaneous production of these radicals results in the rapid formation of peroxynitrite, a strong oxidant with a half-life of less than 1 s in biological systems (Denicola *et al.*, 1993). We hypothesized that this substance might be responsible (at least in part) for the destruction of juvenile and adult flukes by eosinophils in the gut and by macrophages in the liver. Authentic peroxynitrite was applied to the parasite in order to evaluate this assumption.

Murine macrophages stimulated with zymosan/IFN-γ or lipopolysaccharide/IFN-γ killed *Leishmania major* to a similar degree. However, macrophages stimulated with zymosan alone were unable to kill *L. major*. S-nitroso-N-acetyl-penicillamine, which releases nitric oxide, was highly leishmanicidal when added directly to the parasites. Finally, authentic peroxynitrite failed to induce any cytotoxic effect, even at a high concentration. Thus macrophages can produce either nitric oxide, superoxide or both, depending on the stimulus. However, the killing of *L. major* is dependent only on the production of nitric oxide (Assreuy *et al.*, 1994). Peroxynitrite is formed during infection of the susceptible mouse strain, BALB/c, with *Leishmania amazonensis* and may play a role in the aggravation of the disease (Giorgio *et al.*, 1996). The parasiticidal activity of peroxynitrite against *T. cruzi* has been demonstrated. Peroxynitrite was highly trypanocidal, killing *T. cruzi* in a dose-dependent manner. Addition of 500 μM peroxynitrite as a single bolus resulted in 50% inhibition of cell proliferation as followed by growth curves. Other cytotoxic effects of peroxynitrite included cellular swelling and inhibition of cell motility (Denicola *et al.*, 1993). Two critical enzymes in the energy metabolism of *T. cruzi*, succinate dehydrogenase and fumarate reductase, are inactivated by biologically relevant concentrations of peroxynitrite by direct reactions with their sulphhydryl residues (Rubbo *et al.*, 1994).

From data in the literature, Denicola and colleagues (1993) estimated the production of peroxynitrite inside phagolysosomes of activated macrophages to be around 0.5 mM min^{-1}. Concentrations lower than this (i.e. 0.3 mM) used in our study affected fluke viability significantly. Therefore, the present study may have relevance to normal conditions in the body.

In conclusion, our results demonstrate that peroxynitrite may operate *in vivo* as a critical reactive intermediate against liver flukes. Any failure in the activation of hepatic macrophages (so-called Kupfer cells), e.g. in animals treated chronically with corticosteroids, may lead to a decreased production of free radicals and peroxynitrite. Such a failure is likely to deprive the body of a defence tool against liver flukes.

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