Toxicity of nitric oxide and peroxynitrite to bacterial pathogens of fish

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ABSTRACT: The inhibitory effect of the nitric oxide (NO) donor S-nitroso-acetyl-penicillamine (SNAP) and the NO and O_2^- donor 3-morpholino-sydnonimine hydrochloride (SIN-1) was tested in a cell-free assay. Strains of the bacterial fish pathogens *Aeromonas salmonicida*, *Renibacterium salmoninarum* and *Yersinia ruckeri* were exposed to different concentrations of the NO donors for 24 h. The results showed that NO possesses inhibitory properties, while peroxynitrite had no effect. However, when SIN-1 was used in combination with superoxide dismutase (SOD) alone or with catalase, an inhibitory effect comparable to that caused by SNAP was seen. The implications of these results are discussed.

KEY WORDS: SNAP · SIN-1 · Nitric oxide · Peroxynitrite · *Renibacterium salmoninarum* · *Aeromonas salmonicida* · *Yersinia ruckeri*

INTRODUCTION

In mammals, nitric oxide (NO) is produced by the action of a family of enzymes known as nitric oxide synthases (NOS). Two of the isoforms are expressed in endothelial (eNOS) and nervous (nNOS) tissue and have house-keeping roles such as cell-to-cell communication. The third isoform, inducible NOS (iNOS), is known to be expressed during immune responses against pathogens. The reactive nitrogen intermediates (RNI; including NO) so produced are known to inhibit a range of pathogens such as viruses, bacteria, fungi, protozoans and helminths and also tumour cells (Clark & Rockett 1996). However, NO can also combine with reactive oxygen species (ROS), particularly superoxide (O_2^-) produced during the respiratory burst, to produce peroxynitrite, a more potent free radical (Ischiropoulos et al. 1992, Carreras et al. 1994). In mammalian NO research, cell-free assays have been used to investigate the inhibitory properties of NO and peroxynitrite to pathogens. Organisms such as *Escherichia coli* (Zhu et al. 1992, Brunelli et al. 1995),

Mycobacterium avium (Doi et al. 1993), *M. tuberculosis* (O'Brien et al. 1994, Rhoades & Orme 1997) and *Staphylococcus aureus* (Kaplan et al. 1996), the yeast *Candida albicans* (Vazquez-Torres et al. 1995, 1996), the protozoan *Trypanosoma cruzi* (Denicola et al. 1993), and the nematode *Brugia malayi* (Thomas et al. 1997) have been shown to be more susceptible to peroxynitrite than NO itself, whilst the opposite situation has been reported for the protozoan *Leishmania major* (Assreuy et al. 1994) and *C. albicans* (Fierro et al. 1996).

NO is a very versatile molecule, able to attack many enzyme targets such as cytochrome oxidases (Stuehr & Nathan 1989), ribonucleotide reductase (Lepoivre et al. 1991), cytochrome P450 (Wink et al. 1993) and aldolase (Kahl et al. 1978). After NO combines with O_2^- to form peroxynitrite it can also attack enzymes with an iron-sulphur centre, such as hydrogenase (Hyman & Arp 1991) and aconitase (Drapier & Hibbs 1988). All these enzymes have important functions in the physiology of bacteria, parasites and tumour cells. However, this reactivity with enzymes makes NO potentially dangerous for the host cells and tissues. Indeed, NO and peroxynitrite have also been implicated in a series

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of non-infectious conditions such as cerebral ischaemia (Forman et al. 1998), acute myocardial infarction (Shimojo et al. 1998) and heart injury (Wang & Zweier 1996). Moreover, endogenously produced peroxynitrite induces protein oxidation in the mitochondria and nucleus of immunostimulated macrophages (Szabo et al. 1997). Hence, it is not clear whether peroxynitrite formation is beneficial for the host even though it inhibits microbes.

In fish, NOS activity has been reported in several tissues (Schoor & Plumb 1994, Sodestrom et al. 1995, Wong et al. 1998). Moreover, work by Neumann et al. (1995), Wang et al. (1995) and Neumann & Belosevic (1996) has shown that a macrophage cell line and primary macrophage cultures from goldfish produce NO when stimulated with macrophage activating factor (MAF) and bacterial lipopolysaccharide (LPS). Wiegertjes et al. (1998) reported NO production *in vitro* by carp macrophages stimulated with LPS and with the intracellular parasite *Trypanosoma borrelli*. Buentello & Gatlin (1999) also showed that medium composition exerts an influence in the NO production of channel catfish macrophages stimulated by LPS. The inducible form of NOS, iNOS, has been cloned and partially sequenced from a goldfish macrophage cell line and primary cultures of rainbow trout macrophages (Laing et al. 1999). Previous experiments have demonstrated that rainbow trout injected with *Renibacterium salmoninarum* showed an increase of NO (as measured by nitrite/nitrate concentration) in serum, and that iNOS is expressed in kidney and gill tissue (Campos-Pérez et al. 2000), suggesting that the produced NO may have a role in defence in this organism. Hence, in the present work the effectiveness of NO and peroxynitrite to inhibit strains of bacterial pathogens known to infect rainbow trout (*Aeromonas salmonicida*, *R. salmoninarum* and *Yersina ruckeri*) is assessed by using the NO donor S-nitroso-acetyl-penicillamine (SNAP) and the NO and O_2^- donor 3-morpholino-sydnonimine hydrochloride (SIN-1) in a cell-free assay.

MATERIALS AND METHODS

Bacteria. The species and strains used were *Aeromonas salmonicida* MT 004 (A-layer negative and avirulent) and MT 423 (A-layer positive and virulent) (Sharp & Secombes 1993); *Renibacterium salmoninarum* MT 426 (non-autoaggregating and avirulent), MT 405, MT 251 (non-autoaggregating, virulence unknown) and MT 1729 (autoaggregating and virulent) (Campos-Pérez 1998); and *Yersinia ruckeri* MT 223. All strains were obtained from the Marine Laboratory (Aberdeen) culture collection and grown on Tryptic soy agar (*A. salmonicida* and *Y. ruckeri*) or Mueller-

Hinton agar with L-cysteine (*R. salmoninarum*). The *A. salmonicida* strains were stored in agar containing 10% glycerol at –60°C. *Escherichia coli* was grown in Tryptic soy broth (TSB) and was used as a positive control for peroxynitrite killing.

Chemicals. SNAP and SIN-1 were obtained from Alexis Corporation (San Diego, CA). Both chemicals were used according to the instructions provided by the supplier. All solutions were freshly prepared before each experiment and used immediately. N-acetyl-penicillamine, the parental compound of SNAP which does not release NO, was used as a control at 1 mM.

Bactericidal assay. Bacteria were taken from the culture media, counted and suspended in Mueller-Hinton broth at 1×10^9 cells ml^{-1} (*Renibacterium salmoninarum*) or in TSB at 1×10^6 cells ml^{-1} (*Aeromonas salmonicida* and *Yersinia ruckeri*). Starting concentrations for *R. salmoninarum* were higher due to the slow growth characteristic of the species. SNAP and SIN-1 were then added in serial dilutions from 0 to 1 mM (Brunelli et al. 1995, Fierro et al. 1996). A total of 100 ml of the bacterial suspension plus NO donors were pipetted into 96-well plates in quadruplicate wells per dilution. An incubation period of 24 h was chosen for comparative purposes with a macrophage bactericidal assay against *R. salmoninarum* (Campos-Pérez 1998). After the incubation period, bacteria were centrifuged ($150 \times g$), washed twice and allowed to grow for 7 d (*R. salmoninarum*) or 24 h (*A. salmonicida* and *Y. ruckeri*). Superoxide dismutase (SOD, 300 U ml^{-1}) alone or with catalase (Cat, 300 μg ml^{-1}) were added to some of the wells containing SIN-1. After the incubation period, 10 ml of 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) solution at 5 mg ml^{-1} distilled water were added per well (Secombes 1990). Plates were read 15 min later in a multiscan spectrophotometer at 600 nM. The percentage of bacterial survival was calculated as:

$$\frac{\text{OD treated wells}}{\text{OD untreated wells}} \times 100$$

where OD = optical density.

RESULTS

Cell-free NO toxicity

SNAP was inhibitory for all species and strains tested. All *Renibacterium salmoninarum* strains except MT 1729 showed a decrease in growth of between 35 and 40% at the highest SNAP concentration, compared to non-treated controls (Fig. 1), with a significant ($p < 0.001$) dose effect being apparent. No significant differences were found between strains MT 426, MT 405 and MT 251. However, the virulent strain MT 1729
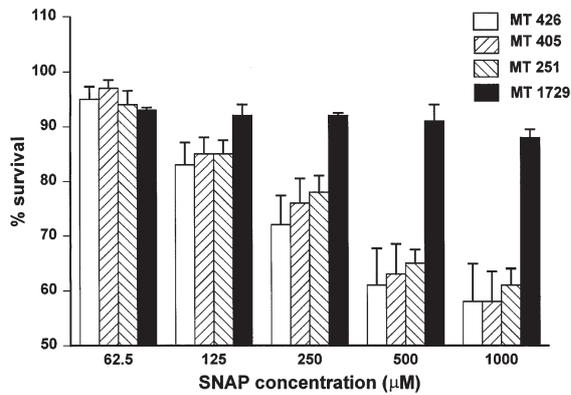
Fig. 1. Effect of SNAP on strains of *Renibacterium salmoninarum*. Bars represent mean + SE of 3 separate experiments

Fig. 3. Effect of SIN-1 on *Renibacterium samoninarum* MT 251. Bars represent mean + SE of 3 separate experiments

showed a higher growth which was significant ($p < 0.001$) compared to the other strains. MT 1729 showed only 10% inhibition at the highest SNAP concentration, and no significant dose effect was found. The *Aeromonas salmonicida* strains also showed a significant dose effect ($p < 0.001$) in their susceptibility to SNAP, though no significant differences were found between strain MT 004 and MT 423 (Fig. 2). By comparison, *Yersinia ruckeri* was relatively resistant to NO inhibition, showing less than 25% decrease in growth even at the highest SNAP concentration (Fig. 2). However, the inhibition did show a significant ($p < 0.05$) dose effect. No strain was inhibited when incubated with 1 mM N-acetyl-penicillamine (data not shown).

Cell-free peroxynitrite toxicity

SIN-1 alone was not inhibitory for *Renibacterium salmoninarum* and the results from these experiments were the same for all 4 strains tested. The data shown in Fig. 3 for strain MT 251 are representative of all the
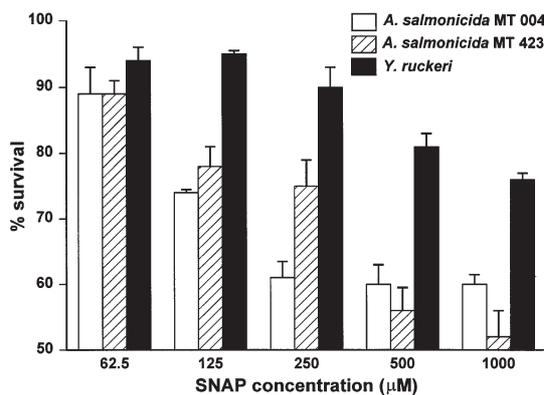
strains. Interestingly, when SOD alone or in combination with catalase was added, a significant ($p < 0.001$) dose-related inhibitory effect was seen and this effect was not very different to that seen with SNAP. The fact that the inhibition caused by SOD in combination with catalase showed no statistical differences compared to that by SOD alone suggests that hydrogen peroxide was not involved in the inhibitory effect. Furthermore, hydrogen peroxide has not been detected during SIN-1 decomposition at the same concentrations used here (Brunelli et al. 1995). *Aeromonas salmonicida* MT 004 showed a slight susceptibility to SIN-1, although no significant dose effect was apparent, with approximately 15% decrease in growth at the highest dose (Fig. 4). Interestingly, this strain showed about 100% growth when SOD was added to SIN-1, which was significantly ($p < 0.05$) higher than SIN-1 alone. This strain also showed a slight but non-significant decrease in growth when catalase was added together with SOD. *A. salmonicida* strain MT 423 showed a pattern similar to that seen for *R. salmoninarum* (Fig. 5). SIN-1 alone did not show inhibitory effects, but after

Fig. 2. Effect of SNAP on *Aeromonas salmonicida* and *Yersinia ruckeri*. Bars represent mean + SE of 3 separate experiments

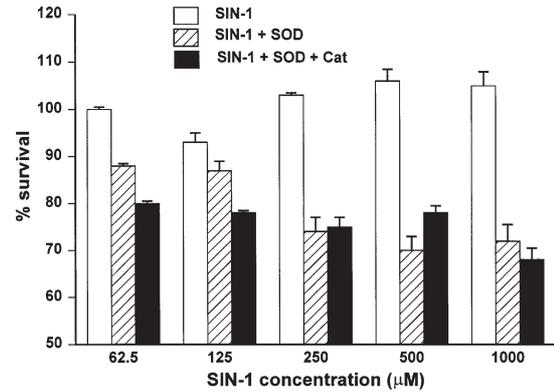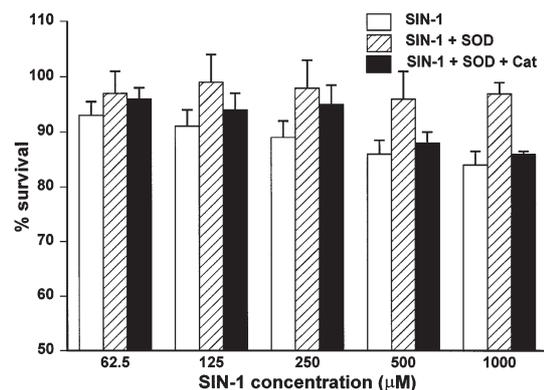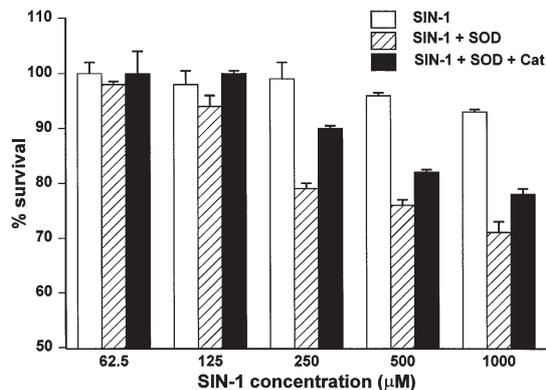Fig. 4. Effect of SIN-1 on *Aeromonas salmonicida* MT 004. Bars represent mean + SE of 3 separate experiments

Fig. 5. Effect of SIN-1 on *Aeromonas salmonicida* strain MT 423. Bars represent mean + SE of 2 separate experiments

adding SOD, a significant (p < 0.001) dose-effect decrease in growth was apparent. Addition of catalase slightly decreased the inhibition seen with SIN-1 + SOD, though there was still a significant (p < 0.001) dose-effect. *Escherichia coli*, however, showed a dose-related inhibitory effect which was significant (p < 0.001) (Fig. 6), and this occurred under conditions identical to those used for the other bacterial species. *E. coli* has been reported to be peroxynitrite sensitive (Brunelli et al. 1995) and hence it was used as a positive control.

DISCUSSION

It has recently been shown that fish macrophages possess the inducible form of NOS, and that they are able to release NO after stimulation with certain molecules (Wang et al. 1995, Laing et al. 1999). The first experiments carried out to compare the bactericidal properties of NO and peroxynitrite suggested the latter molecule possessed a higher toxicity than NO for
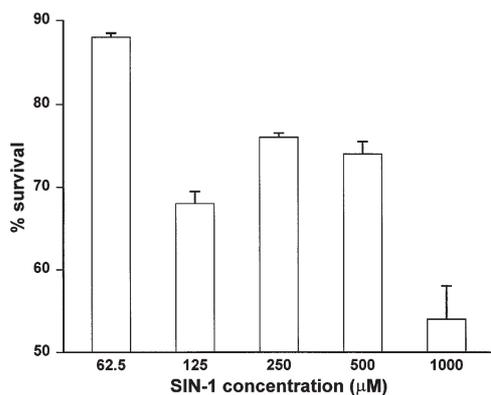

Fig. 6. Effect of SIN-1 on *Escherichia coli*. Bars represent mean + SE of 2 separate experiments

Escherichia coli (Brunelli et al. 1995, using SIN-1 and gaseous NO), *Candida albicans* (Vazquez-Torres et al. 1995, 1996, using SIN-1 and chemically synthesized peroxynitrite and sodium nitroprusside as NO generator) and *Brugia malayi* (Thomas et al. 1997, using SIN-1 and SNAP). Other authors, however, found the opposite situation to be true, with peroxynitrite showing some toxic effect, but the killing by NO alone being much higher for *Leishmania major* (Assreuy et al. 1994) and *C. albicans* (Fierro et al. 1996). In both studies SNAP and SIN-1 were used as generators and, interestingly, when SOD was added in combination with SIN-1, the inhibitory effect was significantly enhanced. The present results with a range of fish pathogens agree with these observations, although a difference was apparent, namely that no inhibition at all was seen using SIN-1. Furthermore, Kaplan et al. (1996) showed that SNAP-generated NO slightly impaired *Staphylococcus aureus* killing by O_2^- at early time points (1 to 5 h) following exposure. At later time points (24 h), exposure to NO correlated with decreased staphylococcal survival. The authors suggested that NO may be more useful under conditions when ROI release is compromised.

Studies of mammalian pathogens show that bacterial species depend on different mechanisms to protect themselves against RNI. Nagata et al. (1998) found that NO inhibits the respiration of *Escherichia coli* in a reversible fashion, whereas peroxynitrite also inhibits *E. coli* respiration but the effect is irreversible. In contrast, both compounds irreversibly inhibit the respiration of *Helicobacter pylori*. The authors hypothesised that NO reacted with endogenous superoxide radicals in *H. pylori*, resulting in peroxynitrite-mediated killing. Membrillo-Hernandez et al. (1999) have found evidence that upregulation of *E. coli* flavohemoglobin (as produced when in contact with NO releasing agents) plays a role in protection involving detoxification of NO-related species and sensing of oxidative stress. Other species of bacteria pathogenic to mammals are also known to express a range of enzymes and proteins which are associated with protection against RNI: *Salmonella typhimurium* lacking glucose 6-phosphate dehydrogenase (G6PD) are more susceptible to ROI and RNI (Lundberg et al. 1999), and a role of flavohemoglobins in protection against RNI has also been suggested (Crawford & Goldberg 1998a,b). Exposure of *Mycobacterium tuberculosis* to a series of NO donors (including SNAP) induced the synthesis of a heat shock protein, sHsp16 (Garbe et al. 1999). Interestingly, SIN-1 failed to produce a pattern consistent with differential gene expression. Unfortunately, there is a paucity of information about the production of enzymes and proteins by fish bacterial pathogens under oxidative stress by RNI compared to mammalian

microbes. Hence, the study of the proteins produced under oxidative stress and the effect of RNI in mutants lacking the genes which produce such proteins would help to identify the mechanisms used by fish pathogens to protect themselves from NO and NO derivatives.

In spite of the lack of information, it is tempting to speculate that the apparent resistance to NO of *Renibacterium salmoninarum* MT 1729 is due to some protective effect caused by the presence of the p57 protein. The presence of this protein is correlated with autoaggregation and virulence (Bruno 1988, 1990) and with immunosuppression (Wood & Kaattari 1996). Moreover, it is a potent inhibitor of the respiratory burst (Densmore et al. 1998). The p57 is not known to possess protective properties but the A-layer of *Aeromonas salmonicida* is also correlated with virulence and protection against the products of the respiratory burst (Karczewski et al. 1991). Why *Yersinia ruckeri* is less susceptible to inhibition by NO is less clear, although the species is known to be a facultative anaerobe, and James (1995) has suggested that some parasites may switch from aerobic to anaerobic metabolism to avoid dependence on enzymes which may be deactivated by NO.

The inhibition seen when SOD was added to the SIN-1 system may be the result of 1 of 2 possible scenarios: CuZnSOD does not bind NO (Gorren et al. 1987) and so remains capable of scavenging O_2^-. In the absence of O_2^- the half life of NO may be enhanced, preserving its reactivity and toxic effects (Oury et al. 1992). Thus, the normal half life of NO (4 to 50 s) may be prolonged, allowing it to reach molecules deep in the target cell. The second possibility is that, if the exogenous SOD contains Cu, peroxynitrite will react with it to form a potent nitrating agent with the reactivity of a nitronium ion (NO_2^+) which nitrates tyrosine residues (Beckman & Crow 1993). In cells, this results in impairment of intracellular signal transduction (Berlett et al. 1996). Why *Aeromonas salmonicida* MT 004 showed an increased growth when exposed to SIN-1 and SOD is not clear, as this strain has been reported to be susceptible to oxygen radicals (Karczewski et al. 1991). However, it has been shown that when *A. salmonicida* is exposed to low concentrations of hydrogen peroxide the bacterium expresses a periplasmic MnSOD and increased levels of cytoplasmic catalase, conferring resistance to ROS (Barnes et al. 1999). MnSOD has been related to protection against SNAP-produced NO (Sano et al. 1997). This may explain the apparent resistance of strain MT 004 to inhibition by SIN-1 in the presence of SOD, which would generate hydrogen peroxide from the O_2^-, which in turn may have induced the expression of MnSOD and catalase.

The results shown here do not differentiate if the effect seen is bactericidal or bacteriostatic. However, the fact that all bacterial species and strains tested were susceptible to SNAP shows that NO itself is a potent inhibitory agent and its potential value in an *in vivo* situation is supported by the fact that the final product of NO decomposition is mainly NO_2^-. NO_2^- can be converted back to active NO by dismutation at low pH (Stuehr & Nathan 1989), as present in a phagocytic vacuole inside a macrophage. In this acidic environment NO would be much more effective than peroxynitrite, which decays to form NO_3^-, in a reaction accelerated at an acidic pH decreasing the toxicity of peroxynitrite (Zhu et al. 1992). Moreover, NO kept inside these phagocytic vacuoles may accumulate until it reaches the concentration required to produce bacterial killing (Chan et al. 1992).

Finally, in several *in vitro* models of macrophage bactericidal activity it has been shown that while producing NO the killing capacity of macrophages was not affected when SOD was added to the system (Denis 1991, Chan et al. 1992). Interestingly, the microorganisms used in these studies were intracellular pathogens of the genus *Mycobacterium*. This further supports the notion that, for intracellular bacteria, O_2^- has no part in the inhibitory effect and that peroxynitrite may not be involved. Put together, the present data suggest that, while peroxynitrite formation is inhibitory for certain species of microbes, the fish pathogens used in the present study appear to be more susceptible to NO alone, with the acidic environment of intracellular vacuoles possibly favouring its inhibitory action.

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