Nisin depletes ATP and proton motive force in mycobacteria

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H.-J. CHUNG, T.J. MONTVILLE AND M.L. CHIKINDAS. 2000. This study examined the inhibitory effect of nisin and its mode of action against Mycobacterium smegmatis, a non-pathogenic species of mycobacteria, and M. bovis-Bacill Carmette Guerin (BCG), a vaccine strain of pathogenic M. bovis. In agar diffusion assays, 2.5 mg ml⁻¹ nisin was required to inhibit M. bovis-BCG. Nisin caused a slow, gradual, time- and concentration-dependent decrease in internal ATP levels in M. bovis-BCG, but no ATP efflux was detected. In mycobacteria, nisin decreased both components of proton motive force (membrane potential, ΔΨ and ΔpH) in a time- and concentration-dependent manner. However, mycobacteria maintained their intracellular ATP levels during the initial time period of ΔΨ and ΔpH dissipation. These data suggest that the mechanism of nisin in mycobacteria is similar to that in food-borne pathogens.

INTRODUCTION

Nisin is a small antimicrobial protein produced by Lactococcus lactis subsp. lactis. It belongs to the group I lantibiotics and has the unusual amino acids, such as dehydroalanin, dehydrobutyrine and lanthionin (Liu and Hansen 1990). Nisin has a broad inhibitory spectrum against Gram-positive food-borne pathogens such as Listeria monocytogenes and Clostridium botulinum (Delves-Broughton 1990). Its status is generally recognized as safe (GRAS) and it is being used as a food biopreservative in more than 50 countries (Federal Register 1988).

Mycobacteria are Gram-positive, aerobic, rod-shaped micro-organisms. The complex hydrophobic cell walls and the ability to survive in host phagocytes are the two salient features of mycobacteria (Connell 1994). Recently, mycobacteria have become very problematic due to the resistance to multiple antibiotics along with the shortage of effective antitubercular drugs. In addition, Mycobacterium paratuberculosis causes John’s disease in cattle and is likely to be associated with Crohn’s disease in humans (1984b; Chiodini et al. 1984a).

In vitro antimycobacterial activities have been reported for defensins (Miyakawa et al. 1996). These antimicrobial peptides are similar to bacteriocins in spatial structures and mode of action (Westerhoff et al. 1989; Juretic et al. 1994), which led us to investigate the mode of action of nisin against mycobacteria. Previously, we reported that low concentrations of nisin inhibited M. smegmatis and caused depletion of intracellular ATP (Montville et al. 1999).

The objectives of this study were to further investigate the action of nisin against M. smegmatis and to study the mode of action of nisin against M. bovis-Bacill Carmette Guerin (BCG). In this study, M. smegmatis and M. bovis-BCG were used as surrogates for studying pathogenic mycobacteria. Mycobacterium smegmatis is a fast-growing and non-pathogenic micro-organism, whereas M. bovis-BCG is a non-pathogenic vaccine strain of pathogenic M. bovis and belongs to the slowly growing mycobacteria, which are closely related to M. tuberculosis (Frothingham et al. 1994).

MATERIALS AND METHODS

Bacterial culture and media

Mycobacterium smegmatis strain MC²155 and M. bovis-BCG were provided by N. Connell (University of Medicine and Dentistry of New Jersey, National Tuberculosis Center, Newark, NJ, USA). Mycobacterium smegmatis cells were grown and maintained in trypticase soy broth without dextrose (BBL, Cockeysville, M.D., USA), supplemented with 0-6% yeast extract (Difco Laboratories, Detroit, MI, USA), 2% bovine serum albumin (Sigma Chemical Company, St. Louis, MO, USA) and 0-1% Tween 80 (Sigma) as previously described (Montville et al. 1999). Mid-log phase cells (O.D. 0.660 0·8–0·9; UV 160 U; Shimadzu, Japan) were used for experiments.

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Mycobacterium bovis-BCG cells were grown in Middlebrook 7H9 (Difco) broth supplemented with 0.5% Tween 80 and 10% acid dextrose catalase (ADC) at 37 °C with very gentle agitation (≈40 rev min⁻¹) for aeration. The ADC solutions were prepared by dissolving 50 g bovine serum albumin, 20 g glucose and 8.5 g NaCl in 11 distilled water and filter sterilizing. The broth was stored at 4 °C.

Listeria monocytogenes Scott A was used in this study as a positive control in the proton motive force assay. Listeria monocytogenes Scott A stock culture was maintained as previously described (Bruno and Montville 1993). Listeria monocytogenes Scott A cells were grown to mid-log phase (OD₆₀₀ 0.6 ~ 0.8) at 30 °C in trypticase soy broth, supplemented with 0.6% yeast extract and 0.5% glucose (TSBYEG) broth.

Preparation of nisin

Pure nisin (Ambicin®) was the gift of AMBI (Tarrytown, NY, USA) and contained 40 IU nisin µg⁻¹ protein. Nisin stock solutions were prepared in nisin diluent (0.02 N HCl + 0.75% NaCl, pH 2.0), autoclaved at pH 3.0 and then adjusted to pH 6.0 with 0.5 N NaOH. Working solutions (10⁻⁵ or 10⁻⁴ IU ml⁻¹) were prepared before every experiment to obtain appropriate final concentrations of nisin in the agar plates or broth. The same volume of nisin diluent (pH 6.5) was used as a control in the experiments.

Cell dry weight determination

The cell dry weights of M. smegmatis and M. bovis-BCG cells were determined according to Gerhardt (1994).

ATP assay

Mycobacterium bovis-BCG cells in mid-log phase were harvested and resuspended in 50 mmol l⁻¹ 2-(N-morpholino)-ethanesulphonate buffer (pH 6.5) supplemented with 0.06% Tween 80, 1.0% glycerol and 10 mmol l⁻¹ KCl. Total and external ATP levels were determined by the bioluminescence method described by Guihard et al. (1993) and Chen and Montville (1995) with modification (Montville et al. 1999) using a Lumac/3 M Biocounter M2010 (Schaeberg, The Netherlands). Internal ATP levels were calculated by subtracting external ATP from total ATP.

ΔpH measurement

The internal pH of cells was measured with the fluorescent pH indicator 2,7-bis-(2-carboxyethyl)-5-(and-6)-carboxy fluorescein, acetoxymethyl ester (Molecular Probe) as described by Molenaar et al. (1991).

Assays were performed using a spectrofluorometer with excitation of 502 nm with slit width of 5.0 nm and emission of 525 nm with slit width of 15.0 nm. Two ml 50 mmol l⁻¹ KPi buffer (pH 6.0) was added to a transparent cuvette, which was placed into the spectrofluorometer; the buffer was constantly stirred during the experiment. Five µl 20% glucose stock solution were added to energize cells and 10 µl 5 mmol l⁻¹ nigericin in 95% ethanol (final concentration 5 µmol l⁻¹) were added to convert ΔpH into ΔΨ. After the signal was stabilized, nisin was added and the effect of nisin concentration on the dissipation of ΔΨ was tested. Valinomycin in 95% ethanol was used as a control for complete dissipation of ΔΨ.

RESULTS AND DISCUSSION

Influence of nisin on intracellular and extracellular ATP levels

Nisin acts on the cytoplasmic membrane of Gram-positive bacteria (Ruhr and Sahl 1985; Kordel and Sahl 1986; Winkowski et al. 1994) by binding to the membrane, inserting into the membrane and forming pores (Winkowski et al. 1994). Based on the antimycobacterial activity of human and rabbit defensins (Ogata et al. 1992), we hypothesized that nisin might act against mycobacteria in a similar manner. Our previous report showed that nisin acts on the cytoplasmic membrane of fast-growing M. smegmatis and depletes intracellular ATP in about 4 h (Montville et al. 1999). In

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slowly growing *M. bovis*-BCG, nisin caused a slower, gradual decrease in internal ATP over time (Fig. 1). No ATP efflux was detected in *M. bovis*-BCG. Despite the thick cell wall of mycobacteria, nisin caused a concentration- and time-dependent decrease in intracellular ATP levels in *M. bovis*-BCG. Much higher nisin concentrations and longer incubation times were required to decrease total ATP levels of *M. bovis*-BCG than of *M. smegmatis*. This is consistent with the agar diffusion assay results. Ten µg ml⁻¹ and 2.5 mg ml⁻¹ nisin caused more than a 90% decrease in intracellular ATP levels in *M. smegmatis* and *M. bovis*-BCG, respectively, agreeing well with their minimum inhibitory dose (MID) values (Montville et al. 1999). Compared with the rapid action of nisin against *L. monocytogenes*, where complete ATP depletion occurs within minutes, significant ATP depletion was observed in *M. smegmatis* only after several hours of treatment with nisin and almost 10 h were required to deplete ATP in nisin-treated *M. bovis*-BCG cells.

We previously observed that only 3.8 ± 2.9% of the total intracellular ATP was lost due to efflux from *M. smegmatis* cells (Montville et al. 1999). There was no detectable efflux of ATP from *M. bovis*-BCG. Perhaps the pores formed in *M. bovis*-BCG membranes are large enough to leak protons but too small for ATP. Similar results were reported for *Enterococcus faecalis* cells treated with lactacin F (Abee et al., personal communication). The intracellular hydrolysis of ATP could be responsible for the decrease in internal ATP in both *M. smegmatis* and *M. bovis*-BCG cells. This observation is supported by previous reports of Winkowski et al. (1994) and Chen and Montville (1995) that cell death in *L. monocytogenes* treated with nisin A and pediocin PA-1 is mainly due to ATP hydrolysis, with only partial efflux of intracellular ATP.

**Effect of nisin on the membrane potential (ΔΨ) and transmembrane pH (∆pH) of sensitive cells**

The cytoplasmic membrane is also the site of energy transduction. Measuring the influence of nisin on the proton motive force provides another way of investigating its mode of action. The effect of nisin on the transmembrane potential (ΔΨ) and transmembrane pH (∆pH) components of the proton motive force can be measured separately. In food-borne pathogens, nisin induces the dissipation of ΔΨ and ∆pH and causes a depletion of intracellular ATP levels as well as a reduction in glycolysis (Bennik et al. 1997). Gao et al. (1991) and Bruno and Montville (1993) demonstrated that nisin dissipates both components of proton motive force in liposomes made from *Escherichia coli* and in *L. monocytogenes* cells, respectively. The direct measurement of ΔΨ in lantibiotic-treated cells showed that minimum membrane potential was required to cause pore formation (Bruno et al. 1993).

The experiments on ΔΨ and ∆pH dissipation were conducted on *M. smegmatis* and *M. bovis*-BCG; *L. monocytogenes* was used as a positive control since it has been previously used in mechanistic studies of bacteriocins (Bruno et al. 1993).

Figure 2 demonstrates the influence of nisin on the ΔΨ of whole cells of *L. monocytogenes* (Fig. 2a), *M. smegmatis* (Fig. 2b) and *M. bovis*-BCG (Fig. 2c), respectively. Nigericin causes the electroneutral exchange of a proton for a potassium ion, converting ∆pH to ΔΨ and was used to dissipate the pH gradient. Valinomycin (5 µmol l⁻¹) was used to confirm the dissipation of ΔΨ. It equilibrates the potassium ion concentration across the membrane. All three figures were normalized by subtracting the baseline curve of nigericin from the curves of nisin and valinomycin treatments. As shown in Fig. 2, the addition of nisin to the energized cells of all three strains caused partial dissipation of ΔΨ in a concentration-dependent manner. The extent of dissipation increased with higher concentrations of nisin. Valinomycin was less effective in the dissipation of ΔΨ in *L. monocytogenes* compared with the high concentrations of nisin, which showed complete collapse of ΔΨ (Fig. 2a). The ΔΨ dissipation by nisin in *M. smegmatis* and *M. bovis*-BCG (Figs 2b and 2c) was similar to that in *L. monocytogenes* and was concentration dependent.

The nisin-driven dissipation of ΔΨ was concentration dependent and appeared similar in *L. monocytogenes*, *M.
smegmatis and M. bovis-BCG cells regardless of their different sensitivities (in agar diffusion assays) to nisin. However, the extent of DC dissipation in the three microorganisms as a function of different nisin concentrations was relative to the sensitivity of the strains to nisin. A much lower nisin concentration was required to disturb the membrane potential of L. monocytogenes. The DC dissipation in M. smegmatis and M. bovis-BCG cells by nisin at three different concentrations (0-625, 12.5 and 50 μg ml⁻¹) at pH 7-0 were of similar rates, but the extent in M. smegmatis was somewhat higher.

Gao et al. (1991) reported that the rate of ΔΨ dissipation increased with the magnitude of the ΔΨ. The internal ATP concentration of three micro-organisms was highest in M. smegmatis (16.6 nmol mg⁻¹ cell dry weight (CDW)), then M. bovis-BCG (7.0 nmol mg⁻¹ CDW) and L. monocytogenes (2.5 nmol mg⁻¹ CDW) had the lowest energy levels (Chen and Montville 1995). Consistent with this report, the rate and extent of dissipation of ΔΨ were more pronounced in M. smegmatis and M. bovis-BCG cells than in L. monocytogenes cells.

The influence of nisin on the ΔpH in M. bovis-BCG was also investigated. Figure 3 shows the effect of nisin on the ΔpH of M. bovis-BCG at pH 6.0. Subtracting the valinomycin curve from the curves of nisin and nigericin treatment normalized the baselines. At pH 7.0, the ΔpH was generated on the addition of valinomycin; 50 μg ml⁻¹ nisin was required to cause partial dissipation of ΔpH but the effect of nisin was not very pronounced (data not shown). In contrast, at pH 6.0, increasing concentrations of nisin led to increasing dissipation of ΔpH. The ΔpH generated in M. bovis-BCG cells by valinomycin treatment was completely dissipated by the addition of 12.5 μmol l⁻¹ nigericin. This demonstrated that nisin dissipated the ΔpH in M. bovis-BCG cells (Fig. 3). The rate and degree were concentration dependent.

Interestingly, the effect of nisin on the ΔΨ and ΔpH dissipation in both M. smegmatis and M. bovis-BCG occurred within 5 min, similar to that in L. monocytogenes, despite the different time needed for nisin to deplete internal ATP levels in listeria and mycobacteria. Exposure of listeria and mycobacteria to nisin resulted in similar concentration-dependent ΔΨ dissipation. However, during the

![Fig. 2](image)

**Fig. 2** Concentration-dependent effect of nisin on the membrane potential of energized cells of (a) *Listeria monocytogenes*, (b) *Mycobacterium smegmatis* and (c) *M. bovis*-Bacill Carmette Guerin at pH 7.0. Nigericin (25 μmol l⁻¹) was used to dissipate ΔpH. The ΔΨ was measured with fluorescent probe 3,3’-dipropylthiadicarbocyanine (DisC35). Arrows indicate addition of valinomycin and nisin at concentrations of 0-625, 1.25, 6.25, 12.5, 25 and 50 μg ml⁻¹.

![Fig. 3](image)

**Fig. 3** Concentration-dependent effect of nisin on the ΔpH of energized *Mycobacterium bovis*-Bacill Carmette Guerin cells at pH 6.0. Valinomycin (5 μmol l⁻¹) was used to dissipate ΔΨ. The ΔpH was measured with the fluorescent pH indicator 2,7-bis-(2-carboxyethyl)-5-(and-6)-carboxy fluorescein acetoxyethyl ester. Arrows indicate addition of nigericin and nisin at concentrations of 0-625 and 50 μg ml⁻¹.
initial time period of 15 min after the nisin treatment, no changes in internal and external ATP levels were observed in either \textit{M. smegmatis} or \textit{M. bovis}-BCG (data not shown). Despite the dissipation of the $\Delta \psi$ within 5 min, \textit{M. smegmatis} and \textit{M. bovis}-BCG were able to maintain the high levels of intracellular ATP for a long period. This suggests that the kinetics of the events in mycobacterial cell death differ from those in food-borne pathogens.

On the basis of the data presented here, it can be concluded that nisin acts against mycobacteria and food-borne pathogens in a similar manner: it dissipates two components of proton motive force, the $\Delta \psi$ and the $\Delta \phi$, and causes loss of the permeability barrier of the cytoplasmic membrane. Therefore, the collapse of proton motive force and low intracellular ATP levels due to the hydrolysis and loss of intracellular ions, would contribute to the bactericidal action of nisin, leading to the death of mycobacteria.

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