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Microcin J25 triggers cytochrome *c* release through irreversible damage of mitochondrial proteins and lipids

María V. Niklison-Chirou[a], Fernando Dupuy[a], Liliana B. Pena[b], Susana M. Gallego[b], Maria Laura Barreiro-Arcos[c], Cesar Avila[a], Clarisa Torres-Bugeau[a], Beatriz E. Arcuri[a], Augusto Bellomio[a], Carlos Minahk[a], Roberto D. Morero[a,*]

[a] *Departamento de Bioquímica de la Nutrición, Instituto Superior de Investigaciones Biológicas (Consejo Nacional de Investigaciones Científicas y Técnicas—Universidad Nacional de Tucumán), Instituto de Química Biológica "Dr. Bernabe Bloj", Chacabuco 461, San Miguel de Tucumán 4000, Argentina*
[b] *Departamento de Química Biológica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina*
[c] *Centro de Estudios Farmacológicos y Botánicos CEFYBO-CONICET, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina*

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ABSTRACT

We previously showed that the antimicrobial peptide microcin J25 induced the over-production of reactive oxygen species with the concomitant release of cytochrome *c* from rat heart mitochondria via the opening of the mitochondrial permeability transition pore. Here, we were able to demonstrate that indeed, as a consequence of the oxidative burst, MccJ25 induces carbonylation of mitochondrial proteins, which may explain the irreversible inhibition of complex III and the partial inhibition of superoxide dismutase and catalase. Moreover, the peptide raised the levels of oxidized membrane lipids, which triggers the release of cytochrome *c*. From *in silico* analysis, we hypothesize that microcin would elicit these effects through interaction with heme *c*1 at mitochondrial complex III. On the other hand, under an excess of L-arginine, MccJ25 caused nitric oxide overproduction with no oxidative damage and a marked inhibition in oxygen consumption. Therefore, a beneficial anti-oxidative activity could be favored by the addition of L-arginine. Conversely, MccJ25 pro-oxidative–apoptotic effect can be unleashed in either an arginine-free medium or by suppressing the nitric oxide synthase activity.

1. Introduction

Microcin J25, a 21-amino acid antimicrobial peptide produced by an *Escherichia coli* strain, is active against close-related bacteria including certain human pathogens as some *Salmonella* and *Shigella* strains (Salomon and Farias, 1992). The peptide has an unusual lasso distinctive structure (Bayro et al., 2003; Rosengren et al., 2003; Wilson et al., 2003) and it is bacteriostatic agent in *E. coli* by inhibiting RNA polymerase causing an impaired transcription of genes encoding cell division proteins (Delgado et al., 2001). An alternative mechanism of action has been described on *Salmonella* serovar Newport cells (Rintoul et al., 2001). It was demonstrated that this antibiotic disrupts membrane integrity and therefore causes a dissipation of the membrane electrical potential in *Salmonella* serovar Newport cells. Furthermore, MccJ25 inhibits NADH and succinate dehydrogenase and alters the oxygen consumption rate (Rintoul et al., 2001). As a result, MccJ25 is a bactericidal peptide rather than a bacteriostatic one in these bacteria. In addition, an superoxide anion production was described in *E. coli* (Bellomio et al., 2007). It was recently shown that ROS was generated as a result of MccJ25–plasma membrane interaction. The ability of MccJ25 to interact with bacterial membranes is supported by studies carried out in liposomes (Rintoul et al., 2000). Moreover, MccJ25 is able to penetrate phospholipid monolayers at air–water interface in the absence of energy driven transport mechanism (Bellomio et al., 2005).

Recently, the effect of MccJ25 on intact rat heart mitochondria was explored (Niklison Chirou et al., 2004). The peptide displays a potent effect as inhibitor of the complex III, disrupts the $\Delta\Psi$ and drastically diminishes the internal ATP level. Moreover, we confirmed that MccJ25 induces superoxide overproduction, thus increasing the mitochondrial inner membrane permeability and activating the mitochondrial transition pore, resulting in swelling and cytochrome *c* release (Niklison Chirou et al., 2008).

Abbreviations: MTP, mitochondrial transition pore; MccJ25, microcin J25; ROS, reactive oxygen species; RNS, reactive nitrogen species; HbO_2, oxyhemoglobin; BSA, bovine serum albumin; TCA, trichloroacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; L-NMMA, L-NG-monomethyl-L-arginine; RCR, respiratory control rate; $\Delta\Psi$, transmembrane electrical potential; ATP, adenosine-5′-triphosphate; ADP, adenosine-5′-diphosphate; SMP, submitochondrial particles; Cu–Zn SOD, copper–zinc superoxide dismutase; 2,4 DNPH, 2,4 dinitrophenyl hydrazine.

* Corresponding author. Tel.: +54 0381 4248921; fax: +54 0381 4248025.
E-mail address: rdmorero@fbqf.unt.edu.ar (R.D. Morero).

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In the present work, we were able to show that MccJ25 induced carbonylation of mitochondrial proteins. Moreover, the peptide was able to oxidize membrane lipids, in particular cardiolipin, which would trigger the release of cytochrome *c*. A preliminary approach using molecular docking let us speculate that microcin may locate in close contact to heme *c*1 unleashing the oxidative damage as well as preventing cytochrome *c* reduction. However, under an excess of L-arginine, MccJ25 switched targets and stimulated mtNOS with no mitochondrial damage.

2. Materials and methods

2.1. Materials

MccJ25 was routinely purified from the supernatants of *E. coli* MC4100 cultures as described elsewhere (Bellomio et al., 2003). Rabbit anti-cytochrome *c*, ADP and ATP were purchased from Sigma (St. Louis, MO, USA). Cardiolipin was purchased from Avanti Polar Lipids (Alabaster, AL, USA). All other reagents used were of analytical grade or the purest available commercial form.

2.2. Mitochondrial isolation and preparation of submitochondrial membranes

Wistar rats (250–300 g) were killed by CO_2 inhalation, in accordance with the European directive for protection of vertebrate animals for scientific research. Rat hearts were homogenized in an ice-cold homogenization medium consisting of 0.23 M mannitol, 70 mM sucrose, 1 mM EGTA and 10 mM Tris–HCl (pH 7.4) with an Omni-Mixer (Sorvall, Norwalk, CT, USA). The homogenate was centrifuged at 900 × *g* for 10 min to discard nuclei and cell debris, and the supernatant was centrifuged at 17,000 × *g* for 10 min. The pellet, was washed and resuspended in the isolation buffer without EGTA (Niklison Chirou et al., 2008). In order to prepare submitochondrial particles, the mitochondrial pellet was resuspended (20 mg/ml) in 50 mM Tris–HCl (pH 7.6), 230 mM mannitol, 70 mM sucrose and sonicated three times, each consisting of a 30-s pulse burst, with 1-min intervals at 4 °C. The sonicated mitochondria were centrifuged at 8500 × *g* for 10 min to remove the unbroken organelles. The supernatant was centrifuged again at 100,000 × *g* for 60 min, and the resulting pellet washed and resuspended in the same buffer (Boveris, 1984). Protein concentration was assayed by the Folin reagent with bovine serum albumin as standard (Lowry et al., 1951).

2.3. Mitochondrial oxygen consumption

Oxygen uptake was determined with a Clark electrode in a 2 ml chamber at 30 °C, in an air-saturated reaction medium consisting of 0.23 M mannitol, 70 mM sucrose, 20 mM Tris–HCl, pH 7.4, 5 mM potassium phosphate, 4 mM $MgCl_2$ and 0.5 mg mitochondrial protein/ml. Respiratory rates were determined with 10 mM succinate as substrate for state 4, whereas state 3 was established by addition of 10 mM succinate and 1 mM ADP. Oxygen uptake was expressed as nanograms of oxygen per minute per milligram of membrane protein (Navarro et al., 2005).

2.4. Enzyme assays

Complex III activity was spectrophotometrically assayed following the changes of cytochrome *c* absorption at 550 nm (Niklison Chirou et al., 2004). Mitochondria were incubated with or without MccJ25 during 60 min at 37 °C with constant stirring. Then activities were measured before and after washing the organelles in order to determine the reversibility of the inhibition.

SOD activity was analyzed using nitroblue tetrazolium and riboflavin (Beauchamp and Fridovich, 1971). Catalase activity was tested using Amplex red reagent. Briefly, mitochondria were treated with or without MccJ25 as described above, then organelles were sonicated and incubated with 10 μM H_2O_2 for 10 min at 37 °C. After incubation, 200 μM Amplex red and 0.4 U/ml horseradish peroxidase were added and incubated 5 min. The resofurin fluorescence produced by remaining H_2O_2 was detected at 590 nm. Excitation wavelength was set at 530 nm.

2.5. Biochemical markers of oxidative stress

Carbonylated proteins were determined in mitochondrial membranes as originally described by Reinheckel et al. (2000). Briefly, mitochondria (2 mg protein/ml) suspended in 100 mM potassium phosphate buffer containing 10 mM succinate were supplemented with 50 μl of 10% trichloroacetic acid; the precipitated proteins were suspended in 50 μl of 0.2% 2,4-dinitrophenyl hydrazine, incubated 1 h at 37 °C, precipitated again with TCA. The precipitated was washed with ethanol:ethyl acetate (50:50), dissolved in 6 M guanidine hydrochloride in phosphate buffer (pH 6.5), and the absorbance determined at 370 nm (ε: 21 mM^{-1} cm^{-1}). Protein carbonyls were expressed as picomoles per milligram of mitochondrial protein.

For Western blot detection of carbonylated proteins, mitochondrial extracts (250 μg protein) that were previously derivatized with 2,4-dinitrophenylhydrazine (DNPH) (Levine et al., 1994) were separated by SDS-PAGE using 12% (w/v) running and 4% (w/v) stacking polyacrylamide gels, respectively (Laemmli, 1970). Two gels were run simultaneously: one for protein staining with Coomassie Brilliant Blue R-250 and the other for immunodetection. Derivatized proteins were transferred onto nitrocellulose membranes and were detected with rabbit anti-DNP primary antibody from Sigma–Aldrich (St. Louis, USA). Bands corresponding to oxidized proteins were visualized by secondary goat anti-rabbit immunoglobulins conjugated with horseradish peroxidase (DAKO), using 3,3′-diaminobenzidine (DAB) as substrate. Gels and membranes were photographed with Fotodyne equipment.

Carbonylated cytochrome *c* was investigated as follows: mitochondrial proteins (250 μg) derivatized with DNPH as mentioned above, were separated by affinity chromatography. Antibodies anti-DNP (50 μl) were linked to cyanogen bromide activated Sepharose 4% agarose matrix (100 μg) from Sigma–Aldrich. Samples were incubated overnight at 4 °C with an excess of anti-DNP-agarose resin and then centrifuged for 5 min at 10,000 × *g*. Resin beads were washed 3 times with Tris-buffered saline and finally re-suspended in 100 mM glycine–HCl (50 μl, pH 2.5). After centrifugation, the pellets were discarded, the supernatants adjusted to pH 6.8 with 0.5 M Tris–HCl buffer (5 μl, pH 8.8) and used for immunodetection of the cytochrome *c*. DNPH derivatized proteins were separated by 15% (w/v) SDS-PAGE. The proteins were electrotransfered onto polyvinylidene difluoride membranes and the cytochrome *c* was detected using anti-cytochrome *c* primary antibodies (Santa Cruz) and goat anti-rabbit immunoglobulins horseradish peroxidase conjugate (DAKO), with DAB as substrate. Membranes were photographed with Fotodyne equipment.

2.6. Determination of NADPH oxidation

Mitochondrial NADPH was monitored at 25 °C by measuring its intrinsic fluorescence at 450 nm (λ_{exc} 340 nm), in an ISS PC1 spectrofluorometer (Niklison Chirou et al., 2008). Mitochondria were suspended in Tris–potassium phosphate (pH 7.4), 150 mM sucrose, 50 mM KCl, 10 mM succinate, 1 μM rotenone and preincubated for 1 min in the presence or absence of 0.5 μM stigmatellin. Oxidation of NADPH was started by adding 20 μM MccJ25.