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Sphingosine impairs mitochondrial function by opening permeability transition pore

Sidi Mohamed Hassoun [a,b], Steve Lancel [a,b], Patrice Petillot [a], Brigitte Decoster [b], Raphael Favory [a,b], Philippe Marchetti [c], Remi Neviere [a,b,*]

[a] *EA 2689, CHRU and Université de Lille 2, IFR 114 IMPRT, Lille 59045, France*
[b] *Département de Physiologie, Faculté de Médecine, Université de Lille 2, Lille, France*
[c] *INSERM U459 U524, IMPRT, 1, Place Verdun, Lille 59045, France*

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Abstract

Growing evidence suggest that, in the heart, sphingosine participates to contractile dysfunction by altering calcium transients and mitochondria function. However, mechanisms underlying sphingosine-induced cardiac mitochondria dysfunction are poorly understood. Here, we studied the effects of sphingosine on isolated cardiac mitochondria of either wild-type or Bcl-2 overexpressing transgenic mice. Sphingosine induced reductions in ADP-coupled respiration, membrane potential, mitochondrial cytochrome *c* content and ATP production, which were partially prevented by cyclosporine A and mitochondrial Bcl-2 overexpression. These data suggest that sphingosine promotes mitochondrial permeability transition pore opening, which may result in uncoupled respiration and participate in cardiac contractile dysfunction.

Keywords: Sphingosine; Heart; Mitochondria; Cyclosporine; Bcl-2

1. Introduction

Pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, are involved in many common cardiac pathologies including myocardial reperfusion injury, chronic heart failure, and sepsis-induced myocardial dysfunction (Prabhu, 2004). In sepsis, myocardial depression is largely attributed to the effects of TNF-α on the heart. Within minutes, TNF-α reduces cardiomyocyte contractility by disturbing intracellular calcium homeostasis, and sphingomyelinase pathway activation has been suggested as underlying mechanism (Oral et al., 1997). Sphingomyelinases yield ceramide from membrane-bound sphingomyelin, which may be converted by ceramidase into sphingosine. This agent was shown in vitro to exert negative inotropic effects by altering cardiac excitation–contraction coupling (Oral et al., 1997). Precisely, sphingosine reduces calcium transients by inhibiting both L-type calcium channel conductance (McDonough et al., 1994) and sarcoplasmic reticulum calcium release (Sabbadini et al., 1992).

Apart from calcium homeostasis perturbations, acylated sphingosine, so-called ceramide, is known to suppress respiratory chain activity (Gudz et al., 1997) and modulate ionic permeability of the lipid component of the inner mitochondrial membrane (Di et al., 2004, 2000). Numerous reports have suggested that the opening of a permeability transition pore could be a primary event in initiation of cytochrome *c* release and in increased solute permeability of the inner mitochondrial membrane in presence of ceramide (Green and Kroemer, 2004; Halestrap et al., 2002). Mitochondrial permeability transition pore is a voltage-dependent, high conductance channel located in the inner mitochondrial membrane. The molecular identity of permeability transition pore has not been elucidated yet. Although the role of cyclophilin D (CyP-D) has now been well established, more controversial is the nature of the membrane components that undergo conformation changes to produce pore. The more

Abbreviations used MPTP, mitochondrial permeability transition pore; WT, wild-type; RCR, respiratory control ratio; TPP^+, tetraphenylphosphonium; NOE, *N*-oleoylethanolamine.

* Corresponding author. Address: Département de Physiologie, Faculté de Médecine, Université de Lille 2, 1, Place de Verdun, Lille Cedex 59045, France. Tel.: +33 3 20 62 69 68; fax: +33 3 20 62 69 93.
E-mail address: rneviere@univ-lille2.fr (R. Neviere).

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widely accepted candidate for the inner membrane protein is adenine nucleotide translocase (ANT). The pharmacological molecule cyclosporine A (CsA) prevents cyclophilin D binding to ANT and thus inhibits the permeability transition pore opening (Halestrap et al., 2002). Anti-apoptotic Bcl-2 proteins, such as Bcl-2 and Bcl-X_L, maintain the MPTP in a closed conformation (Green and Kroemer, 2004).

Our group has provided important results showing that cardiomyocyte sphingosine exposure induces impaired cell fractional shortening, reductions of which were timely related to rapid and sustained loss of mitochondrial membrane potential $\Delta\Psi_m$ (Favory et al., 2004). Hence, the general purpose of the present study was to unravel mechanisms by which sphingosine alters key functions of cardiac mitochondria. First, by measuring mitochondrial respiration, membrane potential, calcium retention capacity, cytochrome *c* content and ATP production, we described for the first time the direct effects of sphingosine on mitochondria isolated from wild-type mouse heart. Second, by the use of CsA and transgenic mice overexpressing the anti-apoptotic protein Bcl-2, we explored whether sphingosine induced MPTP opening and consequently mitochondrial dysfunction.

2. Materials and methods

2.1. Animals used

Wild-type (WT) C57Bl/6 6- to 10-week-old female mice and C57Bl/6 transgenic mice overexpressing Bcl-2 mice were used. All experiments were conducted in accordance with the European Institutes of Health guidelines for the use of laboratory animals.

2.2. Reagents

D-sphingosine and *N*-acetylsphingosine were purchased from Biomol (TEBU-BIO, France). *N*-oleylethanolamine was purchased from Sigma (Sigma, France).

2.3. Mitochondria isolation

Following mouse anaesthesia, heart was excised and rinsed in buffer A (in mM: sucrose 300, TES 5, EGTA 0.2, pH 7.2). After homogenization, lysate was first centrifuged at $800\times g$ for 10 min, the supernatant was centrifuged at $8800\times g$ for 10 min. Pellet was suspended in buffer A and centrifuged at $88{,}000\times g$ for 10 min. The final mitochondria pellet was suspended either in the respiration medium or in the medium used for mitochondria membrane potential evaluation. Purity and integrity of isolated mitochondria were assessed by measuring specific activities of NADPH-cytochrome *c* reductase, as a specific reticulum marker enzyme (Plonne et al., 1999), and cytochrome *c* oxidase, as an inner membrane marker enzyme (Storrie and Madden, 1990).

2.4. Mitochondrial respiration

Mitochondria were suspended in MiR05 (Renner et al., 2003) at 25 °C at the concentration of 200 μg/mL. Respiration parameters were evaluated by using high resolution Oxygraph 2k (Oroboros, Innsbruck, Austria): state 4 respiration rate (oxygen uptake with glutamate 5 mM malate 2 mM in the absence of exogenous ADP), state 3 respiration rate (oxygen uptake with 5 mM malate 2 mM and ADP 500 μM) and respiratory control ratio (RCR) as ratio of states 3 and 4 oxygen uptake rates.

2.5. Cytochrome c content

After respiration experiments, mitochondria were centrifuged at $15{,}000\times g$ for 5 min at 4 °C and stored at −80 °C until use. Fifty micrograms were used for western-blot analysis with mouse monoclonal anti-cytochrome *c* (BD Biosciences, Erembodegem, Belgium).

2.6. ATP production

Hundred micrograms of mitochondria were incubated in respiration medium containing 5 mM glutamate, 2 mM malate and 500 μM ADP for 2 min, at 25 °C. Then, ATP content was determined in 1 μg of mitochondria by the use of the ATP Bioluminescence HSII kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions.

2.7. Mitochondrial membrane potential measurement and calcium retention capacity

In brief, isolated mitochondria (1 mg/mL proteins) were suspended in buffer *c* (in mM): sucrose 250; Tris-MOPS 10; glutamate-Tris 5; malate-Tris 2; Pi-Tris 1; EGTA-Tris 0.02; pH 7.4 at 25 °C in a multiport measurement chamber (NOCHM-4, WPI, Aston, UK) equipped with Ca^{2+}, TPP^+-selective microelectrodes and reference electrodes (WPI, Aston, UK), as previously described (da Silva et al., 1998).

First, mitochondria were gently stirred for 1.5 min in buffer *c* containing 1.5 μM tetraphenylphosphonium TPP^+ (Sigma, Saint Quentin, France). Mitochondrial transmembrane potential $\Delta\Psi_m$ was estimated according to transmembrane distribution of TPP^+ (Kamo et al., 1979) and calculated as $59\log(v/V) - 59\log(10^{\Delta E/59} - 1)$, where v is mitochondrial matrix volume (1.1 μl/mg mitochondrial protein), V is volume chamber (1 mL), and ΔE is voltage difference (mV) between before and after 2,4 dinitrophenol (DNP) treatment (50 μM), used to fully induce $\Delta\Psi_m$ dissipation.

In another series of experiments, mitochondria calcium retention capacity was assessed as previously described (Basso et al., 2005). In brief, mitochondria were gently stirred for 1.5 min in buffer *c* and 20 μM $CaCl_2$ addition was performed every 90 s using a micro syringe injector adapted to a Micro4 pump controller (UMPII and Micro4, WPI, Aston, UK). Each 20 μM $CaCl_2$ pulse was detected as a peak of