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Mitochondrial calcium handling in normotensive and spontaneously hypertensive rats: Correlation with systolic blood pressure levels

Alejandro Ciocci Pardo, Gustavo J. Rinaldi, Susana M. Mosca [1]

Centro de Investigaciones Cardiovasculares, CCT-CONICET, Facultad de Ciencias Médicas, Universidad Nacional de La Plata, La Plata, Buenos Aires, Argentina

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ABSTRACT

The aim was to study the mitochondrial Ca^{2+} handling of mitochondria isolated from normotensive Wistar Kyoto (WKY) and spontaneously hypertensive rats (SHR) hearts and to establish a possible correlation with systolic blood pressure (SBP). Mitochondrial swelling after Ca^{2+} addition, Ca^{2+}-retention capacity (CRC) by calcium green method, and membrane potential (ΔΨm) was assessed. BP was 124 ± 1 (WKY) and 235 ± 6 mm Hg (SHR). CRC, Ca^{2+} response and ΔΨm were lower in SHR than WKY mitochondria. The conclusion is: the more depolarized state of SHR than WKY mitochondria results in an abnormal Ca^{2+} handling and this event is closely associated with the SBP.

1. Introduction

Previous studies show that opening of the permeability transition pore of mitochondria (mPTP) is critically involved in regulating cell death by inducing a sustained and irreversible loss of inner mitochondrial membrane potential, coinciding with mitochondrial swelling and rupture (Javadov and Karmazyn, 2007; Kroemer et al., 2007). The proteins involved in mPTP formation are localized in cytoplasm (hexokinase), outer membrane (VDAC: voltage-dependent anion channel), inner membrane (ANT: adenine nucleotide translocator), and mitochondrial matrix (cyclophilin D) (Baines, 2009). In the presence of oxidative stress and mitochondrial Ca^{2+} overload protein components are assembled such as a pore-forming radius of 1.0 to 1.3 nm, which allows the non-selective passage of molecules smaller than 1.5 kDa (Halestrap, 2009).

Homeostasis of mitochondrial Ca^{2+} is well maintained by a balance of Ca^{2+} uptake, sequestration and release mechanisms (Griffiths, 2009; Gunter and Sheu, 2009; Santo-Domingo and Demaurex, 2010). The main route for Ca^{2+} uptake is the ruthenium red-sensitive Ca^{2+} uniporter (Graier et al., 2007; Gunter and Pfeifer, 1990), which is in large part driven by the negative mitochondrial membrane potential (ΔΨm). The main route for release of mitochondrial Ca^{2+} is the mitochondrial Na^{+}/Ca^{2+} exchanger (NCX) (Dedkova and Blatter, 2008; Gunter and Pfeifer, 1990; Hoppe, 2010).

The mitochondrial dysfunction has been increasingly associated with the development of hypertension (Lopez-Campistrous et al., 2008). It has been proposed that oxidative stress (Dikalov and Ungvari, 2013) and abnormalities in Ca^{2+} handling (Failli et al., 1997; Sugiyama et al., 1990; Williams et al., 2014) play important role in the development of hypertension. However, there is no agreement regarding the disorders of Ca^{2+} management in a model of compensatory hypertrophy. Thus, in earlier studies, Postnov's group reported an increase in the Ca^{2+}-accumulating capacity of cardiac mitochondria from spontaneously hypertensive rats (SHR) (Orlov et al., 1980). A subsequent study (Aguilera-Aguirre et al., 2002) showed that mitochondrial Ca^{2+} uptake and accumulation capacity of SHR are lower compared to normotensive rats.

Therefore, the aim of the present study was to characterize the Ca^{2+} handling and changes of potential (ΔΨ) of mitochondria isolated from SHR and to its control normotensive Wistar Kyoto (WKY) and to establish a possible correlation with the systolic blood pressure.

2. Material and methods

2.1. Animals

We used normotensive (Wistar Kyoto, WKY) and spontaneously hypertensive rats (SHR) of 5 months of age. All procedures followed during this investigation conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and to the guidelines laid down by the Animal Welfare Committee of La Plata School of Medicine.

E-mail address: smosca@med.unlp.edu.ar (S.M. Mosca).
[1] Centro de Investigaciones Cardiovasculares, Universidad Nacional de La Plata, 60 y 120, 1900 La Plata, Argentina. Tel./fax: +54 221 425 5861.

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2.2. Systolic blood pressure measurement

For indirect systolic blood pressure (SBP) readings, the rats were placed in a chamber at 37 °C for 10 min, and then transferred to a standard setup with heating pad and acrylic restrainer, tail cuff and pulse sensor (Narco Biosystems, Houston, TX). The tail cuff was connected to a cylinder of compressed air through an arrangement of inlet and outlet valves that permitted inflation and deflation of the cuff at a constant rate. The tail cuff pressure was continuously recorded with a solid state pressure sensor (Sensym, Honeywell Sensing & Control, Inc.). The signals from the pulse and pressure sensors were conveniently amplified and then digitized with an analogy-digital board (DT16EZ, Data Translation, Inc., Marlboro, MA) mounted in a desktop computer. On-line display for controlling the procedure, and files for later processing, were obtained with appropriate software (Labtech Notebook Pro, Laboratory Technology Corp., Wilmington, MA). For each BP determination the inflation and deflation readings were always recorded, as well as the compression interval (Fritz and Rinaldi, 2008).

2.3. Isolation of rat heart mitochondria

Rat heart mitochondria were obtained by differential centrifugation (Mela and Seitz, 1979). Left ventricle (LV) was washed and homogenized in ice-cold isolation solution (IS) consisting of 75 mM sucrose, 225 mM mannitol, and 0.01 mM EGTA neutralized with Trizma buffer at pH 7.4. After the tissue pieces were settled, the entire supernatant was discarded and fresh IS (5 ml) was added, and the mixture was transferred to a hand homogenizer. Proteinase (0.8 mg, bacterial, type XXIV, Sigma, formerly called Nagarse) was added just before starting the homogenization procedure. The whole homogenization procedure took no longer than 14 min in two steps of 7 min each (with 5 ml addition of fresh IS each). The homogenate was carefully transferred after each step to a polycarbonate centrifuge tube. After 5 min of 750 ×*g* of centrifugation to discard unbroken tissue and debris, the supernatant was centrifuged at 8000 ×*g* for 10 min to sediment the mitochondria. The mitochondrial pellet was washed twice with IS and the last one with suspension solution (IS without EGTA) at 8000 ×*g* for 5 min each. The residue was washed and re-suspended in a cold solution containing mannitol and sucrose. The mitochondrial protein concentration was evaluated by the Bradford method (1976) using bovine serum albumin as standard. The purity of preparation was determined by immunodetection of the mitochondrial outer membrane voltage dependent anion channel (VDAC) and by the absence of the cytosolic glyceraldehyde phosphate dehydrogenase (GAPDH) (data not shown).

2.3.1. mPTP resistance to opening Ca^{2+}-mediated

The mPTP resistance to opening was assessed by incubating 0.3 mg/mL of isolated mitochondria from WKY and SHR in a buffer containing (in mmol/L): 120 KCl, 20 MOPS, 10 Tris HCl, and 5 KH_2PO4 adjusted to pH = 7.4. After 5-min preincubation, in the mitochondria energized with the addition of 5 mmol/L succinate was assessed the resistance to opening of mPTP with 20, 50, 100, 200, 500 and 1000 μmol/L $CaCl_2$. If mPTP is open, solutes will be free to enter the inner matrix, causing the mitochondria to swell. These changes are observed as decreases of light scattering and followed using a temperature-controlled Hitachi F4500 spectrofluorometer operating with continuous stirring at excitation and emission wavelengths of 520 nm (Baines et al., 2003). Light scattering decrease (LSD) was calculated for each sample by taking the difference of scattered light between before and after the addition of $CaCl_2$.

2.3.2. Calcium retention capacity

Calcium retention capacity (CRC) was defined here as the amount of Ca^{2+} required triggering a massive Ca^{2+} release by isolated cardiac mitochondria (Obame et al., 2008). It is used as an indicator of the resistance of the mPTP to opening after matrix Ca^{2+} accumulation and expressed as nmol $CaCl_2$ per mg of mitochondrial proteins. Extramitochondrial Ca^{2+} concentration was recorded with 0.5 μmol/L calcium green-5N (Invitrogen, Carlsbad, CA, USA) with excitation and emission wavelengths set at 506 and 532 nm, respectively. Isolated mitochondria from WKY and SHR (0.3 mg/ml) were suspended in 2 mL buffer (150 mmol/L sucrose, 50 mmol/L KCl, 2 mmol/L KH_2PO4, and 5 mmol/L succinate in 20 mmol/L Tris/HCl, pH 7.4). At the end of the preincubation period (300 s), successive pulses of 10 μM Ca^{2+} were added. When mitochondria cannot hold more Ca^{2+}, the mPTP is opened and the concentration of this ion increases in the incubation medium. After sufficient calcium loading, extramitochondrial calcium concentration abruptly increased, indicating a massive release of Ca^{2+} by mitochondria as a result of mPTP opening as previously described (Tang et al., 2007). Number of pulses, total time (TT, sec), pulse time (PT, sec) and velocity of Ca^{2+} efflux after mPTP opening were also measured.

2.3.3. Mitochondrial membrane potential

Mitochondrial potential changes were evaluated by measuring rhodamine-123 (RH-123) fluorescence quenching under the buffer described above containing RH-123 0.1 μM. As suggested by Emaus et al. (1986), the experimental work has been performed by exciting RH-123 at 503 nm and detecting the fluorescence emission at 527 nm. During the measurements, the reaction medium containing mitochondria (0.1 mg/ml) was continuously stirred. Mitochondrial membrane potential (ΔΨm) was calculated following the instructions previously detailed (Scaduto and Grotyohann, 1999) using the Nernst–Guggenheim equation. According to those authors, RH-123 uptake is in proportion to ΔΨm, therefore the rate of fluorescence quenching has also to be a function of ΔΨm, as well as the steady-state level of fluorescence decrease. Changes of ΔΨm produced by Ca^{2+} 20 and 200 μM addition with and without cyclosporine A (CsA) were also calculated.

2.4. Statistical analysis

Data were expressed as means ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Newman–Keul's post-test used for multiple comparisons among groups. Values of $p < 0.05$ were considered to indicate statistical significance.

3. Results

Fig. 1 shows a typical trace of pulse and pressure used for systolic blood pressure (SBP) measurement in WKY (A) and SHR (B). Mean data of SBP, plus the values of body weight (BW, g), heart weight (HW, mg) and hypertrophic index (HI) calculated as HW and BW ratio of both rat strains are displayed in Table 1. SBP and HI were significantly higher in SHR than WKY, indicating the presence of hypertrophy associated with high pressure as one recognized characteristic of hypertensive animals.

In Fig. 2 the mitochondrial swelling, measured as light scattering decrease (LSD), after different Ca^{2+} concentrations in samples derived from WKY and SHR hearts were depicted. The LSD produced by the addition of Ca^{2+} 20 μM was similar in both rat strains whereas that at higher Ca^{2+} concentrations significant differences between WKY and SHR were found. Thus, mPTP of mitochondria from SHR hearts shows a lesser response to Ca^{2+} than WKY. Additionally, the number of Ca^{2+} 10 μM pulses to produce the cessation of mitochondrial Ca^{2+} uptake and mPTP opening was significantly lower in hypertensive than normotensive rats (Fig. 3). Consequently, Ca^{2+} retention capacity (CRC) and total time (TT, sec) were significantly lesser whereas the time of each pulse (PT) was higher for SHR compared to WKY mitochondria. Thus, CRC acquired values of 180 ± 14 vs. 520 ± 40 nmol Ca^{2+}/mg prot,

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