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Changes in mitochondrial functionality and calcium uptake in hypertensive rats as a function of age

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Abstract

We studied whether mitochondrial functions and Ca^{2+} metabolism were altered in Wistar Kyoto normotensive (WKY) and spontaneous hypertensive rats (SHR). Ca^{2+} uptake was decreased in SHR compared to WKY rats. Accumulation of Ca^{2+} was more efficient in WKY than in SHR rats. $m\Delta\Psi$ was lower in SHR compared to WKY rats. Basal complex IV activity was higher in SHR than WKY rats, whereas basal L-citrulline production, an indicator of nitric oxide synthesis, was decreased in SHR and dependent on Ca^{2+} concentration ($p < 0.05$). Impact of Ca^{2+} was counteracted by EGTA. These data show an age-dependent decreased mitochondrial functions in brain mitochondria during hypertension.

Keywords: Brain mitochondria; Calcium uptake; Nitric oxide; Cytochrome *c* oxidase; Hypertension; Aging

1. Introduction

Intracellular free calcium ($[Ca^{2+}]_i$), plays an important role in cellular signaling. Accordingly, $[Ca^{2+}]_i$, is a second-messenger, implicated in the action of growth factors, hormones via transmitting signals to various kinases and enzymes (McCormack and Denton, 1993; Brini, 2003). Regulation of $[Ca^{2+}]_i$ concentration is controlled by the uptake and release via Ca^{2+} channels of plasma membrane, endoplasmic reticulum, and inner mitochondrial membrane (Nicholls, 1986). Intramitochondrial Ca^{2+} ($[Ca^{2+}]_m$) concentrations are maintained by active transport. It has been shown that rat liver and heart mitochondria contain 1–2 nmol Ca^{2+} per milligram of mitochondrial protein (cited in Ghafourifar and Saavedra-Molina, 2006). Mitochondria contain tightly regulated transport systems to maintain mitochondrial Ca^{2+} levels. The Ca^{2+} uptake takes place via an electrophoretic uniporter that is driven by the transmembrane potential (Nicholls, 2004, 2005). Mitochondrial release of Ca^{2+} ions is achieved by reversal of the influx carrier, and by a Na^+ dependent (Ca^{2+}/Na^+) or independent (Ca^{2+}/H^+) exchanger. The Ca^{2+}/Na^+ pathway predominates in mitochondria of heart, brain, skeletal muscle, adrenal cortex, brown fat, and most tumor tissues. The Ca^{2+}/H^+ system is important in liver, kidney, lung, and smooth muscle mitochondria (McCormack and Denton, 1993; Richter, 1997; Bringold et al., 2000).

The hypertension is an established risk factor for cerebrovascular disease. A mechanism contributing to maintain high blood pressure during acute hypertension is the increased generation of superoxide ($O_2^{\cdot -}$) in the vessel wall, which leads to vascular injury (Hougaku et al., 1992; Ohtsuki et al., 1995; Halliwell and Gutteridge, 1999). The chronic hypertension selectively causes cell damage and injury in hypertensive-vulnerable organs like brain. Brain and nervous tissue are particularly prone to oxidative

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damage for several reasons: the high Ca^{2+} traffic across neuronal membranes, the presence of excitotoxic amino acids; some neurotransmitters are autoxidizable molecules and could generate $O_2^{\cdot -}$, the neuronal membrane lipids contains a high content of polyunsaturated fatty-acids, which are targets of oxygen-derived free radicals, the brain is relatively deficient in antioxidants defenses and the high rate of O_2 consumption in the tissue (Ohtsuki et al., 1995; Halliwell and Gutteridge, 1999). Brain mitochondria consume about 90% of the oxygen used by cells, and the mitochondrial respiratory chain generates high concentrations of reactive oxygen species (ROS) that attack cellular macromolecules, oxidize membranous phospholipids, proteins and DNA (Ohtsuki et al., 1995).

Spontaneously hypertensive rats (SHR) exhibit several abnormalities regarding Ca^{2+} metabolism. These abnormalities include decrease in Ca^{2+} level in the serum, hypercalciuria, and alteration in intestinal Ca^{2+} transport. An increment in $[Ca^{2+}]_I$, had been reported in platelets and lymphocytes from hypertensive humans and in SHR (Arab et al., 1990). In mitochondria the basal matrix Ca^{2+} concentration is significantly lower in heart and kidney tissues from SHR compared to normotensive control animals (Aguilera-Aguirre et al., 2002). Over production of ROS in respiratory chain of brain mitochondria during hypertension can impair progressively mitochondrial energy metabolism and may be implicated in the vulnerability of SHR to cerebral ischemia, resulting in a progressive neuronal cell death (Ohtsuki et al., 1995).

Mitochondrial enzymes, such as pyruvate dehydrogenase, $NAD(P)^+$-dependent isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase, and mitochondrial nitric oxide synthase (mtNOS) can be activated by increase in Ca^{2+} level (Hansford and Zorov, 1998; Ghafourifar and Cadenas, 2005). mtNOS catalyzes the oxidation of L-arginine to nitric oxide ($NO^{\cdot}$) and citrulline. $NO^{\cdot}$ is a free radical that binds to cytochrome *c* oxidase (complex IV) of the mitochondrial electron transport chain to inhibit respiration by competitive action with oxygen in complex IV, and therefore regulates the membrane potential ($\Delta\Psi$) and ATP synthesis (Giulivi et al., 1998; Ghafourifar and Richter, 1999; Aguilera-Aguirre et al., 2002; Calderón-Cortés et al., 2006). mtNOS and the enzymes mentioned above regulate ATP synthesis to support cellular energetic requirements (Bringold et al., 2000; Traaseth et al., 2004).

The goal of this study was to address the hypothesis that during hypertension, $[Ca^{2+}]$m and Ca^{2+}-regulated NOS activity are altered in brain mitochondria by the impairment on $\Delta\Psi$ establishment, due to decreased mitochondrial electron transport chain at the level of complex IV.

In this study we utilized healthy young, pre-hypertensive (1-month) and adult (7-months) rats with established hypertension (middle age, spontaneously hypertensive: SHR). In control experiments, the normotensive Wistar Kyoto (WKY) rats were used (1- and 7-months-old), We studied whether Ca^{2+}-driven $NO^{\cdot}$-mediated inhibition of complex IV activity could alter calcium transport-energy dependent mitochondrial function in brain of rats, which may be related to augmentation of blood pressure.

2. Materials and methods

2.1. Chemicals

EGTA, KH_2PO_4, $MgCl_2$, mannitol, sucrose, bovine serum albumin (fatty acid free), MOPS, sodium deoxycholate, percoll, calcium chloride, KH_2PO_4, succinic acid, arsenazo III, safranine, rotenone, antimycin-A, ascorbic acid, ruthenium red (RR), *N*,*N*,*N*,*N*-tetramethyl-*p*-phenylenediamine (TMPD), carbonylcyanide-*m*-chlorophenylhydrazone (CCCP), and potassium cyanide (KCN) were purchased from Sigma Chemical Co. Stigmatellin was obtained from Fluka Biochemika. 4-(6-Acetoxymethoxy-2,7-dichoro-3-oxo-9-xanthenyl)-4′-methyl2,2′-(ethylene dioxy)dianiline-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis (acetoxymethyl) ester (Fluo-3/AM), *S*-nitroso-*N*-acetylpenicilamine (SNAP), and pluronic acid were purchased from Molecular Probes.

2.2. Animals

Normotensive genetic control (WKY) and SHR rats of 1- and 7-months of age were fed *ad libitum* and kept under controlled conditions of light:darkness in our animal facilities. All animal procedures were conducted in accordance with our Federal Regulations for the Use and Care of Animals (NOM-062-ZOO-1999, Ministry of Agriculture, Mexico), and were approved by the Institutional Committee of the Universidad Michoacana de San Nicolás de Hidalgo, on the use of animals. Systolic blood pressure was determined by plethysmography.

2.3. Isolation of rat brain mitochondria

Brain mitochondria were isolated by differential centrifugation in a Percoll gradient as described (Thakar and Hassan, 1988; Sims, 1990). Briefly, rats were decapitated and the brain was extracted and placed in a cold medium that contained 210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 0.5% bovine serum albumin, 10 mM MOPS (pH 7.4). The brain was homogenized manually in a glass homogenizer and centrifuged at 400*g*; the supernatant was centrifuged at 9000*g*. Centrifugations were carried out for 10 min at 4 °C. The pellet was suspended in 15% Percoll and placed in a discontinuous Percoll gradient (23 and 40%). The gradient was centrifuged at 30,700*g*, 15 min. at 4 °C, and mitochondria were isolated, diluted 1:4, centrifuged and washed at 16,700*g* in the isolation medium to which 0.5% bovine serum albumin had been added, followed by centrifugation at 6900*g* for 10 min. Mitochondrial protein concentration was measured by the Lowry technique (Lowry et al., 1951). The purity of mitochondrial preparations was assessed by Western blotting. The endoplasmic reticulum marker calreticulin was