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Brain Region-Specific, Age-Related, Alterations in Mitochondrial Responses to Elevated Calcium

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An age-related Ca^{2+} dysregulation and increased production of reactive oxygen species (ROS) may contribute to late-onset neurodegenerative disorders. These alterations are often attributed to impaired mitochondrial function yet few studies have directly examined mitochondria isolated from various regions of the aged brain. The purpose of this study was to examine Ca^{2+}-buffering and ROS production in mitochondria isolated from Fischer 344 rats ranging in age from 4 to 25 months. Mitchondria isolated from the cortex of the 25 month rat brain exhibited greater rates of ROS production and mitochondrial swelling in response to increasing Ca^{2+} loads as compared to mitochondria isolated from younger (4, 13 month) animals. The increased swelling is indicative of opening of the mitochondrial permeability transition pore indicating impaired Ca^{2+} buffering/cycling in aged animals. These age-related differences were not observed in mitochondria isolated from cerebellum. Together, these results demonstrate region specific, age-related, alterations in mitochondrial responses to Ca^{2+}.

KEY WORDS: Mitochondria; aging; calcium; reactive oxygen species; rat; mitochondrial permeability transition.

INTRODUCTION

Mitochondria serve a number of functions including maintenance of cellular bioenergetics, Ca^{2+} regulation, control of cell death cascades, and represent the major intracellular source of reactive oxygen species (ROS) (Fiskum, 2000). Although numerous studies have demonstrated increased oxidative stress and ROS production in the aged brain (Floyd and Hensley, 2002; Joseph *et al*., 2000) the underlying cause is uncertain. The elevated oxidative damage could represent cumulative damage due to increased ROS production, a decline in antioxidant defenses, or a decrease in the degradation of oxidized proteins and lipids.

Previous studies have demonstrated an age-related increase in ROS production using rat brain homogenates (Baek *et al*., 1999; Driver *et al*., 2000), cortical slices (Kannurpatti *et al*., 2004), or synaptosomal preparations (Choi, 1995). However, it is not known if the source of increased ROS production is mitochondrial in orgin. To determine if alterations in mitochondria contribute to the age-related increases in ROS production and mitochondrial permeability transition (mPT), we examined mitochondria isolated from the cortex, hippocampus, and cerebellum of rats of 4, 13, and 25 months of age.

MATERIALS AND METHODS

Mitochondrial Isolation

All experimental protocols involving animals were approved by the University of Kentucky Animal Use and Care Committee and the reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

This mitochondrial isolation protocol has been previously described (Brown *et al*., in press; Sullivan *et al*., 2000, 2003, 2004) and all procedures were performed on ice. Briefly, male Fischer 344 (F344) rats of

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3 different ages were decapitated and the brains rapidly removed. The cortices, hippocampi, and cerebellum were dissected out and separately placed in an all-glass dounce homogenizer containing five times the volume of isolation buffer with 1 mM EGTA (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 1 mM EGTA, pH 7.2). The tissues were homogenized and mitochondria were isolated by differential centrifugation. The homogenate was spun twice at 1300 $\times$ g for 3 min in an eppendorf microcentrifuge at 4°C and the supernatant was transferred to new tubes. The resulting supernatant was topped off with isolation buffer with EGTA and spun at 13,000 $\times$ g for 10 min. The supernatant was discarded; the pellet was resuspended in 500 μL of isolation buffer with EGTA; and put under a pressure of 1000 psi for 5 min (or up to a maximum of 1200 psi for 10 min) inside the nitrogen cell disruption bomb from Parr Instrument Company (Moline, IL) at 4°C. After bursting of synaptosomes, the samples were brought up to a final volume of 2 mL with isolation buffer with EGTA, and centrifuged at 13,000 $\times$ g for 10 min. The pellet was resuspended in isolation buffer without EGTA and centrifuged at 10,000 $\times g$ for 10 min. The final mitochondrial pellet was resuspended in isolation buffer without EGTA to yield a concetration of $\sim$10 mg/mL. The protein concentration was determined using the bicinchoninic acid protein assay kit from Pierce (Rockford, IL) by measuring absorbance at 560 nm with a Biotek Synergy HT plate reader (Winooski, VT). The respiratory activity of isolated mitochondria was measured using a Clark-type oxygen electrode (Hansatech Instruments, Norfolk, England) and only mitochondrial preparations with respiratory control ratios above 5 were used in the studies.

Mitochondrial Reactive Oxygen Species

Mitochondrial reactive oxygen species formation was measured using 2′-7′-dichlorodihydro-fluorescein (DCF) diacetate (Molecular probes) as described previously with some slight modifications (Sensi *et al*., 2003; Sullivan *et al*., 2003). Isolated mitochondria (1 mg/mL) were placed in a thermostatically controlled, constantly stirred cuvette in a total volume of 1.5 mL containing respiration buffer (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 2 mM MgCl, 2.5 mM KH_2PO_4 at pH 7.2) with 5 mM pyruvate, 2.5. mM malate, 25 U/mL horseradish peroxidase and 10 μM DCF. The arbitrary fluorescence units were measured using a Shimadzu RF-5301PC spectrofluorophotometer (excitation 485 nm, emission 530 nm). Each run was performed identically with a baseline reading of mitochondria in buffer followed by a bolus of 50 μM $CaCl_2$ and then a bolus of 100 μM $CaCl_2$ for a total run time of 5 min per sample per region. The slope of DCF fluorescence was quantified for the respective conditions including baseline, after addition of 50 μM Ca^{2+}, and after 150 μM total exogenous Ca^{2+} using the Shimadzu Hyper RF software. Results are expressed as mean percent of baseline slope $\pm$ SEM from five animals in each age group. Statistical analyses were performed using a oneway analysis of variance with the Bonferroni/Dunn post hoc analysis, $p < 0.05$.

Mitochondrial Swelling

Isolated mitochondria (1 mg/mL) were incubated at 37°C in de-energizing buffer of 150 mM KCl, 20 mM MOPS, 10 mM Tris, 2 μM ionomycin, 1 μM rotenone, and 1 μM antimycin-A as described previously (Sensi *et al*., 2003; Sullivan *et al*., 2003). After 5 min, calcium (or only buffer in controls) was added to mitochondria and the absorbance (540 nm) was measured. Mitochondrial swelling represents a decrease in absorbance, normalized, averaged and expressed in arbitrary units (AU). All measurements were made with a Biotek Synergy HT plate reader. This data represent six animals from each age group with three replicates per animal.

RESULTS

In this study, male F344 rats were used from three different age groups (groups, age, mean weight): young, 4 months old, 206.5 g; middle, 13 months old, 450.7 g; and aged, 25 months, 426.5 g. From each animal, total mitochondria were isolated separately from the cortex, hippocampus, and cerebellum. We observed increased rates of DCF fluorescence in response to elevated [Ca^{2+}] in mitochondria isolated from the cortex and hippocampus (Fig. 1(a) and (b)), but not cerebellum (Fig. 1(c)), of aged rats as compared to younger animals. Specifically, isolated cortical mitochondria from the aged rat had significantly increased rates of DCF fluorescence as compared to the young- and middle-age groups at both concentrations of calcium. In addition, the aged hippocampal mitochondria had significantly greater rates of ROS generation than the young-age group at the 50 μM and 150 μM [Ca^{2+}]. Aged cortical and hippocampal mitochondria (Fig. 2(a) and (b)) underwent more rapid and larger magnitude swelling than corticla and hippocampal mitochondria from younger rats. In contrast, cerebellar mitochondria showed no age-related changes in the ROS generation (Fig. 1(c)) or mitochondrial swelling (Fig. 2(c)) across the three age groups.

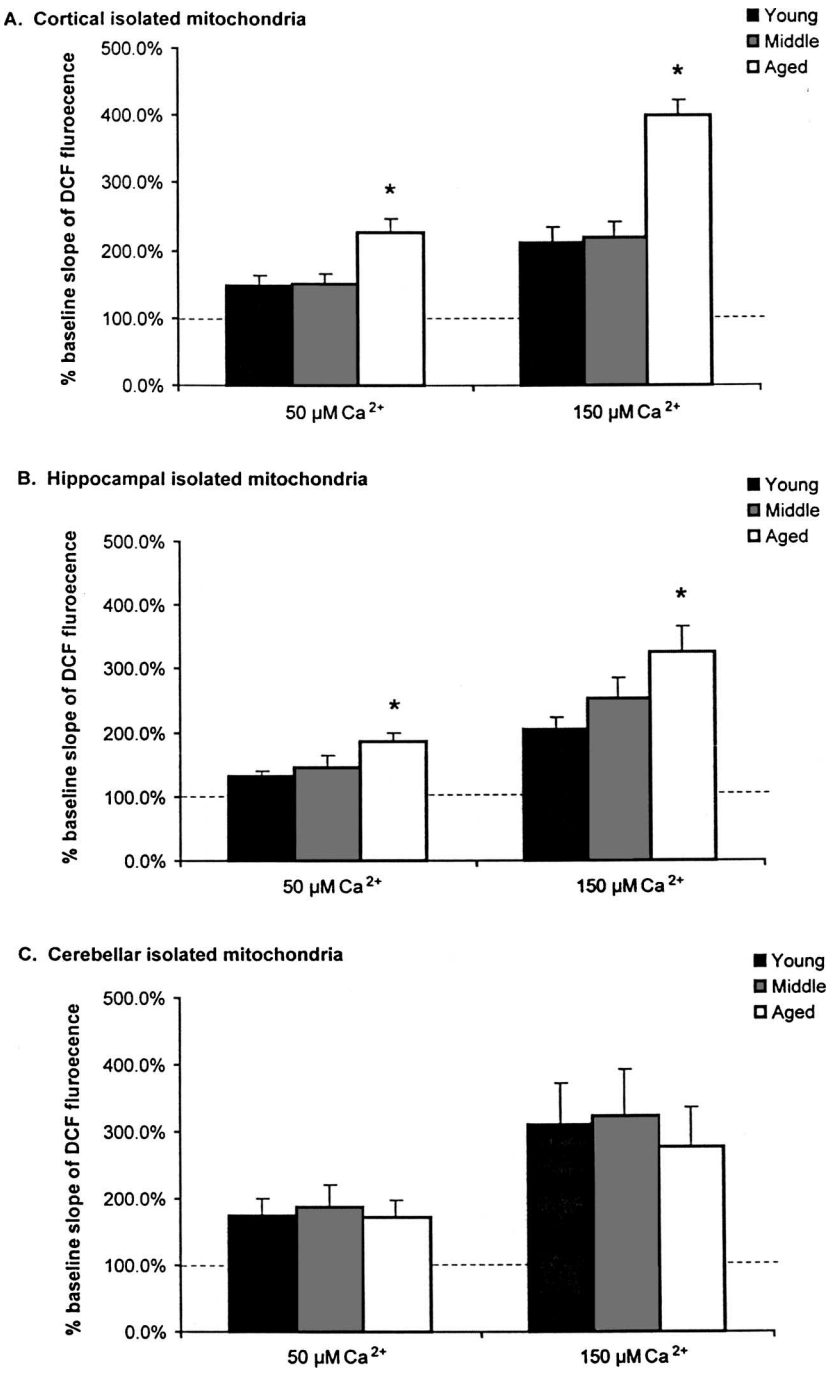

Fig. 1. Age-related differences in the rate of DCF fluorescence from three regions of the rat brain. There was an increased rate of DCF fluorescence in isolated cortical (a) and hippocampal (b) mitochondria from aged rats as compared to young- and middle-age rats after the addition of increasing concentrations of Ca^{2+} (cumulative concentration of 50 μM and 150 μM). However, there was no age dependant increase in the rate of DCF fluorescence in isolated cerebellar (c) mitochondria after the addition of increasing concentrations of calcium. The dotted line represents the baseline slope of DCF fluorescence (of 100%) on each graph. Statistical analyses were performed using one-way analysis of variance with Bonferroni/Dunn post hoc analysis. $p < 0.05$. Results are expressed as percent of baseline slope $\pm$ SEM from five animals in each age group with two replicates per animal.

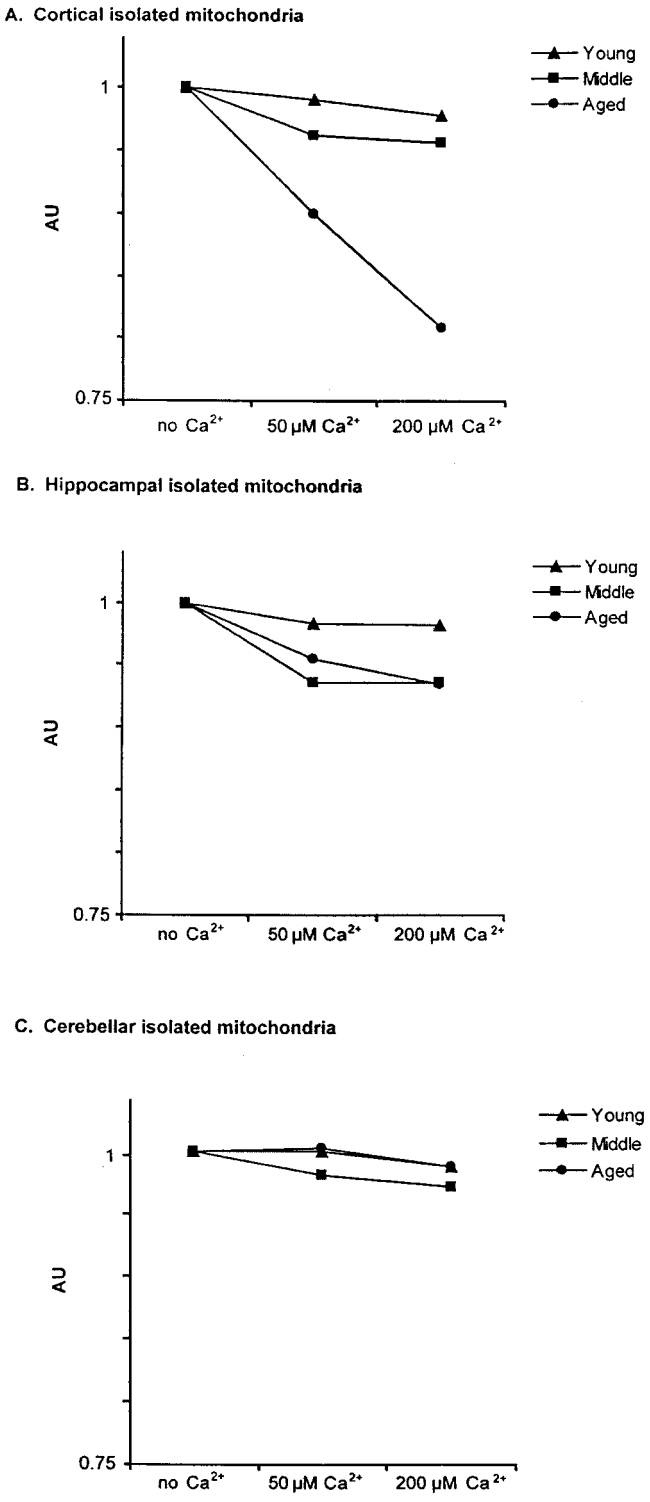

Fig. 2. Ca^{2+}-induced swelling of mitochondria changes with age in isolated cortical (a) and hippocampal (b) mitochondria from F344 male rats, but not in cerebellar (c) mitochondria. Aged cortical mitochondria underwent increased swelling compared to younger rat cortical mitochondria. This data represents six animals from each age group with three replicates per animal.

DISCUSSION

The results demonstrate an increased rate of Ca^{2+}-stimulated ROS production in cortical and hippocampal, but not cerebellar, mitochondria as a function of age. The localization of the increased mitochondrial ROS production to brain regions vulnerable to late-onset neurodegenerative disorders (cortex, hippocampus) differs slightly from results obtained with homogenates in which ROS production increased with age in the cortex, but not in hippocampus or cerebellum (Back *et al*., 1999). The mechanism(s) underlying the regional changes in Ca^{2+} stimulated mitochondrial ROS production are uncertain,but are hypothesized to reflect an age-related accumulation of oxidative damage to the mitochondria. The cortex and hippocampus both contain relatively high densities of *N*-methyl-D-aspartate receptors and may be more prone to glutamate-induced elevations in intraneuronal Ca^{2+} and the subsequent mitochondrial damage. However, the cell types in which the age-related mitochondrial alterations occurred were not examined in the present study. Current studies in our laboratory are examining ROS production in synaptic versus nonsynaptic mitochondrial fractions.

The mechanism underlying the increased rate of ROS production in response to Ca^{2+} is not clear (Galindo *et al*., 2003; Gordeeva *et al*., 2003; Sousa *et al*., 2003). A previous view was that Ca^{2+} influx uncoupled the proton gradient from ATP synthase, resulting in increased flux through the electron transport chain and therefore increased ROS production. However, proton ionophores or chemical uncouplers such as FCCP result in decreased mitochondrial ROS production (Hansford *et al*., 1997; Sullivan *et al*., 2003). Recent data suggest that Complex I is a major source of ROS production (Liu *et al*., 2002), either through succinate oxidation and reverse electron flow from Complex II or via the oxidation of NADH-dependent respiratory susbtrates by Complex I (Starkov and Fiskum, 2003). Importantly, in our studies we utilized the NADH-linked substrates malate and pyruvate to mimic more physiologically relevant conditions than succinate driven respiration. By inducing mitochondrial swelling and the release of cytochrome *c* from mitochondria, Ca^{2+} may disrupt the efficiency of electron transport and thereby elevate ROS production. Mitochondrial nitric oxide synthase (mtNOS) is another likely source of ROS/RNS in response to Ca^{2+}. Importantly, the DCF used to measure ROS production in this study and many other studies also reacts with reactive nitrogen species (Rao *et al*., 1992). There is evidence of a mitochondrial, Ca^{2+}-dependent, nitric oxide synthase that is distinct from the brain or neuronal NOS isoform (Ghafourifar and Richter, 1997; Lacza *et al*., 2003) although nisic intentions (Gao *et al*., 2004). We are currently investigating the contribution of NOS activity to the Ca^{2+}-induced elevation of DCF fluorescence using mitochondria-enriched preparations.

Although neurons are largely maintained in the aging brain, they become less capable of regulating intracellular Ca^{2+}, display evidence of increased oxidative stress, and contain damaged and dysfunctional mitochondria. These alterations have each spawned their own theories of aging including the "calcium hypothesis" (Khachaturian, 1987; Landfield, 1987; Thibault *et al*., 1998), the "free radical/oxidative stress" hypotheses (Ames and Shigenaga, 1992; Butterfield *et al*., 1999; Harman, 1973), and the "damaged mitochondria" hypothesis (Fleming *et al*., 1982; Kowald, 2001; Nicholls, 2002). These hypotheses are interrelated, making it difficult to differentiate between cause and effect. For example, mitochondrial Ca^{2+} levels are closely linked to free cytosolic Ca^{2+}, and any condition that elevates cytosolic Ca^{2+} would likely increase mitochondrial Ca^{2+} and ROS production. Therefore, it was not clear if age-related increases in ROS production were the result of mitochondrial or nonmitochondrial mechanisms. The results of the present study however clearly demostrate that mitochondria isolated from the cortex and hippocampus of aged rats have greater rates of ROS production in response to elevated Ca^{2+} than do mitochondria isolated from young animals. Additionally mitochondria isolated from older animals swell in response to much lower levels of Ca^{2+} than mitochondria from younger animals. These data would indicate that age-related changes in mitochondrial function to Ca^{2+} could contribute directly to cellular Ca^{2+} dysregulation and increased oxidative stress associated with senescence.

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