Experimental Neurology 223 (2010) 557–565

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Lifelong protection from global cerebral ischemia and reperfusion in long-lived *Mclk1*$^{+/-}$ mutants

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ARTICLE INFO

Article history:
Received 22 October 2009
Revised 1 February 2010
Accepted 9 February 2010
Available online 17 February 2010

Keywords:
Mclk1
Coq7
clk-1
Ubiquinone
Ischemia
Reperfusion
Aging
Age-related diseases
BCCAO
Oxidative stress

ABSTRACT

To achieve a long life span, animals must be resistant to various injuries as well as avoid or delay lethality from age-dependent diseases. Reduced expression of the mitochondrial enzyme CLK-1/MCLK1 (a.k.a. Coq7), a mitochondrial hydroxylase that is necessary for the biosynthesis of ubiquinone (UQ), extends lifespan in *Caenorhabditis elegans* and in mice. Here, we show that long-lived *Mclk1*$^{+/-}$ mutants have enhanced resistance to neurological damage following global cerebral ischemia–reperfusion (I/R) injury induced by transient bilateral common carotid artery occlusion (BCCAO). Both young (~100 days old) and relatively aged (~450 days old) mutants display increased resistance as indicated by a significant decrease in the amount of degenerating cells observed in forebrain cortex and in hippocampal areas after ischemia and reperfusion. Furthermore, less oxidative damage resulting from the procedure was measured in the brain of young *Mclk1*$^{+/-}$ animals. The finding that both young and old mutants are protected indicates that this is a basic phenotype of these mutants and not a secondary consequence of their slow rate of aging. Thus, the partial resistance to I/R injury suggests that *Mclk1*$^{+/-}$ mutants have an enhanced recovery potential following age-dependant vascular accidents, which correlates well with their longer survival. By relating this neuroprotective effect to previously reported characteristics of the *Mclk1*$^{+/-}$ phenotype, including altered mitochondrial metabolism and increased HIF-1α expression, this study establishes these mutants as useful models to analyze the mechanisms underlying tolerance to ischemia, particularly those associated with ischemic preconditioning, as well as to clarify the relation between aging and age-dependent diseases.

Introduction

CLK-1/MCLK1 is a mitochondrial hydroxylase that is necessary for the biosynthesis of ubiquinone (coenzyme Q or UQ), the essential electron transporter of the mitochondrial respiratory chain that is also known for its crucial antioxidant properties (Levavasseur et al., 2001). We have previously reported that inactivation of *clk-1* in *Caenorhabditis elegans* (Wong et al., 1995) and partial inactivation of its orthologue in *Mclk1*$^{+/-}$ mice (Liu et al., 2005), resulted in a significantly prolonged lifespan. By studying various aspects of the mitochondrial and metabolic phenotype of the long-lived *Mclk1*$^{+/-}$ mutants, we have found that mitochondria of young *Mclk1*$^{+/-}$ mice display slow electron transport, contain low levels of ATP, and sustain high oxidative stress (Lapointe and Hekimi, 2008). Despite the early mitochondrial dysfunction, the function of *Mclk1*$^{+/-}$ mitochondria declines less rapidly with age than that of the wild type, and there is a slower accumulation of global oxidative biomarkers of aging in these mutants (Lapointe et al., 2009). Furthermore, we succeeded in demonstrating a causal link between the altered mitochondrial function of young mutants and the improved age-dependent phenotypes of aged mutants, by showing that the *Mclk1*$^{+/-}$ condition fully prevents the accelerated deterioration of mitochondrial function and the increased mitochondrial oxidative stress observed in *Sod2*$^{+/-}$ mutants (Lapointe et al., 2009). Recently, we reported that the altered mitochondrial phenotype of *Mclk1*$^{+/-}$ mutants modulates the immune response by enhancing basal and stimulated expression of HIF-1α in liver and macrophages, in association with elevated expression of inflammatory cytokines (Wang et al., 2010).

To increase lifespan, it is generally assumed that a genetic manipulation such as the reduction of *Mclk1* expression must be able to reduce the prevalence or severity of diseases that normally limit lifespan (Hekimi, 2006). This could be achieved by slowing down the development of the cellular dysfunctions that result from the aging process (e.g., loss of mitochondrial function, increased oxidative stress, or chronic inflammation) and that typically exacerbate a number of diseases including endothelial dysfunction and stroke. These kinds of diseases are frequently called age-dependent or age-associated diseases. A prolongation of lifespan could also be observed if the mutants were simply resistant to an age-dependent pathology without necessarily having induced a change in the age-dependency of the pathology. To address these questions in the *Mclk1*$^{+/-}$ mutant context, we have tested here whether both young and old animals showed some degree

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doi:10.1016/j.expneurol.2010.02.002

of tolerance to cerebral ischemia–reperfusion (I/R) injury, which is linked to severe diseases connected to aging, mitochondrial function, oxidative stress, and immune function.

When blood flow to a tissue is interrupted or severely reduced as occurs during ischemia, the essential oxygen supply needed to maintain cellular homeostasis becomes insufficient. This can arise in a variety of situations; in particular as a consequence of obstruction of arteries, with the heart and the brain have been found to be the most commonly affected (Doyle et al., 2008; Lesnefsky and Hoppel, 2003). If tissue ischemia occurs for a prolonged period, cell death is the main consequence, but even when the ischemic episode is relatively brief, the reestablishment of blood flow at the time of reperfusion and the resulting oxygen delivery is known to induce a highly harmful process that leads to cellular damage and cell death (Warner et al., 2004). Indeed, I/R brain injury is similar to the pathophysiology of stroke and brain trauma, which are among the leading causes of death and disability in people (Rosamond et al., 2007). The vascular obstructions at the origin of ischemic insults are typically the consequence of age-dependent processes such as atherosclerosis and heart arrhythmias and are also strongly favored by endothelial dysfunctions and general vascular deterioration that are hallmarks of aging (Matsumoto et al., 2007; Park et al., 2007; Schaller, 2007; Westrick et al., 2007). Thus, the prevalence of I/R injury as well as its outcome has been shown to be profoundly influenced by age (Yager et al., 2005). It is not yet well understood how exactly I/R episodes act to damage cells. However, it has been demonstrated that it involves mitochondria and particularly the toxic properties of the mitochondrial reactive oxygen species (ROS) (Fiskum et al., 2004; Friberg et al., 2002; Lesnefsky et al., 2001).

To test whether the long life of *Mclk1*$^{+/-}$ mutants involved a resistance to the damaging processes underlying I/R injury, we used a surgical procedure, transient bilateral common carotid occlusion (BCCAO), to induce cerebral I/R in young and old animals and scored the resultant neuronal damage. We find that the mutants have an enhanced resistance to I/R at all ages as revealed by decreased levels of degenerating cells and less oxidative damage after the procedure. This indicates that the resistance is due to the basic changes in physiology induced by the reduction in MCLK1 levels, but not to the fact that *Mclk1*$^{+/-}$ and *Mclk1*$^{+/+}$ animals age at different rates (Lapointe et al., 2009). Although it is unknown whether vascular accidents limit the lifespan of caged mice, the resistance we observe is at the very least likely to help aged animals to survive for longer the development of other types of age-related pathologies.

Materials and methods

Animals

Heterozygous *Mclk1* mutant mice (*Mclk1*$^{+/-}$) and their wild type littermates (*Mclk1*$^{+/+}$) were produced as previous reported (Levavasseur et al., 2001; Liu et al., 2005) and were maintained in the C57BL/6 J and Balb/c backgrounds by inbreeding. A total of 62 male mice in the C57BL/6 J background (young animals) and 56 female mice in the Balb/c background (aged animals) were used. Experimental groups with their corresponding sample sizes are listed in Table 1. All procedures were approved by McGill's Animal Care and Ethics committees.

Global cerebral ischemia protocol

Experimental animals were subjected to transient global ischemia by bilateral common carotid artery occlusion (BCCAO), a well-defined I/R procedure for mimicking cerebrovascular accidents, including in transgenic mice models (Cho et al., 2007; Kelly et al., 2001). Before the operation, mice were anesthetized with a specific ketamine cocktail containing 50% ketamine, 25% xylazine, 10% acepromazine, and 15 % saline by intraperitoneal injection using 1 μl/mg body weight. Body temperature was strictly kept at 37 ± 0.5 °C by a feedback homeothermic blanket with a rectal probe (Harvard Apparatus, South Natick, MA). A midline skin incision on the ventral surface of the neck was made. The salivary glands were moved laterally, and the carotid sheath was exposed bilaterally. The common carotid arteries were then carefully separated from adjacent vagus and sympathetic nerves with a microdissector and forceps. Microvascular clips (B-2, Fine Science Tools) were applied to both isolated common carotid arteries to block brain blood flow for the desired time: 10 minutes for young mice and 5 minutes for aged mice. After the operation, the animals were kept on the heating blanket until they awoke from anesthesia and then transferred to their home cage at room temperature. Control animals (sham-operated) underwent the same operation procedures but were not subjected to the carotid occlusion. Aged animals were treated with only 5 minutes of ischemia because it was found that longer treatment resulted in inacceptable number of deaths before 24 hours of reperfusion. Animals that died within less than 24 hours after the beginning of reperfusion were excluded.

Table 1
Experimental groups and animals.

	Groups	Controls (sham-operated)	BCCAO-operated	Average age (days)	Body weight (g)
Young animals	*Mclk1*$^{+/+}$	11	20	107 ± 13	27.9 ± 2.7
(males/C57BL/6)	*Mclk1*$^{+/-}$	11	20	104 ± 12	27.1 ± 2.7
Aged animals	*Mclk1*$^{+/+}$	12	18	548 ± 10	28.6 ± 3.6
(females/Balb/c)	*Mclk1*$^{+/-}$	9	15	550 ± 16	28.7 ± 3.9

Cerebral blood flow determination

Cerebral blood flow changes were monitored by a Doppler flow meter (BLF21, Transonic Systems Inc, Ithaca, NY). The flow meter was connected to a data acquisition systems (MP150, BIOPAC Systems Inc, Goleta, CA), which is itself connected to a computer and operated by the Acknowledge 3.8 software. A needle probe was connected to the flow meter and held by a micromanipulator. To measure brain blood flow, the anesthetized mouse was put in a prone position. A 0.5-cm cross-line incision was then made, and the skull was exposed. The calibrated flow meter was warmed up for 10 minutes, and the probe was put on the skull surface at the middle right hemisphere. Brain blood flow was measured for 10 seconds at 3 time points, namely before ischemia, after 5 minutes of arteries occlusion (young mice) or 3 minutes (aged mice), and 5 minutes after ischemia, to record normal blood flow, blood flow reduction during occlusion, and brain blood flow resumption after ischemia, respectively. Cerebral blood flow was reported as milliliters per milligram per minute. Mean blood flow during the 10-second recording was used for each measurement.

Histopathology

After 24 hours of reperfusion, animals were anesthetized by using the previously described ketamine cocktail and sacrificed by neck dislocation. Brain was washed by transcardiac infusion of saline, then carefully extracted from the skull and cut into two halves through the midline. The left hemisphere was snap frozen in liquid nitrogen and stored at −80 °C for further biochemical studies. The right hemisphere was fixed in 4% paraformaldehyde overnight and then cut coronally into 4 blocks at preselected levels which are located at +1.5 mm from bregma (layer 1), bregma (layer 2), −1.5 mm from bregma (layer 3), and −3 mm bregma (layer 4), before being embedded in paraffin. Coronal sections of 4 μm thicknesses were then generated and subsequently stained with hematoxylin–eosin (HE).

For estimating brain pathological changes following I/R, we have used 4 HE stained sections per preselected level. To quantify brain damage, degenerated cells were counted using a computer-assisted