

Delaying the Mitochondrial Decay of Aging with Acetylcarnitine

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ABSTRACT: Oxidative mitochondrial decay is a major contributor to aging. Some of this decay can be reversed in old rats by feeding them normal mitochondrial metabolites, acetylcarnitine (ALC) and lipoic acid (LA), at high levels. Feeding the substrate ALC with LA, a mitochondrial antioxidant, restores the velocity of the reaction (K_m) for ALC transferase and mitochondrial function. The principle appears to be that, with age, increased oxidative damage to protein causes a deformation of structure of key enzymes with a consequent lessening of affinity (K_m) for the enzyme substrate. The effect of age on the enzyme-binding affinity can be mimicked by reacting it with malondialdehyde (a lipid peroxidation product that increases with age). In old rats (vs. young rats), mitochondrial membrane potential, cardiolipin level, respiratory control ratio, and cellular O_2 uptake are lower; oxidants/ O_2 , neuron RNA oxidation, and mutagenic aldehydes from lipid peroxidation are higher. Ambulatory activity and cognition decline with age. Feeding old rats ALC with LA for a few weeks restores mitochondrial function; lowers oxidants, neuron RNA oxidation, and mutagenic aldehydes; and increases rat ambulatory activity and cognition (as assayed with the Skinner box and Morris water maze). A recent meta-analysis of 21 double-blind clinical trials of ALC in the treatment of mild cognitive impairment and mild Alzheimer's disease showed significant efficacy vs. placebo. A meta-analysis of 4 clinical trials of LA for treatment of neuropathic deficits in diabetes showed significant efficacy vs. placebo.

KEYWORDS: acetylcarnitine (ALC); L-carnitine; mitochondrial decay; aging; lipoic acid (LA); brain

MITOCHONDRIAL DECAY WITH AGE

Mitochondrial dysfunction may be a principal underlying event in aging, including brain degeneration.¹⁻³ Mitochondria provide energy for basic metabolic processes, and their decay with age impairs cellular metabolism and leads to cellular decline. Mitochondrial membrane potential, respiratory control ratios, and cellular oxygen consumption decline with age, and oxidant production increases.³⁻⁷ Both genetic and epigenetic changes to mitochondria and to cells may be involved. Mutations in genes that encode mitochondrial proteins could compromise mitochondria by altering components of the electron transport chain,³ resulting in inefficient electron trans-

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Ann. N.Y. Acad. Sci. 1033: 108–116 (2004). © 2004 New York Academy of Sciences.
doi: 10.1196/annals.1320.010

port and increased superoxide production.⁵ The resultant oxidative damage to mitochondria may compromise their ability to meet the steady energy demands of the brain. Oxidized proteins accumulate with age,⁸ and these may also cause mitochondrial inefficiencies, leading to oxidant formation, perhaps due, in part, to deformation of enzyme structure leading to a poorer K_m for substrate, which could be ameliorated by higher substrate levels.⁹ For example, the mitochondrial complexes III and IV show a significant increase in K_m and decrease in V_{max} with age.¹⁰ Oxidants may also cause increased damage and consume critical metabolites such as ubiquinone or small-molecular-weight antioxidants. The significant loss of cardiolipin in aging may be in part because of greater oxidative damage and/or reduced biosynthesis. Loss of cardiolipin, coupled with oxidation of critical thiol groups in key proteins, may adversely affect transport of substrates and cytochrome *c* oxidase activity¹¹ necessary for mitochondrial function. These changes could directly impact the ability of mitochondria to maintain their membrane potential.

Mitochondrial function during aging was assessed in isolated rat hepatocytes to avoid the problem of differential lysis when old, fragile mitochondria are isolated.⁴ Rhodamine-123, a fluorescent dye that accumulates in mitochondria on the basis of their membrane potential, was used as a probe to determine whether this key function is affected by aging. A marked fluorescent heterogeneity was observed in hepatocytes from old rats (20–28 months), but not young rats (3–5 months), suggesting age-associated alterations in mitochondrial membrane potential, and the driving force for ATP synthesis. Three distinct cell subpopulations were separated by centrifugal elutriation; each exhibited a unique rhodamine-123 fluorescence pattern, with the largest population from old rats having significantly lower fluorescence than that seen in young rats. This apparent age-associated alteration in mitochondrial membrane potential was confirmed by measurements with radioactive tetraphenylphosphonium bromide. Cells from young rats had a calculated membrane potential of 154 mV, in contrast to that of the three subpopulations from old rats of 70 mV (the largest population), 93 mV, and 154 mV. Production of oxidants was examined using 2,7-dichlorofluorescein, a dye that forms a fluorescent product upon oxidation. The largest cell subpopulation and a minor one from old animals produced significantly more oxidants than cells from young rats. To investigate the molecular cause(s) for the heterogeneity, we determined the levels of an age-associated mtDNA deletion. No significant differences were seen in the three subpopulations, indicating that the mitochondrial decay is due to other mutations, epigenetic changes, or both.¹²

ACETYL-L-CARNITINE/L-CARNITINE

L-Carnitine has been described as a conditionally essential nutrient for humans. L-Carnitine is a betaine required for the transport of long-chain fatty acids into the mitochondria for fuel. It also facilitates the removal from the mitochondria of the excess short- and medium-chain fatty acids that accumulate during fat metabolism. Tissue levels of carnitine in animals, including humans, decrease with age, thus reducing the integrity of the mitochondrial membrane.

Acetylcarnitine (ALC) is more widely used than L-carnitine in animal research and clinical trials to gain metabolic benefits to the brain, heart, and other organs. In aging or in conditions of disease, ALC is better absorbed and more efficiently

crosses the blood-brain barrier as compared to L-carnitine.¹³ Mitochondrial function and ambulatory activity were monitored after feeding old rats the mitochondrial metabolite ALC.¹⁴ ALC supplementation significantly reverses the age-associated decline of mitochondrial membrane potential as assessed by rhodamine-123 staining. Cardiolipin, which declines significantly with age, is also restored. ALC increases cellular oxygen consumption, which declines with age, to the level of young rats. Ambulatory activity, a measure of general metabolic activity, defined as mean total distance traveled, in old rats is almost threefold lower than that in young animals. ALC supplementation increases ambulatory activity significantly in both young and old rats, with the increase being larger in old rats. ALC supplementation to old rats markedly reverses the age-associated decline in many indices of mitochondrial function and general metabolic activity, but does not decrease oxidative stress.¹⁴ Our results are consistent with the work of Paradies and colleagues that shows that ALC stabilizes the inner mitochondrial membrane, increases cardiolipin levels in the heart, and reverses the decline in activity of a number of mitochondrial translocases and of cytochrome *c* oxidase.^{6,11,15,16}

In animals, the effect most studied is improvement of age-associated cognitive dysfunction, including tests of the Morris water maze for spatial memory,¹⁷ active avoidance learning,¹⁸ discrimination learning,¹⁹ radial maze,²⁰ and long-term memory performance in the split-stem T-maze.²¹ These tests show that ALC improves the age-associated decline of learning and memory in old animals. Long-term ALC feeding decreases mortality, does not interfere with food and water intake, and increases longevity.²² ALC affects physical activity in different ways, depending on the experiment. Onofrij *et al.*²³ found that ALC-treated rats show heightened arousal, while Blokland *et al.*²⁴ found that ALC-treated old rats defecate more, make fewer crossings, and spend more time in the corner squares in an open-field test; they interpreted this in terms of an enhanced emotional reactivity of old rats treated with ALC.

ALC improves nerve regeneration in rats²⁵ and protects neurons from the toxicity of mitochondrial uncouplers or inhibitors.²⁶ Feeding old rats ALC restores levels of this metabolite to those found in tissues of young rats.²⁷ Paradies *et al.* fed old rats ALC and studied the effects on heart mitochondrial function: they showed that ALC feeding increases cardiolipin content; elevates activities of cytochrome *c* oxidase and adenine nucleotide translocase; and increases the rates of phosphate and pyruvate transport, palmitoylcarnitine-supported respiration, and carnitine-carnitine and carnitine-palmitoylcarnitine exchange reactions.^{6,11,15,28–30} ALC also attenuates neurologic damage after brain ischemia and reperfusion in canines;³¹ elevates levels of glutathione (GSH) and γ -aminobutyric acid in the brains of mice;³² and increases the activities of NADH-cytochrome *c* oxidoreductase, succinate cytochrome *c* oxidoreductase, and cytochrome *c* oxidase in synaptosomes isolated from spf mice, an animal model to study the neuropathology of congenital ornithine transcarbamylase deficiency.³³ ALC also seems to possess antiapoptotic properties.³⁴

Clinical trials with ALC showed some improvements in cognitively impaired alcoholics³⁵ and in those with Alzheimer's disease (AD) or dementia-associated cognitive dysfunction. A meta-analysis of 21 double-blind, placebo-controlled prospective, clinical trials of 3- to 12-month duration on the efficacy of ALC in mild cognitive impairment and early AD concluded that ALC had a significant beneficial response compared to placebo;³⁶ beneficial effects of ALC supplementation were seen on both the clinical scales and the psychometric tests at the time of the first

assessment at 3 months, and the benefits increased over time.³⁶ Another analysis of 11 clinical trials of AD patients found that there was evidence for benefit of ALC on clinical global impression, but there was no evidence using objective assessments in any other area of outcome.³⁷ The significant effect on early AD and mild impairment and the less significant effect on AD patients may suggest that ALC is more effective in preventing and slowing the progression of AD than in treating severe AD.

One of the mechanisms for improvement of cognition is that ALC helps the brain maintain a constant supply of energy by boosting the levels of phospholipid precursors for membrane synthesis.³⁸ ALC treatment in old rats delays progression in hearing loss, and reduces age-associated mtDNA deletions and presbycusis by up-regulating mitochondrial function and improving energy-producing capabilities.³⁹ Kuratsune *et al.* showed that patients with chronic fatigue syndrome have a deficiency of serum acylcarnitine, and ALC supplementation improves daily activity and reduces symptoms.^{40,41} They investigated brain uptake in rhesus monkeys of ALC labeled in different positions by positron emission tomography and found a high uptake of [²⁻¹¹C]ALC into the brain, suggesting that endogenous serum ALC has some role in conveying an acetyl moiety into the brain, especially under an energy crisis.⁴²

ALC DOSE-RESPONSE STUDY

Our previous study showed that feeding old rats ALC converted the mitochondria of liver to a more youthful state, both structurally and functionally, and increased ambulatory activity in the old rats, but caused an increase in oxidants.¹⁴ Increased oxidants were found to be a side effect of the very high dose used. We carried out a dose-response study on the effects of lower doses of ALC on rat brain function, mitochondrial morphological change, and oxidative stress in old rats.²⁷ ALC was administered at 0.15%, 0.5%, and 1.5% in drinking water for 4 weeks. We found that there was an age-related decrease in carnitine levels in the brain and plasma, with an age-related increase in the liver. The increased level of carnitines in liver may suggest an impaired net transport of carnitine from the liver to the blood in old animals because there is an age-dependent decrease in the plasma. All the doses of ALC (4 weeks) showed significantly increased levels of carnitine, dependent on dose, in the brain and plasma, without apparent changes in the liver. The high dose (1.5%) for a shorter term (2 weeks) also seems effective in elevating the carnitine levels in the brain and plasma. Administration of carnitine, as well as ALC, also effectively elevated the carnitine levels in the brain and plasma.²⁷

The lower concentrations of ALC (0.15% and especially 0.5%) ameliorated the age-associated decline in ambulatory activity and mitochondrial crista loss in the dentate gyrus of the hippocampus more effectively than the 1.5% dose.²⁷ The lower doses had no effect on protein oxidation, in contrast to the 1.5% dose, which caused an increase in protein carbonyls in the brain. Furthermore, lower doses (0.15%) also reduced the age-dependent increase in malondialdehyde, an end product of lipid peroxidation, more effectively than the 1.5% dose (data not shown). These results suggest that (1) oxidative stress in the brain, a side effect, only occurs at very high doses of ALC administration to old rats and (2) a lower dose of ALC administered to old rats can improve brain function by partially reversing the age-associated mito-

chondrial decay, by repairing mitochondrial structure, and by reducing oxidative stress.^{27,43–46} Our accompanying paper⁴⁷ discusses this issue in greater detail.

LIPOIC ACID

R-Lipoic acid (LA) can be reduced in mitochondria to form the coenzyme dihydro-lipoic acid. LA is also an effective inducer^{48,49} of phase-2 enzymes, over 200 of them, including GSH synthesis, which protect against sulfhydryl oxidation and alkylation.⁵⁰

DELAYING THE MITOCHONDRIAL DECAY OF AGING

Oxidative mitochondrial decay is a major contributor to aging.^{3,4,51–53} We are making progress in reversing some of this decay in old rats by feeding them normal mitochondrial metabolites, ALC and LA, at high levels. The principle behind this effect appears to be that, with age, increased oxidative damage to protein causes a deformation of structure of key enzymes with a consequent lessening of affinity (K_m) for the enzyme substrate.⁵⁴ The effect of age on the enzyme-binding affinity of ALC transferase can be mimicked by reacting it with malondialdehyde (a lipid peroxidation product that increases with age). Feeding the substrate ALC with LA, a mitochondrial antioxidant, restores the velocity of the reaction (K_m) for ALC transferase and mitochondrial function.⁵⁴ In old rats (vs. young rats), mitochondrial membrane potential, cardiolipin level, respiratory control ratio, and cellular O₂ uptake are lower; oxidants/O₂, neuron RNA oxidation, and mutagenic aldehydes from lipid peroxidation are higher.^{4,14,54–60} Ambulatory activity and cognition decline with age.^{55,56} Feeding old rats ALC with LA for a few weeks restores mitochondrial function; lowers oxidants, neuron RNA oxidation, and mutagenic aldehydes; and increases rat ambulatory activity and cognition (as assayed with the Skinner box and Morris water maze).^{54–56} A recent meta-analysis of 21 double-blind clinical trials of ALC in the treatment of mild cognitive impairment and mild AD showed significant efficacy vs. placebo.³⁶ A meta-analysis of 4 clinical trials of LA for treatment of neuropathic deficits in diabetes showed significant efficacy vs. placebo.⁶¹

DELAYING BRAIN AGING

Accumulation of oxidative damage to mitochondria, protein, and nucleic acid in the brain may lead to neuronal and cognitive dysfunction. The effects on cognitive function, brain mitochondrial structure, and biomarkers of oxidative damage were studied after feeding old rats ALC [0.5% or 0.2% (w/v) in drinking water] and/or LA [0.2% or 0.1% (w/w) in diet].⁵⁵ Using Morris water maze–assessed spatial memory, temporal memory was tested by using the peak procedure (a time-discrimination procedure). Dietary supplementation with ALC and/or LA improved memory, the combination being the most effective for two different tests of spatial memory ($P < 0.05$; $P < 0.01$) and for temporal memory ($P < 0.05$). Immunohistochemical analysis showed that oxidative damage to nucleic acids (8-hydroxyguanosine and 8-hydroxy-2'-deoxyguanosine) increased with age in the hippocampus, a region important for

memory. Oxidative damage to nucleic acids occurred predominantly in RNA. Dietary administration of ALC and/or LA significantly reduced the extent of oxidized RNA, the combination being the most effective. Electron microscopic studies in the hippocampus showed that ALC and/or LA reversed age-associated mitochondrial structural decay. These results suggest that feeding ALC and LA to old rats improves performance on memory tasks by lowering oxidative damage and improving mitochondrial function.⁵⁴

To clarify the mechanism for the beneficial effects of ALC plus LA, we tested whether the dysfunction with age of carnitine acetyltransferase (CAT), a key mitochondrial enzyme for fat oxidation, is due to decreased binding affinity for substrate and whether this substrate, fed to old rats, restores CAT activity.⁵⁴ The kinetics of CAT were analyzed by using the brains of young and old rats and of old rats supplemented for 7 weeks with the CAT substrate, ALC, and/or the mitochondrial antioxidant precursor, LA. Old rats, compared with young rats, showed a decrease in CAT activity and in CAT-binding affinity for both substrates, ALC and CoA. Feeding ALC or ALC plus LA to old rats significantly restored CAT-binding affinity for ALC and CoA, as well as CAT activity. To explore the underlying mechanism, lipid peroxidation and total iron and copper levels were assayed: all increased in old rats. Feeding old rats LA or LA plus ALC inhibited lipid peroxidation, but did not decrease iron and copper levels. *Ex vivo* oxidation of young rat brain with Fe(II) caused loss of CAT activity and binding affinity. *In vitro* oxidation of purified CAT with Fe(II) inactivated the enzyme, but did not alter binding affinity. However, *in vitro* treatment of CAT with the lipid peroxidation products, MDA or 4-hydroxynonenal, caused a decrease in CAT-binding affinity and activity, thus mimicking age-related change. Preincubation of CAT with ALC or CoA prevented MDA-induced dysfunction. Thus, feeding old rats high levels of key mitochondrial metabolites can ameliorate oxidative damage, enzyme activity, substrate-binding affinity, and mitochondrial dysfunction.⁵⁴

ACKNOWLEDGMENTS

This work was supported by the Ellison Medical Foundation Grant SS-0422-99, the National Institute on Aging Grant AG17140, the Wheeler Foundation Fund of the University of California, the National Institute of Environmental Health Sciences Center Grant ES01896, and the National Center for Complementary and Alternative Medicine Research Scientist Award K05 AT001323-4. We thank Sigma-Tau for acetylcarnitine and Viatris for *R*-lipoic acid.

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