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Perspective

Current Thoughts on the Role of Mitochondria and Free Radicals in the Biology of Aging

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THE charge of this discussion group was to assess what we know about the role of mitochondrial oxidative stress in aging and to determine potential directions for future research in this area. The Free Radical or Oxidative Stress Theory of Aging posed by Harman (1) just more than 50 years ago has been one of the leading and most studied theories in aging research. The basis of this theory is that free radical species generated by normal cellular processes translate to gradual and cumulative damage over the life span of an organism and contribute to age-related declines in physiological function and an increase in age-related pathologies. Despite the attractive and logical nature of this tenet, the existing evidence in support of the role of oxidative stress in aging remains essentially correlative. In addition, some recent studies using transgenic and knockout mouse models have provided data that do not support the theory as originally stated. For example, mice deficient in manganese superoxide dismutase (MnSOD) show increased oxidative damage to DNA, increased pathology, and yet no reduction in life span (2). Likewise, a number of other mouse models with increased or reduced levels of key antioxidant enzymes do not show changes in life span (3–5; Y. Zhang et al., in preparation). Part of the reason for the lack of conclusive evidence in support of this theory is that experimental approaches to date have not provided clear proof or tests of the principles underlying the theory. Below, we discuss some potential problems that may have contributed to the lack of conclusive evidence in this area to date, and we suggest strategies to more definitely investigate the role of oxidative stress in aging.

MITOCHONDRIAL DYSFUNCTION AND THE FREE RADICAL THEORY OF AGING

Because mitochondria are a primary source of cellular reactive oxygen species (ROS), it is logical that mitochon-

dria and mitochondrial dysfunction have been identified as key components behind the free radical theory. In fact, the pivotal role of mitochondria in age-related increases in oxidative stress was suggested by Harman himself several years after his original theory was proposed (6). For the past 25 years, the role of mitochondrial oxidative stress in aging has been a major focus of research in aging under the premise that mitochondrial dysfunction would lead to an increase in mitochondrial ROS generation, damage to mitochondrial components, and as a result a further increase in ROS generation. However, this brings up the key challenge of defining mitochondrial dysfunction and identifying which components of mitochondrial function will affect aging when compromised. Mitochondria have a wide variety of functions, including adenosine triphosphate (ATP) production, ROS generation, calcium homeostasis, apoptotic signaling, use of each of the macronutrient fuels (fats, carbohydrates, amino acids; anabolic functions), and control of energy efficiency. To date, most studies addressing mitochondrial dysfunction in aging have used measures of ATP production or increased oxidant generation as a readout of mitochondrial (dys) function. These studies have been conducted in a variety of tissues and organisms, using a wide array of assay techniques. The data that have been generated thus far are not conclusive; many studies show evidence of mitochondrial dysfunction as predicted by the Free Radical Theory, but many studies show no change. One potential explanation for the disparate results in this area is that the assays for mitochondrial function have many potential sources of error, beginning with the isolation of the mitochondria themselves. Is there a problem with mitochondrial fragility that complicates the use of isolated mitochondria from aging animals? It is possible that the isolation procedure itself can lead to a selective isolation of only the most “healthy” or the isolation of a population of mitochondria that have been compromised by the isolation process. This is further confounded by the fact that some tissues (eg,

skeletal muscle and heart) have more than one population of mitochondria, distinguished by location as well as function. Do mitochondria differ within a given cell? Is there an age-related effect in only one subcellular mitochondrial population? These questions underscore the need for improved means to evaluate mitochondrial function *in vivo*. One important recent advance in this area is the development of technology that allows investigators to directly measure oxygen consumption in cultured cells in response to treatment with agents that alter electron transport. Such methodology allows analysis of mitochondrial function while leaving the mitochondria intact and avoiding confounding factors resulting from isolation procedures and thus is a promising new tool for studying mitochondrial function in aging.

NEED FOR IMPROVED ASSAYS TO EVALUATE THE ROLE OF MITOCHONDRIA IN AGING

Another potential reason for the lack of conclusive evidence in support or rejection of the Free Radical Theory is the need for more sensitive and reproducible assays to measure oxidative damage and mitochondrial function. Oxidative damage assays have been particularly problematic. One major confounding factor is the potential for introducing oxidative damage during the isolation and sample preparation. Many oxidative modifications, particularly to lipids, are very unstable and therefore difficult to assay with reliability and sensitivity. Assays for modifications to DNA and proteins have also suffered from limitations of sensitivity and reproducibility. Another issue in measuring modifications to protein and DNA is the fact that these assays have typically focused on only a few specific modifications (eg, nitrotyrosine adducts or protein carbonyls for oxidative modification of proteins and oxo-8-dG adducts for damage to DNA). Clearly, we are only measuring a small fraction of the types of damage that are generated. Some advances have been made in the technologies available to measure oxidative damage, but much of the research still uses older, less sensitive assays.

Assays for mitochondrial function have also suffered from lack of sensitivity and scope. Until recently, the assays for measuring mitochondrial oxidant generation in particular have suffered significantly from lack of sensitivity. In fact, in many early studies, increases in ROS generation could only be measured in the presence of electron transport chain inhibitors. Assays of mitochondrial oxidant generation have typically used a horseradish peroxidase-coupled system with homovanillic acid, *p*-hydroxyphenylacetic acid, or scopoletin to detect hydrogen peroxide. Recently, improvements in detection systems (ie, the fluorescent probe Amplex Red [Molecular Probes, Invitrogen, Carlsbad, CA]) and the use of electron paramagnetic resonance have allowed measurement of H₂O₂ and superoxide release from isolated mitochondria with significantly enhanced

sensitivity. Continued advances in probes for detection of mitochondrial oxidant generation and other aspects of mitochondria function (eg, ATP generation, redox regulation, calcium homeostasis) are necessary to allow more accurate assessment of the role of mitochondrial function in aging. In addition, there is a need to generate reporter mice that provide readouts of mitochondrial function. These could be used to compare specific mitochondrial changes in existing models.

TESTING FOR A DEFINITIVE LINK BETWEEN MITOCHONDRIAL FUNCTION, OXIDATIVE DAMAGE, AND AGING

This leads us to an important question underlying the lack of a definitive link between mitochondrial function, oxidative damage, and aging. Is there hard evidence for an intervention that improves mitochondrial function and affects aging? The key question that needs to be answered is whether mitochondrial dysfunction correlates with tissue dysfunction that is ultimately a causal factor in determining life span. To do this in a meaningful way, we need a systematic approach that measures several types of mitochondrial function and measures of mitochondria oxidant production in a variety of tissues in a model organism during aging. These studies can be even more powerful if measured in models of increased longevity such as caloric restriction, or compared between long- and short-lived species. One powerful approach might be to produce transgenic models that will allow us to titrate antioxidant levels. The majority of mouse studies using overexpression or reduction of antioxidant protection to study aging have used null or heterozygous knockouts or transgenics with moderate (two- to fourfold) increases in expression of primary antioxidant enzymes. Using modern transgenic approaches, it may be possible to titrate antioxidant levels rather than using the more traditional all or none approaches. Finally, we have a critical need for the development of functional reporter mice that can be used for *in vivo* detection of proteins involved in pathways of cell signaling, inflammatory processes, apoptosis, transcription factor activation, and other cell processes. By pairing with investigators in other fields, we may be able to gain access to established reporter tools that could help us advance research on aging and oxidative stress.

Along these lines, there is also a clear need for better ways to quantitatively define mitochondrial function with age. Because mitochondria have multiple functions, it is quite reasonable that some functions may be preserved while others change. For example, ATP production may be preserved while oxidant production is increased. Which aspects of this mitochondria function are important in aging and in which tissue? Does an alteration in only one aspect of mitochondrial function result in a meaningful consequence for aging? Furthermore, the situation may vary from tissue to tissue.

NEED TO UNDERSTAND THE ROLE OF MITOCHONDRIAL GENOMIC STABILITY IN AGING

Another key question we considered is whether evidence exists for clonal expansion of mitochondrial DNA damage leading to an aging phenotype? Recent studies using polymerase γ mutant mice show early death with the appearance of several phenotypic characteristics of accelerated aging. However, there is not complete agreement that this premature aging caused by mitochondrial DNA damage is relevant to natural aging. Thus, there is a need for models that do not have this caveat, that is, a need for models to extend life span by decreasing cumulative damage and not shorten life by increasing damage. Some suggestions by which this could be accomplished are conditional variation in DNA replication or repair to control changes in adult tissues and in a tissue-specific manner.

UNDERSTANDING THE CONTRIBUTION OF UNIVALENT AND DIVALENT PATHWAYS OF OXIDATIVE STRESS TO AGING

Other aspects of mitochondrial oxidant generation with aging have been limited by methodology. For instance, mitochondria produce different oxidants, including superoxide anion, hydrogen peroxide, and organic peroxides. The distinction between superoxide and hydrogen peroxide could be very important because the permeability characteristics across membranes differ. Thus, there is a need for improved methods to discriminate between superoxide anion and hydrogen peroxide as mitochondrial products. More generally, there is a need to address the role of univalent electron transfer (free radical) as opposed to divalent electron transfer (nonradical) reactions in mitochondrial function and dysfunction. For instance, the ability to discriminate species could allow studies to test whether specific ROS serve as redox signaling agents in mitochondria, analogous to established signal transduction mechanisms of hydrogen peroxide in the cytoplasm. Using such approaches in mouse models with genetically controlled differences in abundance of Mn-superoxide dismutase (*Sod2*) could provide an understanding of the apparently contradictory effects of different levels of MnSOD on life span. These methods could further be used to address the contribution to aging of reactive species for redox signaling as opposed to generation as an uncontrolled and toxic byproduct. Applications could include studies to determine whether some electron transfer sites generate ROS for signaling, whereas other sites represent uncontrolled generation contributing to aging.

CONTRIBUTION OF MITOCHONDRIAL UNCOUPLING TO AGING

The rate of generation of ROS by mitochondria has been linked to the magnitude of the mitochondrial membrane potential, which is, at least in part, controlled by uncou-

pling proteins. Models in which the extent of uncoupling is systematically varied could provide a means to separate energy production and oxidant production, as well as allow studies of links to other mitochondrial functions in aging, including Ca^{2+} homeostasis, lipid metabolism, protein import, mitochondrial biogenesis, and turnover, and so forth. Both genetic and pharmacological models could be useful.

UTILIZING INFORMATION-RICH TECHNOLOGIES OF GENOMICS, PROTEOMICS, AND METABOLOMICS TO DISCRIMINATE REVERSIBLE AND ADAPTIVE CHANGES FROM PRIMARY CAUSAL EVENTS OF AGING

There is a need for application of newer genomic, proteomic, and metabolomic methods to address macromolecular damage and functional changes of mitochondria in more detail. Evidence is available for changes in function of specific proteins, and some of these changes are linked to modifications of particular residues on these proteins, some of which are reversible and some irreversible. However, there is little understanding of primary versus secondary changes, that is, which events are causal and which are consequence. There is also a need to understand whether irreversible changes are determined by changes within the mitochondria, as indicated by mitochondrial genomic damage, or by extramitochondrial changes. Such studies could include studies to address the importance of extramitochondrial ROS generation and nuclear genomic changes. Studies of oxidant-generating enzymes that change with aging, such as nicotinamide adenine dinucleotide phosphate oxidases, and so forth, would also be informative.

NEED FOR A CONCEPTUAL FRAMEWORK TO DISCRIMINATE DELETERIOUS VERSUS PROTECTIVE CHANGES OF AGING

There is little conceptual framework to evaluate whether changes are inherently negative contributors to aging or are, in fact, protective mechanisms that help maintain normal function despite aging. In other words, some changes may function in signaling, whereas others represent damage or impaired function. Application of newer and more powerful profiling approaches could improve detail of functional changes with aging, and identification of critical macromolecular targets of aging could guide efforts for therapeutics to enhance healthy aging. Detailed profiling of mitochondria-related gene expression and proteomic and metabolomic changes with aging could also provide the basis for development of systems biology descriptions of mitochondrial aging. Importantly, such approaches also could provide an improved basis for integration of well-characterized cell culture models, in which aging-related genes and proteins are modified, with more relevant but costly animal models used in life span/health span studies.

In summary, research in the 50 years since the Free Radical Theory was first proposed has resulted in a significant

amount of correlative data consistent with the theory, yet definite proof is still lacking. Several studies in the past few years have yielded results inconsistent with the theory as originally proposed and suggest a need for a reevaluation or modification of the theory. Recent improvements in our ability to measure mitochondrial function, oxidative stress and damage with higher accuracy and sensitivity, and continued advances in genetic approaches to modify transgenic mouse models and generate reporter mouse models will allow us to more directly and definitively test the role of mitochondria and oxidative stress in aging.

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