DIETARY RESTRICTION AUGMENTS RESISTANCE TO OXIDANT-MEDIATED INHIBITION OF MITOCHONDRIAL TRANSCRIPTION

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ABSTRACT

The exquisite sensitivity of mitochondrial transcription to oxidant stress suggests that chronic, low level oxidative stress may impair mitochondrial gene expression during the aging process. In this study, we assessed the effects of age and of life-prolonging, anti-oxidative dietary restriction (DR) regimens on sensitivity of mitochondrial transcription to oxidant stress. Studies were carried out using liver mitochondria isolated from male Fischer 344 rats of different ages (6, 12, 18, or 24 months) fed *ad libitum* (AL) or maintained on DR. Transcriptional capacity was assessed in isolated mitochondria challenged with different doses of either hydrophilic or hydrophobic peroxy radicals generated by AAPH [2,2'-azobis-(2-amidino-propane) hydrochloride] or AMVN [2, 2'-azobis-(2,4,-dimethyl-valeronitrile), respectively].

The most striking effect was that DR increased resistance to AMVN nearly 400% at 6 months and nearly 700% at 24 months, relative to resistance in AL rats. Results also suggest that resistance to both AAPH and AMVN was decreased slightly in older AL rats, but was maintained in the DR rats. These results show that DR augments the defense systems that protect one of the mitochondria's most vulnerable systems. This augmentation is one of the largest magnitude effects of DR yet observed against oxidative challenge.

KEY WORDS: Mitochondria, transcription, oxidative stress, free radicals, dietary restriction, aging

INTRODUCTION

The mechanisms by which dietary restriction (DR) regimens increase longevity and decrease morbidity remain one of the most intriguing problems in modern gerontological research.^{1,2} One of DR's most striking effects is its ability to reduce the free radical-mediated damage, e.g., lipid peroxidation, that occurs throughout

the aging process.^{1,3} This ability of DR to decrease oxidative damage to critical targets, such as DNA and mitochondria, has led to the possibility that the suppression of oxidative stress is the mechanism by which DR increases life span and decreases morbidity.^{4,5} This hypothesis is strengthened by growing evidence consistent with free radical and oxidative stress-based hypotheses of aging.⁵⁻⁸ DR attenuates the effects of oxidative stress by reducing free radical production, by increasing resistance to oxidant-mediated damage, or by increasing detoxification and repair processes.³

Both the ability of DR to reduce oxidant-mediated damage and an appreciation for the interaction between reactive oxygen species and mitochondria have led several investigators to examine the ability of DR to protect mitochondrial structure and function.^{9,10} Mitochondria from DR animals have lower basal levels of lipid peroxidation, generate less lipid peroxidation upon challenge, and detoxify harmful lipid peroxidation byproducts at greater rates than do those from AL rats.^{11,12} Mitochondria from DR animals also maintain membrane structure and function (e.g., fluidity and lipid composition) later in life than do those from AL rats.¹³ Further work shows that steady state levels of 8-hydroxydeoxyguanosine¹⁴ and mitochondrial DNA deletions¹⁵ are lower in the livers of DR rats, indicating that restriction protects the mitochondrial genome as well as the lipid bilayer. Together, these and other studies indicate that DR can protect mitochondrial lipids, proteins, and nucleic acids from free radical attack.

As yet untested, however, is the ability of DR to protect mitochondrial DNA (mtDNA) expression. Maintenance of mitochondrial functions, including respiration, calcium regulation, and mtDNA replication are all dependent on proper mitochondrial gene expression. Notably, however, the proximity of the inner mitochondrial membrane to the relatively unprotected mitochondrial genome and the accessory proteins required for transcription suggests that mitochondrial transcription might be susceptible to inhibition by free radicals, by lipid peroxidation byproducts, or by both. In fact, mitochondrial transcription is very sensitive to low levels of

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some reactive oxygen species.¹⁶⁻¹⁸ Peroxyl radicals generated by AAPH [2,2'-azobis-(2-amidino-propane) hydrochloride] or AMVN [2, 2'-azobis-(2,4,-dimethyl-valeronitrile)] impair mitochondrial transcription prior to inducing detectable levels of lipid peroxidation.¹⁶ Transcription is also sensitive to a lipid peroxidation byproduct, 4-hydroxynonenal, but not to malondialdehyde (another lipid peroxidation byproduct) or to high levels of lipid peroxidation generated by ADP/Fe/NADPH.¹⁶ Together, these results suggest that mitochondrial transcription is differentially sensitive to specific modes of free radical reaction.

The sensitivity of the mitochondrial transcription system to oxidative stress suggests that its function is highly dependent on the protective capacity of the mitochondrial antioxidant defense system. We therefore examined the effects of age and DR on the ability of these systems to protect mitochondrial transcriptional capacity.

MATERIALS AND METHODS

Animal Husbandry

Specific pathogen-free male Fischer 344 rats were maintained under barrier conditions at the University of Texas Health Science Center.¹⁹ Animals were fed a soy-protein based diet either ad libitum or restricted to 60% of ad libitum intake from 6 weeks of age onward.^{19,20} DR resulted in a greater than 40% increase in both mean and maximum lifespan.¹⁹

Isolation of Mitochondria

Liver mitochondria were isolated as described previously.¹⁶ Following decapitation, livers were rapidly removed and placed in an ice-cold solution containing 140 μ M KCl and 20 mM HEPES pH 7.4. Following homogenization with a motor driven teflon pestle, samples were centrifuged at 1000 g for 10 min. Supernatants were removed and centrifuged at 10,000 g for 15 min. Pellets were washed twice in KCl/HEPES with recentrifugation at 10,000 g. Following the final wash, mitochondria were resuspended in the same buffer and aliquots were frozen quickly in liquid nitrogen. Protein determinations were carried out using the Sigma Protein assay Kit (P5656).

Mitochondrial Transcription

Mitochondrial transcription reactions and dose response studies were carried out as described previously.^{16-18,21} Control samples received water alone in place of oxidant. Transcriptional capacity following oxidant exposure was expressed as a percentage of the activity in this sample. In most samples, AMVN's initial solvent, methanol, was present at concentrations that did not affect transcription. Methanol does, however, begin to interfere slightly at the concentrations present when the dose of AMVN is >1200 μ M. Therefore, to avoid measuring effects due to the methanol, samples having

an ID₅₀ for AMVN of >1200 μ M were scored as 1200 μ M. Dose response studies used a minimum of 4 samples treated with AMVN, 5 treated with AAPH, and a control. The ID₅₀ for a given sample was determined by regression analysis. Total mitochondrial transcriptional capacity was assessed using the same assay, but in the absence of oxidant. Levels of transcription in the samples used in these experiments were comparable to those seen previously in this laboratory.^{16-18,21}

Transcription reactions were carried out by mixing mitochondria with an equal volume of oxidant diluted in water to twice the desired final concentration. Samples were then incubated for 15 min at 37°C and unreacted oxidant was then removed via centrifugation. Following centrifugation, mitochondria were resuspended in a transcription buffer containing ³²P-UTP and allowed to transcribe for 30 min, after which the RNA was purified and analyzed by gel electrophoresis^{16-18,21}. Radioactivity present in the two major bands was assessed using a Molecular Dynamics Phosphorimager. Because of the similar sizes of many mitochondrial messages, these bands contain most of the major processed mitochondrial RNAs including cyt b, ND1, ND2, ND4, A6/8, Col, ColI, and ColIII. In the presence of ATP, these bands also contain the 12S and 16S mitochondrial ribosomal RNAs, which are poorly expressed in the absence of exogenous ATP. Because these messages are coordinately regulated, and since previous work¹⁶ (and data not shown) suggests no consistent message-to-message variation following oxidant challenge, no individual analysis of transcripts was carried out. No change in oxidant-mediated effects has been observed in the presence and absence of ATP, suggesting that identical mechanisms act to repress the transcription of these ribosomal messages and those of the mRNAs. For this reason, ATP was added in these experiments as it increases the total level of transcription and thereby the sensitivity of the system.

Statistical Analysis

Data were considered statistical outliers if they failed the Dixon test²² and were more than four standard deviations away from the mean for their group. Three data failed these tests and were excluded from the remaining statistical analysis. One 6-month DR rat had an AAPH ID₅₀ of 29 μ M, one 18-month AL rat was resistant to 1200 μ M AMVN, and one 24-month AL rat had an AAPH ID₅₀ of 15 μ M. These points are circled in Figures 1 and 2. Remaining data were analyzed by ANOVA (Student-Newman-Keuls) using the SigmaStat software package from Jandel. Relationships between resistance to AAPH and AMVN were carried out by regression and correlation coefficient analysis using the MacIntosh program Cricket Graph. Additional analyses (median test and t-test) noted in text were carried out using the statistical package SAS. Normality was determined by SigmaStat or SAS, as appropriate.

Figure 1: Resistance to AAPH-Mediated Inhibition of Mitochondrial Transcription. ID₅₀s for AAPH were determined by dose response analysis and are plotted by age and dietary group. Bars represent mean $\pm$ sem. Statistical significance was ascertained by ANOVA (Student-Newman-Keuls) as described in the Methods. No values were statistically significant (but see Results). Circled points were eliminated from statistical analysis (see Methods).

RESULTS

Both AAPH and AMVN thermally decompose at low, constant rates to generate peroxy radicals. This provides a means of delivering a controlled oxidative stress to a sample.²³ An additional advantage of the use of these oxidants is that AAPH and AMVN, and the radicals they generate, are hydrophilic and hydrophobic, respectively. These solubility differences lead to differential distribution and secondary reactions of the radicals they produce, offering the opportunity to challenge the system in different ways.

Resistance to AAPH and AMVN was evaluated in liver mitochondria from 5-7 rats of each age (6, 12, 18, and 24 months) maintained on each dietary regimen (AL and DR). Resistance was determined by constructing dose response curves with both oxidants for each of the 49 animals tested. In general, five doses of AAPH and four doses of AMVN were used to construct each curve. Once the dose response data was obtained, regression analysis was used to determine the approximate dose where 50% of transcriptional capacity was lost (ID₅₀). Data obtained in this way are plotted in Figures 1 and 2.

Mean ID₅₀ for AAPH (Figure 1) displayed no statistically significant changes with either age or diet group. Power analysis revealed a β of 0.23, suggesting that animal-to-animal variation (see especially the 18 AL and 24 DR groups in Figure 1) may underlie the observed lack of statistical significance. Indeed, by the less stringent median test, 24 AL samples had reduced defenses relative to both 18 AL and 24 DR samples at a $p < 0.05$. The median test was used because the data was non-normal. It should be noted, however, that much of the enhanced defense in the 24 DR group was due to

Figure 2: Resistance to AMVN-Mediated Inhibition of Mitochondrial Transcription. ID₅₀s for AMVN were determined by dose response analysis and are plotted by age and dietary group. Bars represent mean $\pm$ sem. Numbers indicate multiple overlapping points. Statistical significance was ascertained by ANOVA (Student-Newman-Keuls) as described in the Methods. Effects of aging on AMVN resistance: 6 DR and 24 DR vs 12 DR or 18 DR, $p < 0.05$. Effects of diet on AMVN resistance, 6 AL vs 6 DR and 24 AL vs 24 DR, $p < 0.05$ (and see Results). Circled point was eliminated from statistical analysis (see Methods).

two samples whose ID₅₀ for AAPH exceeded 20 mM. In the 24-month AL, one sample resisted 15 mM AAPH, but is an outlier statistically, and therefore not included in the statistics (the two high DR samples were not statistical outliers). This suggests that sampling may partially contribute to the extent of the observed difference between AL and DR animals with respect to resistance to AAPH.

DR rats generally maintained a greater resistance to AMVN-mediated inhibition than their AL counterparts (Figure 2). Resistance displays a U-shaped age-dependency in the DR, in contrast with a trend ($p > 0.05$) towards an age-related decline in the AL. DR samples at 6 and 24 months of age have resistances 4- and 7-fold higher than their AL counterparts, respectively. These large differences are statistically significant by ANOVA. The 2-fold difference between 18 DR and 18 AL samples also approaches statistical significance by ANOVA, and secondary analysis reveals that this smaller difference is statistically significant by less stringent tests such as t-test ($p < 0.05$).

The differential modulation of resistance to AAPH and AMVN with age and diet highlights distinctions between both these chemical species and their accessibility to the targets sensitive to their actions, and the ability of an organism to modulate these environments. This is seen clearly in the different patterns evidenced in Figures 1 and 2 and by the lack of correlation between levels of the two defenses in individual animals (Figure 3).

Figure 3: Lack of Correlation Between AMVN and AAPH Resistance. Data from Figure 1 replotted to show lack of correlation between resistance of a given sample to AAPH and to AMVN. Regression: $y = 327.97 + 16.86x$, $r^2 = 0.071$, $p > 0.05$ by analysis of the correlation coefficient²⁹.

DISCUSSION

Mitochondrial roles in calcium homeostasis and energy production are essential for cellular homeostasis, and mitochondrial dysfunction therefore contributes to cell- and system-level pathology. The proper maintenance of mitochondrial function is dependent on mitochondrial gene expression, as four of the five multi-subunit enzyme complexes require components encoded within the mitochondrial genome. We have previously shown that the mitochondrial transcription system is exquisitely sensitive to oxidative stress^{16-18, 21}. This suggests that the oxidant stress that occurs during the aging process may lead to oxidant-mediated repression of mitochondrial transcription, which may, in turn, play roles in aging processes and in chronic disease. This notion is supported by observations of decreased transcription in mitochondria from aged brain,²⁴ and in Alzheimer's disease.²⁵

The susceptibility of mitochondrial gene expression to oxidants would be exacerbated if the defense systems that protect the transcription system gradually weaken over time. Under such conditions, transcription could be impaired by progressively lower levels of oxidative stress. Chronic inhibition may lead to impaired respiration due to an inability to replace respiratory complexes. This possibility has been verified *in vitro*,²⁶ and other studies from our laboratory suggest that this is the major mechanism by which diabetes leads to loss of liver mitochondrial function.²¹ Thus, the experimental system that we have been studying appears relevant to *in vivo* pathology.

In general, mitochondria are protected from free radical- and oxidant-mediated damage by a defense system that includes both small molecular weight antioxidants, such as α -tocopherol and ubiquinol, and

enzymatic free radical scavengers such as MnSOD, catalase, and glutathione-peroxidase. The data presented in Figure 3 show that there is little relationship between the level of resistance to AAPH and that of resistance to AMVN. Coupled with previously published data showing similar findings (i.e., lack of co-variation in resistance) in mitochondria from different tissues¹⁸ and in liver mitochondria isolated from diabetic animals,²¹ these current data indicate that the detrimental effects of AAPH and AMVN are prevented by at least partially independent mechanisms.

We have shown that α -tocopherol, ascorbate, and glutathione all attenuate, but do not prevent, the effects of AAPH and AMVN on transcription.¹⁷ Further data from studies in diabetic rats strongly suggest that the enzymatic free radical scavengers may not be the major means of protecting the mitochondrial gene expression system²¹. These data suggest that the major component of mitochondrial resistance must be an antioxidant with either a greater affinity for radicals than α -tocopherol and ascorbate, a different suborganellar localization, or both.

The current study addressed whether the defenses that protect mitochondrial transcription were compromised by aging and whether life-prolonging, anti-oxidative DR regimens increased mitochondrial resistance to oxidant-mediated inhibition of transcription. The most striking effect noted is that resistance to AMVN-mediated inhibition was augmented to about 400% of AL levels at 6 months, and to about 700% of AL rats at 24 months. These changes rank among the largest known effects of DR on antioxidant functions. Resistance was unchanged at 12 months and elevated about 100% at 18 months. Although the DR-mediated increase in resistance to the hydrophobic AMVN is in accordance with DR's ability to protect other hydrophobic targets (e.g., membranes), the effects of DR described here differ on two counts. First, the timing of the response is very different, with DR having a chronologically U-shaped effect in this system, whereas DR-mediated protection of membranes is relatively constant throughout the lifespan. Second, although protection of membranes by DR is significant, it is relatively small compared with the 700% increases in resistance demonstrated here.

The differential effects of age and diet on resistance to AAPH and AMVN-mediated inhibition indicate that a better understanding of these mechanisms will likely lead to important insights into the means by which DR protects mitochondrial function. This is especially true as the transcription system appears to be one of the most sensitive to oxidant-mediated inhibition. One possible explanation for the differential effects of DR on resistance to the hydrophilic, AAPH-derived peroxyl radicals and the hydrophobic, AMVN-derived peroxyl radicals rests in DR-mediated changes in membranes.^{27,28} Membrane changes may alter the intramitochondrial distribution both of AAPH and AMVN and of the secondary radicals they generate. An equally

attractive possibility is that DR-mediate changes in the antioxidants that protect mitochondria from oxidant-mediated damage.

The evidence presented leads us to speculate that the putative factor protecting mitochondria against AMVN is likely hydrophobic (because of the nature of AMVN and its radicals) and is a more potent free radical scavenger than α -tocopherol and ascorbate, because of the inability of these antioxidants to protect. These and other pieces of preliminary evidence have led us to hypothesize that this factor is ubiquinol, a possibility that we are currently exploring.

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