

Protein stability and resistance to oxidative stress are determinants of longevity in the longest-living rodent, the naked mole-rat

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The widely accepted oxidative stress theory of aging postulates that aging results from accumulation of oxidative damage. Surprisingly, data from the longest-living rodent known, naked mole-rats [MRs; mass 35 g; maximum lifespan (MLSP) > 28.3 years], when compared with mice (MLSP 3.5 years) exhibit higher levels of lipid peroxidation, protein carbonylation, and DNA oxidative damage even at a young age. We hypothesize that age-related changes in protein structural stability, oxidation, and degradation are abrogated over the lifespan of the MR. We performed a comprehensive study of oxidation states of protein cysteines [both reversible (sulfenic, disulfide) and indirectly irreversible (sulfinic/sulfonic acids)] in liver from young and old C57BL/6 mice (6 and 28 months) and MRs (2 and >24 years). Furthermore, we compared interspecific differences in urea-induced protein unfolding and ubiquitination and proteasomal activity. Compared with data from young mice, young MRs have 1.6 times as much free protein thiol groups and similar amounts of reversible oxidative damage to cysteine. In addition, they show less urea-induced protein unfolding, less protein ubiquitination, and higher proteasome activity. Mice show a significant age-related increase in cysteine oxidation and higher levels of ubiquitination. In contrast, none of these parameters were significantly altered over 2 decades in MRs. Clearly MRs have markedly attenuated age-related accrual of oxidation damage to thiol groups and age-associated up-regulation of homeostatic proteolytic activity. These pivotal mechanistic interspecies differences may contribute to the divergent aging profiles and strongly implicate maintenance of protein stability and integrity in successful aging.

cysteine oxidation | *Heterocephalus glaber* | mechanisms of aging | proteasome activity | protein homeostasis

The naked mole-rat (MR; Bathyergidae; *Heterocephalus glaber*), is the longest-living rodent known with a maximum lifespan (MLS) of >28.3 years (1). Evolving in a protected underground milieu, extrinsic mortality is low and this mouse-sized (35 g) rodent lives 8 times longer than laboratory mice. MRs have a similar longevity quotient [LQ = 5; i.e., ratio of observed MLS to that predicted by body mass (2)] to that of humans, another very long-living mammal. MRs show many signs of attenuated aging (3); they exhibit no age-related changes in body composition, physiology, and molecular function from 2 to >20 years (4–6). Old animals (>24 years; >85% MLS), nevertheless, can be differentiated on sight in that they are less active and have a thin translucent parchment-like skin (1). These data support the premise that the MR is an excellent animal model for studying molecular and biochemical mechanisms involved in successful slow aging and sustained health span (7).

The oxidative stress theory of aging predicts that differential rates of aging among species may be caused by inherent differ-

ences in oxidative damage accrual (8). Although widely accepted (9–12), there are a growing number of exceptions to this theory, often contingent on the specific species, strain, and/or tissue under investigation (7, 13–15). Data from long-lived species (such as birds, bats, and MRs), and transgenic mice with altered expression of antioxidants, commonly suggest that oxidative damage is not directly correlated with MLS (15, 16). The MR is one of the notable exceptions to the oxidative stress theory of aging (7, 14, 17, 18). It has similar levels of reactive oxygen species (17, 18) and antioxidant defenses (19) when compared with shorter-living rodents; however, even at a young age it exhibits higher levels of lipid peroxidation, protein carbonylation, and DNA oxidative damage than do mice (14). These measures of oxidative stress do not change with age (20). In addition, MR cells appear to be inherently protected from most types of cytotoxic insults, maintaining greater viability than other rodents (17, 21). These data suggest that the oxidative damage, per se, may not effect longevity, but rather resilience to damage and the mechanisms facilitating this may be a far more important determinant of aging.

Proteins are one of the prime targets for oxidative damage (22), and cysteine residues are particularly sensitive to oxidation because the thiol group in cysteine can be oxidized to both reversible [sulfenic acid (SOH), disulfide bond formation (S—S)] and irreversible oxidative states [sulfinic (SO₂H) and sulfonic acids (SO₃H)] (23, 24). Cysteine residues are important physiologically because they are often found at catalytic and regulatory sites in proteins and enzymes (24). The thiol groups in cysteines are strongly buffered against oxidation by keeping the internal cellular environment in a relatively reduced state (25, 26). However, these intracellular conditions may change with age. Redox ratios in plasma reportedly decrease with age in humans (27, 28), increasing vulnerability of thiol groups to oxidative damage.

Chronic oxidative stress may lead to protein misfolding. The intricate tertiary-folded structure of proteins is maintained with the assistance of various heat shock proteins and molecular chaperones (29). Impairment of functional structure can accelerate formation of toxic protein oligomers or aggregates that

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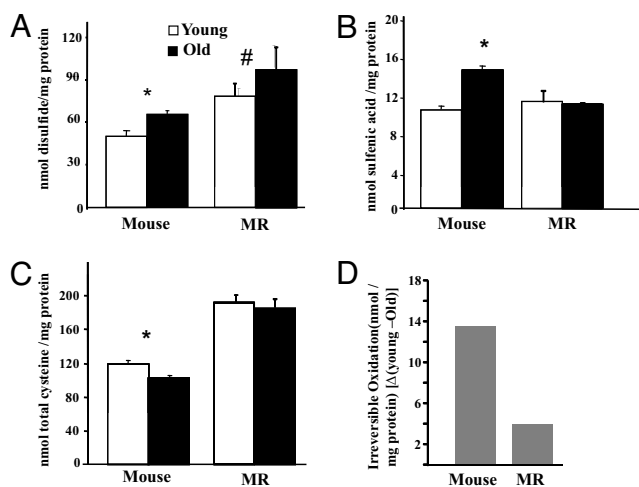


Fig. 1. Age-related changes in cysteine oxidation in cytosolic liver homogenates from young (open bars) and old (solid bars) mice and MRs. Data are the means of 8 (mice) and 10 (MRs) \pm SEM. The * denotes values that are significantly ($P \leq 0.05$) different from young mice as analyzed by nonparametric ANOVA. (A) Disulfide bond content increases 1.3-fold in mice with age, but is unchanged (#, $P = 0.07$) with age in MRs. (B) Sulfenic acid levels increase 1.5-fold with age in mice ($P = 0.04$), but are unchanged with age in MRs. (C) Total cysteine content in MRs is 1.6-fold higher than in mice ($P = 0.05$). Whereas total cysteine declines significantly with age in mice, no age-related changes are evident in MRs. (D) Irreversible oxidation increases 3.4-fold more with age in mice than in MRs. Levels of irreversible cysteine oxidation are obtained from the differences (Δ) between the values of total cysteine in young and old mice and MRs.

contribute to pathologies commonly observed in neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington diseases (30). Thus, we hypothesize that attenuated accumulation of oxidized proteins and maintenance of protein structural stability may be important determinants for slow aging.

In this article, we test this hypothesis by determining whether MRs, when compared with shorter-lived mice, (i) exhibit attenuated age-related changes in protein oxidation (e.g., cysteine oxidation), (ii) have proteins that are more resistant to urea-induced unfolding, and (iii) maintain protein homeostasis as measured by ubiquitination and proteasome activity with age. We report that when compared with mice MRs show (i) no age-related changes in cysteine modification (from reversible to irreversible oxidation state), (ii) resistance to protein unfolding, and (iii) attenuated accumulation of ubiquitinated proteins and sustained proteasomal function during aging. Our comparative findings elucidate a key mechanistic difference that may contribute to disparate longevity among species and strongly implicate maintenance of protein stability in successful aging.

Results

Attenuated Age-Related Changes in Cysteine Oxidation in MRs. The thiol group in cysteine is sensitive to oxidation and exhibits various oxidation states, including reversible and irreversible states. We have developed 3 distinct fluorescence-based assays to quantify the various states of cysteine oxidation; 2 measure reversible states (disulfide and sulfenic acid) (31) and the third measures total cysteine and indirectly indicates the levels of irreversible oxidation [sulfinic/sulfonic/others (23, 32)] that we have used in liver tissues of young and old MRs and mice. The disulfide content detected in young MRs tends ($P > 0.07$) to be higher than that found in young mice (Fig. 1A). Disulfide content in mouse liver proteins is elevated ($\approx 28\%$; $P < 0.05$) with age,

whereas age-associated accumulation is not evident in MR samples.

Similarly, sulfenic acid in MR is unchanged with age, whereas in mice it increases with age (Fig. 1B). These results show that reversibly oxidized cysteine groups accumulate over a 1.7-year period in mice, whereas age-related accrual is absent over a 25-year interval in MRs.

We also measured total cysteine and indirectly the levels of the irreversible oxidized products of cysteine ($-\text{SO}_2\text{H}/-\text{SO}_3\text{H}$) (32, 33). Because the fluorescent thiol alkylating agent [6-iodoacetamidofluorescein (6-IAF)] we use binds selectively to $-\text{SH}$ groups and not to oxidized thiols, a lower fluorescence signal (thus lower levels of incorporation of 6-IAF to the thiol group) indicates increased levels of irreversible oxidation. Young MRs have a 1.6-fold more total cysteine than do young mice (Fig. 1C) that is sustained over the 24-year age interval in our MR samples (Fig. 1C). This titer decreases significantly ($\approx 12\%$) with age in mice, indicative of an overall increase in irreversibly oxidized cysteine residues (Fig. 1D). Lack of age-related changes over a 24-year period in either reversible or irreversible cysteine oxidation suggests that MRs have evolved efficient means of maintaining protein thiols.

Proteins from MRs Are Resistant in Vitro Unfolding. Cysteine thiol groups are critical components for structural maintenance of proteins (24). Because we observed marked interspecies differences in levels of thiol modification, we questioned whether they may influence the stability and structural state of proteins by measuring the relative resistance of proteins to urea-induced unfolding. Urea-induced denaturation exposes the hydrophobic pockets on the surface of proteins. Hydrophobic sites can be photolabeled by the apolar fluorescent probe 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid (BisANS). The intensity of BisANS fluorescence is indicative of the number of hydrophobic pockets exposed and thereby changes in protein conformation can be monitored (34). No significant differences in the fluorescence intensity of untreated (baseline) liver protein samples are evident both among species and with age, although levels of fluorescence tend to be higher in MRs samples than in mice (Fig. 2A). We set these species baseline levels arbitrarily to 100% when testing changes in fluorescence intensity in response to urea-induced unfolding. Increasing concentrations of urea (from 0 to 3 M) result in greater exposure of hydrophobic sites. BisANS incorporation peaks at 1 M and thereafter declines, indicating that surface hydrophobic pockets have collapsed because of denaturation induced by excessively high levels of urea (Fig. 2B). We therefore assessed both interspecies differences and age effects at a urea concentration of 1 M. At this concentration, the young mouse proteome shows more than twice as much BisANS incorporation as that of young MRs, indicating that MRs are more resistant to urea-induced unfolding (Fig. 2B). Age-related interspecific differences are also evident (Fig. 2C). Old MR samples show only a 50% increase in fluorescent intensity after 1 M urea treatment, whereas the old mouse proteome exhibited a 220% increase from basal levels. Clearly the MR proteome is significantly more resistant to unfolding and even 26-year-old MRs are better able to maintain protein homeostasis than can young mice.

We assessed whether resistance to urea-induced protein unfolding is accompanied by sustained protein function by measuring the activity of a key glycolytic enzyme, GAPDH when liver samples are treated with 1 M urea. We specifically chose this enzyme because its catalytic site has critical thiol groups that are vulnerable to unfolding (35). Activity of GAPDH decreased by 40% with age (4 months–2 years) in mice, whereas it was sustained ($P > 0.24$) over a 24-year period in MRs (Fig. 2D). After urea treatment specific activity of GAPDH declined to similar very low levels in both young and old mice. Both young

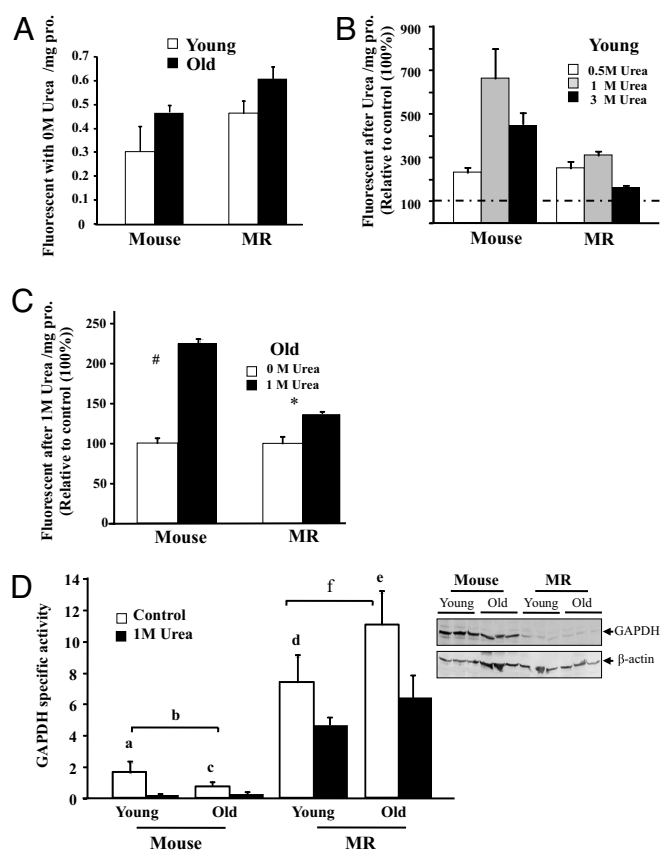


Fig. 2. Proteins from both young and old MRs are extremely resistant to protein unfolding, whereas those from mice are markedly more susceptible. Protein unfolding is measured by incorporation of the BisANS fluorescence probe when hydrophobic pockets are exposed and is expressed as fluorescent units/mg protein as a percentage of control (100%). Data are mean \pm SEM ($n = 6$). The # ($P = 0.0038$) and * ($P = 0.048$) denote those values that are significantly different from untreated samples when analyzed by nonparametric ANOVA. (A) Basal protein unfolding is unchanged with age ($P = 0.094$) in mice and MRs. (B) Young mice have markedly higher levels of protein unfolding after urea treatment than do young MRs with maximal BisANS incorporation at 1 M urea. (C) BisANS incorporation in response to 1 M urea is abrogated with age, although old mice show less resistance to unfolding than do old MRs. (D) GAPDH activity declines less with urea treatment in MRs than it does in mice regardless of age, indicating that MRs are better able to maintain protein structure and function than mice. A representative Western blot of GAPDH expression is shown. Letters a–e represent significant differences among comparative datasets (a = 0.03; b = 0.05; c = 0.05; d = 0.05; e = 0.05), while f (representing age-related differences in untreated GAPDH activity in MRs) is not significant ($P = 0.24$).

and old MRs showed only a 40% decline and maintained a significantly higher level of activity than observed in mice. These data show that proteins of MRs are extremely resilient and effectively maintain both structure and function when faced with unfolding stressors.

Age-Related Increase in Protein Ubiquitination Is Diminished in MRs.

Polyubiquitination is the rate-limiting step for degradation of the oxidized/misfolded proteins by proteasome (36). We measured relative amounts of protein ubiquitination by Western blot analyses. Ubiquitin levels in young mice and MR were similar ($P = 0.09$) (Fig. 3A). Levels increased with age in mice, whereas no age-related changes from 2 to 26 years were evident in MR samples (Fig. 3B and Fig. S1). Absence of age-related accumulation of oxidized thiol groups and ubiquitinated proteins in MR

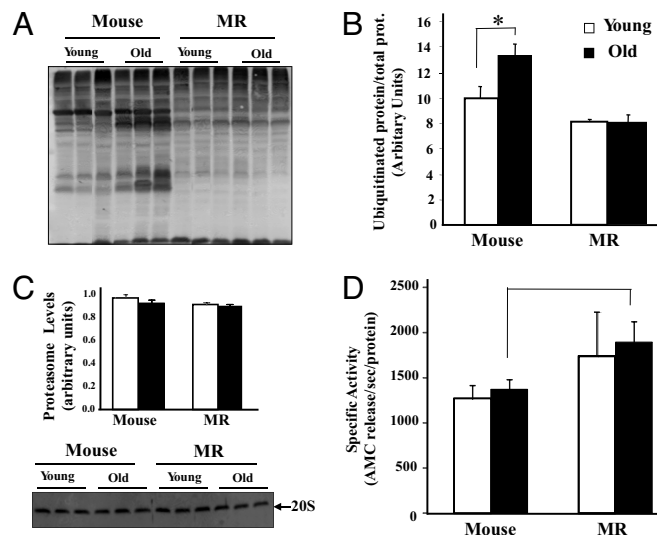


Fig. 3. Ubiquitinated proteins increase with age in mice, but are similar in samples from 2-year-old and 26-year-old MRs. (A) A representative Western blot of ubiquitinated proteins is shown. (B) Levels of ubiquitinated protein as measured by Western blot analysis in cytosolic protein homogenates from the livers of young (open bars) and old (solid bars) mice and MRs are shown. Data (mean \pm SEM; $n = 3$) were analyzed by nonparametric ANOVA. The * denotes values that are significantly ($P \leq 0.05$) different from young mice. (C) The levels of proteasome (20S) are similar in both mouse and MRs. The total amount of 20S proteasome subunit is measured by Western blot analysis. (D) Proteasome activity is greater in old MRs than in old mice (*, $P = 0.052$). Chymotrypsin-like proteasome activity is measured in cytosolic protein homogenates from livers of young (open bars) and old (solid bars) mice and MRs by using the 20S proteasome fluorometric (AMC) assay kit. Data (mean \pm SEM; $n = 3$) were analyzed by nonparametric ANOVA.

most likely reflects rapid removal of oxidized and/or misfolded protein in this species.

We measured age-related changes in 20S proteasomal chymotrypsin-like activity (CLPA) in liver proteins from young and old by fluorescent-labeled substrate cleavage. Rates of activity were normalized to protein levels of 20S (Fig. 3C and refs. 37 and 38). 20S protein content did not change with age in either species (Fig. 3C). CLPA tended to be greater in young MR than in young mice ($P = 0.12$), but was significantly greater ($P = 0.05$) in old MRs relative to old mice (Fig. 3D).

Discussion

Our data show that MR protein structure and integrity are better maintained during aging than that of mice. There are 4 salient findings in this study. When compared with mice MRs show (i) higher levels of total cysteine, (ii) no age-related change in cysteine oxidation over >2 decades, an exceptionally long period for a mouse-sized rodent, (iii) remarkable resistance to protein unfolding, and (iv) lower levels of protein ubiquitination and higher levels of CLPA.

It is an intriguing observation that the proteome of MRs exhibits such high levels of cysteine (Fig. 1C). We do not know with certainty the functional significance of this finding; for cysteine-rich proteins tend to be more vulnerable to oxidation. However, it is unlikely that the MR proteome differs this much from that of other rodents, especially because we routinely and mostly successfully use commercially available antibodies for protein analyses. More likely certain cysteine-rich proteins are in great abundance in MRs; indeed MRs have more cysteine-rich GST (3-fold) and GSH (1.4-fold) than mice (Fig. S2A), suggesting that detoxification processes are more enhanced in these animals. An alternate plausible explanation may be that the

Measurement of Cysteine Oxidation. Cysteine oxidation was measured by detection of disulfides (31, 41). Briefly, liver tissues were homogenized in 50 mM potassium phosphate buffer (pH 8.0) containing 0.5 mM MgCl₂, 1 mM EDTA, 150 mM iodoacetamide, and protease inhibitor mixture [500 μM 4-(2-aminoethyl)benzenesulfonyl fluoride, 150 nM aprotinin, 0.5 mM EDTA, and 1 μM leupeptin hemisulfate] and centrifuged for 30 min at 16,000 × g at 4 °C. For the disulfide assay, samples were incubated in phosphate buffer (pH 8.0) also containing 150 mM iodoacetamide, which reacts with free thiol groups; the free iodoacetamide was removed by protein precipitation with 10% trichloroacetic acid and washed 3 times with 100% ethanol/ethyl acetate (1:1). Samples were resuspended in 8 M urea and incubated with 1 mM DTT for 30 min at 37 °C to reduce disulfide bonds in the samples. Free thiol groups (–SH) arising from the reduced disulfides were labeled with 1 mM 6-IAF, a fluorescent-tagged iodoacetamide. For the sulfenic assay (reversible cysteine oxidation), cytosolic samples were incubated with 6 M Urea and 2 mM DTT for 1 h at 37 °C. Then free thiol groups arising from these reducing conditions were blocked with 200 mM iodoacetamide. AsO₃ (5 mM) was added to reduce the reversible oxidation in cysteines, followed by 1 mM DTT. Free thiol groups arising from these reducing conditions were labeled with 1 mM 6-IAF. For determination of irreversible cysteine oxidation, we measured the total amount of cysteine indirectly (thiol and reversible oxidation) by incubating with 6 M Urea and 2 mM DTT for 1 h at 37 °C. Then 5 mM of AsO₃ was added to reduce all reversible oxidation. Free thiol groups arising from these reducing conditions were labeled with 1 mM 6-IAF. Protein concentration was measured by using the Bradford method, and 10 μg of protein was subjected to gel electrophoresis. After electrophoresis, the image of the fluorescent protein and the total amount of protein (measured by Sypro Ruby staining) were captured by using the Typhoon 9400 scanner (emission filter 526 and 620 nm, respectively; Amersham Bioscience). The intensities of fluorescence and Sypro Ruby were calculated for each protein by using ImageQuant 5.0 software (Molecular Dynamics). Data were expressed as nmol of cystine oxidation/mg of protein.

Measurements of Ubiquitinated Proteins. Levels of ubiquitinated protein were measured in cytosolic protein homogenates from liver. Protein concentration was measured by BCA (bichromic acid) protein assay (Pierce), and equal amounts of protein (15 μg) were separated on a 12% SDS/PAGE and subjected to Western blot analysis. Mouse polyclonal antibody against ubiquitin (Santa Cruz Biotechnology) was used to identify the ubiquitinated proteins. Intensities of the bands in each sample (entire lane from the top to the bottom) were quantified by densitometry using ImageQuant version 5.0. Results were expressed as ubiquitinated protein normalized to the total amount of protein measured by Coomassie blue.

Determination of Resistance to Protein Unfolding. Resistance to protein unfolding was measured by 3 M urea treatment followed by UV-induced photoincorporation of BisANS to proteins as described (34). Briefly, cytosolic protein extracts were diluted to 1 mg/mL in labeling buffer containing 50 mM

Tris-HCl, 10 mM MgSO₄ at pH 7.4, and protease inhibitors. Samples were treated with and without 3 M Urea for 2 h at room temperature, then 100 mM BisANS was added, and samples were immediately vortexed. Sample (200 μL) was added to a clear 96-well plate and incubated on ice (to minimize local heating) for 1 h under direct exposure of a 115-V, 0.16-Amp handheld longwave UV lamp (365 nm; UV Products model UVL-21). After photoincorporation of BisANS, proteins were then dissolved in Laemmli buffer and subjected to SDS/PAGE. After electrophoresis, gels were removed and illuminated with 365-nm UV light, and BisANS fluorescence was captured with an AlphaImage 3400 (Alpha Innotech) for quantification. Data were expressed as fluorescent units/mg protein.

20S Proteasome Activity Assay. Liver samples were homogenized in homogenization buffer [50 mM Tris-CL (pH 8.0), 1 mM EDTA, 0.5 mM DTT], and protein concentrations were measured by BCA protein assay (Pierce). For each sample, 100 μg of total protein was assayed in triplicate in 96-well plates by using 20S proteasome fluorometric [7-amino-4-methylcoumarin (AMC)] assay kit per the vendor's instructions (Calbiochem). In brief, the release of free AMC from the fluorogenic peptide Suc-Leu-Leu-Val-Tyr-AMC was measured over time at 37 °C by using a microplate fluorescence spectrophotometer. 20S activity was calculated by the slope of free AMC release over time, after a ≈10-min period of normalization. Quantity of 20S proteasome was measured by Western blot analysis. Twenty micrograms of total cellular protein extract was subjected to SDS/PAGE followed by Western blot analysis using rabbit polyclonal antibody against 20S proteasome subunit (37, 38). Band intensities were measured by using Image Quant software package (Molecular Dynamics). 20S-specific activity was calculated by normalizing 20S activity to the quantity of 20S proteasome, measured by Western blot analysis. Data were expressed as AMC release/s per protein.

GAPDH Enzymatic Activity Assay. GAPDH activity was measured as described by Rafter *et al.* (42) and Harting and Velick (43). Briefly, liver cytosolic protein extracts were diluted in 15 mM sodium pyrophosphate/30 mM sodium arsenate buffer at pH 8.5 to 0.05 mg of protein/mL, and 0.1 mL of tissue extract was added to a cuvette containing 2.6 mL of pyrophosphate/arsenate buffer containing 0.015 M sodium pyrophosphate, 0.03 M sodium arsenate at pH 8.5, 0.1 mL of 7.5 mM NAD, and 0.1 mL of 0.1 M DTT. Absorbance was monitored at 340 nm, and enzyme units were calculated by using the extinction coefficient of NADH, where 1 unit is equal to the amount of enzyme required to convert 1 μmol of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate/min at 25 °C and pH 8.5. GAPDH protein levels were determined in liver cytosolic samples from mouse and MRs by Western blot analysis using a specific GAPDH polyclonal rabbit anti-human GAPDH (Alamo Laboratories). The intensities of the bands were quantified by using ImageQuant version 5.0 (Molecular Dynamics). β-Actin was used as the loading control.

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