

Aging augments mitochondrial susceptibility to heat stress

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Haak JL, Buettner GR, Spitz DR, Kregel KC. Aging augments mitochondrial susceptibility to heat stress. *Am J Physiol Regul Integr Comp Physiol* 296: R812–R820, 2009. First published January 14, 2009; doi:10.1152/ajpregu.90708.2008.—The pathophysiology of aging is accompanied by a decline in tolerance to environmental stress. While mitochondria are primary suspects in the etiology of aging, little is known about their ability to tolerate perturbations to homeostasis in older organisms. To investigate the role of mitochondria in the increased susceptibility to heat stress that accompanies aging, young and old Fischer 344 rats underwent a heat stress protocol known to elicit exaggerated cellular damage with aging. At either 2 or 24 h after heat stress, livers were removed from animals, and hepatic mitochondria were isolated. Electron microscopy revealed extensive morphological damage to mitochondria from young and, to a greater extent, old rats after heat stress. There was also a significant loss of cytochrome *c* from old, but not young, mitochondria and a persistent increase in 4-hydroxynonenal-modified proteins in old vs. young mitochondria exposed to heat stress. Electron paramagnetic resonance measurements of superoxide indicate greater superoxide production from mitochondria of old compared with young animals and suggest that mitochondrial integrity was altered during heat stress. The mitochondrial stress response, which functions to correct stress-induced damage to mitochondrial proteins, was also blunted in old rats. Delayed and reduced levels of heat shock protein 60 (Hsp60), the main inducible mitochondrial stress protein, were observed in old compared with young mitochondria after heat stress. Additionally, the amount of Hsp10 protein increased in young, but not old, rat liver mitochondria after hyperthermic challenge. Taken together, these data suggest that mitochondria in old animals are more vulnerable to incurring and less able to repair oxidative damage that occurs in response to a physiologically relevant heat stress.

mitochondria; liver; stress response; ROS; cytochrome *c*

AGING IS A CONDITION ASSOCIATED with a gradual decay in physiological function and integrity. While the mechanisms dictating age-associated biological decline are unclear, volumes of research have focused on the free radical theory of aging. This theory suggests that as an organism ages, a progressive production of reactive oxygen and nitrogen species causes uncorrected macromolecular and cellular damage that accumulates and eventually impacts systemic function. Consistent with this theory, several reports have confirmed an aging-related increase in the production of reactive species accompanied by a loss of cellular integrity and function (19, 26, 33).

Along with an increase in free radical production and oxidative damage, one of the consequences of aging is a decrease in stress tolerance at both cellular and whole-organism levels.

Elderly humans, for example, account for the majority of the morbidity and mortality cases observed during exposure to environmental stressors, such as high ambient temperatures. In fact, recent heat waves have disproportionately claimed the lives of thousands of people over the age of 65 years (1, 44, 49, 50), consistent with the tenet that aging is associated with decreased tolerance to stress. These epidemiological studies have also been recapitulated in a range of animal studies. For instance, upon exposure to heat stress, aged rats display increased morphological damage at cellular and tissue levels, along with elevated levels of reactive oxygen species (ROS) production, substantial oxidative damage, and an associated increase in mortality rate (17, 57, 58).

While aged organisms demonstrate increased oxidative damage when exposed to an environmental challenge, the sources of excess ROS have yet to be elucidated. At the cellular level, it is widely accepted that mitochondrial respiration is the primary source of ROS (48). The four enzyme complexes that make up the electron transport chain (ETC) contain several redox centers that may catalyze the univalent reduction of O₂ to form superoxide. Specifically, Complexes I and III are currently viewed as the main contributors to superoxide production in mitochondria of normal cells (14, 15, 28, 32). Aging has been shown to alter mitochondrial ROS production (16, 34, 48), and more specifically, Complex I appears to be the main contributor to increased ROS levels (13, 29, 30, 34, 52). Because mitochondria from aged animals display increased production of superoxide, these organelles could be more susceptible to heat stress, causing them to further increase intracellular ROS levels and enhance macromolecular damage.

Indeed, previous reports have suggested that mitochondria can be affected by heat stress (3, 7, 18, 38, 39, 42, 51), although the results from these studies are difficult to interpret due to the common use of mild heat stress as a preconditioning agent. For instance, experiments in yeast (7), Antarctic bivalves (18), and rat cardiomyocytes (39) suggest that severe heat stress can structurally and functionally damage mitochondria in an *in vivo* setting. Left unchecked, dysfunctional mitochondria can increase ROS production, leading to a cascade of damage that could cause cell death and eventually lead to deficits in organ function (24, 45, 55).

To protect themselves from injury, cells maintain a large arsenal of highly conserved stress proteins that act to repair damaged proteins and prevent dysfunction. Mainly responsive to the denaturation of proteins (9), the cell stress response involves upregulation and translocation of stress proteins that

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act to bind damaged and misfolded proteins to facilitate refolding, transport, and degradation (25). In addition to the cellular stress response, mitochondria have their own specific stress response (6, 31, 59). The main mitochondrial stress proteins are Hsp60, mtHsp70 (mortalin), and Hsp10 (chaperonin 10), all of which perform the vital functions of importing, transporting, refolding, and preventing aggregation of mitochondrial proteins (10, 53, 54). Hsp60 is the main heat-inducible protein, although the expression of all three proteins can be upregulated during mitochondrial and cellular perturbation.

Previous data have shown that mitochondrial protein degradation and import, two key functions of mitochondrial stress proteins, are impaired with aging, implying that the mitochondrial stress response may be diminished in older organisms (4, 6, 40). Whereas earlier reports have suggested that aging causes declines in the broadly defined "cell stress response" (17, 46, 57), the specific effect of aging on the mitochondrial stress response is poorly characterized. Given that aging alters the cellular stress response associated with hyperthermic challenge, one of the aims of this study was to determine whether the mitochondrial stress response was also impaired with age.

The works of this laboratory have focused specifically on the liver, as it is an organ that is critical for the maintenance of homeostasis in the face of environmental challenge. The liver is a primary site of damage with passive heat stress (17, 22, 23, 58). Its location at the body's core, coupled with its high metabolic rate, raises the temperature of the liver above measured core temperature (11, 47). Besides its apparent susceptibility to heat stress, the liver has also been shown to display aging-related changes. Both increased ROS production (56) and delayed regeneration after injury (43) have been noted in liver tissue in aged organisms. Additionally, the liver has been shown to exhibit age-related alterations in mitochondrial function and ROS production that may further implicate them as a source and/or target of heat stress-induced oxidative damage (8, 13, 21, 27, 29, 34, 52).

If environmental challenge produces greater oxidative stress with aging, and mitochondria are less functional in aged organisms, then it is reasonable to expect that mitochondria are a source of the increased cellular damage seen in old rats after heat stress. We, therefore, hypothesized that mitochondria in aged organisms are more susceptible to heat stress-induced injury and less able to respond to damage as it accrues. To pursue these studies, we used a well-established, physiologically relevant *in vivo* heat stress protocol and assayed the impact of a repeated heat challenge on hepatic mitochondria of young and old rats.

METHODS

Animals

Young (6 mo, $n = 14$, 300–400 g) and old (24 mo, $n = 14$, 325–425 g) male Fischer 344 rats (National Institute on Aging) were used in these experiments. Animals were housed at normal room temperature ($\sim 22^\circ\text{C}$) on a 12:12-h light-dark cycle and fed water and standard chow *ad libitum*. All protocols were approved by the Institutional Animal Care and Use Committee at The University of Iowa. Rats of both age groups were randomly assigned to either heated or sham-heated groups.

Heating

Conscious and unrestrained rats were fitted with colonic thermistor temperature probes (Yellow Springs Instruments, Yellow Springs, CO) prior to an experiment to monitor core temperature. Thermistors remained in place throughout the duration of the heating protocol and for an additional 30 min during the recovery period. Each rat was placed in a plastic cage that was situated underneath an infrared lamp. The lamp was turned on intermittently, as a rat's core temperature was raised from $\sim 38^\circ\text{C}$ to 41.0°C over a 60-min period, and then core temperature was maintained at 41.0°C for an additional 30 min. Rats were allowed to recover in their cages at room temperature for 22.5 h, at which point the heating protocol was repeated. Sham control rats were handled exactly as heated rats, except the heat lamps were not turned on. Body core temperature remained stable ($\sim 38^\circ\text{C}$) throughout the protocol in these animals.

Mitochondrial Isolation

At either 2 or 24 h after the end of the second heating, rats were euthanized via an overdose of pentobarbital sodium (80 mg/kg *ip*). Livers were removed, washed in ice-cold PBS, blotted dry, and weighed. The livers were finely minced with razor blades in ~ 10 ml of isolation medium (110 mM mannitol, 20 mM sucrose, 2 mM Tris, 1 mM EDTA, 1% BSA, pH 7.4), then homogenized for 20 strokes in isolation medium (1:10 wt/vol) at speed 1 on a Sonics Vibracell homogenizer. The homogenate was centrifuged at 4°C (500 *g*) for 10 min to remove debris and dead cells. Supernatant was transferred to a new, clean centrifugation tube and cold centrifuged at 8,000 *g* for 10 min. Pellets were resuspended in storage medium (110 mM mannitol, 20 mM sucrose, 2 mM Tris, and 1 mM EGTA) and then washed twice at 7,000 *g* for 10 min. The pellets were again resuspended in storage media and either used immediately for electron paramagnetic resonance (EPR) spectroscopy, added to fixative in preparation for electron microscopy analysis, or frozen and stored at -80°C for future assays.

Electron Paramagnetic Resonance

Freshly isolated mitochondria (300 μg) were first incubated with the spin trap 5-dimethyl-1-pyrroline *N*-oxide (DMPO; 100 mM) for 2 min at room temperature, followed by the addition of substrates for either Complex I (NADH; 200 μM) or Complex II (succinate; 10 mM) of the ETC. In addition to the substrates, one of two ETC inhibitors was also added to incubate with a sample: rotenone (5 μM), a Complex I inhibitor, or antimycin A (5 μM), a Complex III inhibitor. The samples that were analyzed are shown in Table 1. To determine whether EPR signals could be attributed to superoxide, every trial was reassayed with the addition of SOD. SOD (500 units) was added to a sample and incubated for 2 min before the addition of DMPO. Upon addition of the ETC substrates and inhibitors, the

Table 1. Summary of EPR samples

Trial #	DMPO, 100 mM, and Mitochondria, 100 μg	NADH, 200 μM	Succinate, 10 mM	Rotenone, 5 μM	Antimycin A, μM
1	X				
2	X	X			
3	X	X		X	
4	X	X			X
5	X		X		
6	X		X	X	
7	X		X		X

DMPO, 5-dimethyl-1-pyrroline *N*-oxide; NADH, reduced nicotinamide adenine dinucleotide (a complex I substrate); Succinate, complex II substrate; rotenone, electron transport chain (ETC) complex I inhibitor; antimycin A, ETC complex III inhibitor. Each trial was run on every mitochondrial sample. Additionally, every trial was repeated in the presence of SOD (500 units).

sample was incubated for 10 min at 37°C. The sample, which totaled 0.50 ml, was then added to a TM flat cell; spectra were collected using a Bruker (Karlsruhe, Germany) ESP-300 EPR spectrometer in the University of Iowa ESR Facility. Instrument settings were center field, 3478.73 G; sweep width, 80.00 G; microwave power, 40.2 mW; typical klystron frequency, 9.751 GHz; modulation amplitude, 1.00 Gauss; modulation frequency, 1.00 kHz; receiver gain, 1.00×10^5 ; scan rate, 80 G/84 s; and time constant, 82 ms. Spectra are the result of 15 signal-averaged scans.

Immunoblotting

Sample protein concentrations were determined using the Bradford protein assay (Bio-Rad, Carlsbad, CA). Protein (30 μ g) was run and separated on 4–20% SDS-PAGE and then transferred to nitrocellulose membranes. Membranes were then blocked for 2 h at room temperature in a solution of Tris-buffered saline and 0.05% Tween (TTBS) with 5% dry milk and 2% BSA. After washing with TTBS, the primary antibody of interest was applied to the membranes. The following primary antibodies and dilutions were used for these experiments: anti-cytochrome *c* (StressGen, Ann Arbor, MI) 1:500; anti-Hsp60 (StressGen) 1:1,000; anti-mtHsp70 (StressGen) 1:5,000; anti-Hsp10 1:2,500 (StressGen); and anti-4-hydroxynonenal (4HNE) (Axxora, San Diego, CA) 1:1,000. After further washing with TTBS, the membranes were probed with secondary antibodies. For anti-cytochrome *c*, anti-Hsp60, and anti-mtHsp70 antibodies, goat anti-mouse antibody (StressGen) was used diluted 1:5,000. For anti-4HNE and anti-Hsp10, goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used at a dilution of 1:10,000. After exposure to the secondary antibody, membranes were washed again with TTBS. The blots were then developed using an enhanced chemiluminescence-Western blot detection kit (Amersham, Piscataway, NJ) and BioMaxMR film (Kodak, Rochester, NY). Gels were dried in a drying solution of 20% ethanol and 10% glycerol and used as protein loading controls. Densitometry data are presented as ratios to young control, where young control values have the designation of 1.

Electron Microscopy

Immediately after isolation, mitochondria were fixed at 4°C in 2.5% glutaraldehyde. Fixed mitochondria were rinsed in Sorenson's phosphate wash buffer, postfixed in Caulfield's osmium tetroxide plus sucrose, and rinsed again in wash buffer. Samples were dehydrated in ethanol followed by propylene oxide. After dehydration, samples were embedded in BEEM (West Chester, PA) capsules using EMbed 812 resin and polymerized at 60°C overnight. Samples were cut into thin sections (70–80 nm) and mounted on copper grids. They were then stained with 7.7% uranyl acetate; Reynolds lead citrate served as the counterstain. Micrographs were recorded using a transmission electron microscope (Hitachi H-600) operated at 75 kV.

Statistics

Group means were compared via an ANOVA design for the factors of time and age. The post hoc tests of Bonferroni and Tukey were performed to validate the results of the ANOVA. Differences were considered significant at a level of $P < 0.05$. Data are presented as means \pm SE.

RESULTS

Histology

The repeated heat stress protocol caused extensive damage to mitochondria in both young and old animals (Fig. 1). At the 2-h time point, young and old mitochondria displayed significant damage, including swelling, loss of cristae, and interruption in membrane integrity. Twenty-four hours after heating,

mitochondria isolated from old rats showed damage that was similar to the 2-h time point. However, mitochondria from young rat livers at the 24-h time point exhibited no discernible differences from those of the young control animals.

Superoxide Production

EPR was utilized to measure superoxide production from isolated mitochondria. DMPO is a commonly used spin trap that forms a stable compound with a byproduct of superoxide production. Using EPR, we were able to isolate and measure the adducts of superoxide produced by isolated liver mitochondria in a variety of conditions. Two substrates for the electron transport chain were used in combination with two specific chain blockers as a potential means to determine the site(s) of superoxide production in these mitochondria. Under control conditions, liver mitochondria isolated from old animals produced greater amounts of superoxide than mitochondria from young animals when incubated with the Complex I substrate NADH (Fig. 2, *top*). The greater superoxide production remained evident when mitochondrial ETC Complexes I (rotenone) and III (antimycin A) were blocked. Two hours after heat stress, there was a decrease in old and no change in young mitochondrial superoxide production when a Complex I substrate was added. However, 24 h after heating, NADH-linked superoxide production was elevated in young mitochondria above control and the levels at 2-h. At the 24-h time point, superoxide production was also higher in old mitochondria than at 2 h, but it remained lower than old control levels. Two other substrates for Complex I that are sometimes used to measure Complex I function are glutamate and malate. Experiments with NADH were repeated in the presence of 5 mM glutamate/5 mM malate, and no differences in superoxide production were observed between the NADH and glutamate/malate trials (data not shown).

To confirm that DMPO-OH signals were attributable to the production of superoxide in mitochondria, experiments were repeated with the addition of the antioxidant enzyme SOD. SOD functions as a scavenger for superoxide; therefore, inhibition of signals with the addition of SOD signified the contribution of superoxide to the DMPO-OH that was detected. All signals were inhibited when SOD was added to the samples (Fig. 2*D*).

There are four complexes in the ETC that could be responsible for excess superoxide production in mitochondria. The two most common are Complex I and III. To determine whether Complex III was a source of the excess superoxide, the NADH experiments were also performed using a Complex II substrate. If Complex III was involved in the production of excess superoxide, a repeat of the NADH results would be expected. However, when the Complex II substrate succinate was added to mitochondrial samples, no differences in superoxide production were observed among any of the treatment groups (data not shown). In agreement with previous observations, these experiments indicate that Complex I produces more superoxide in old compared with young mitochondria (30, 34), though Complex II and, in this case, Complex III, appear to be unaltered with aging.

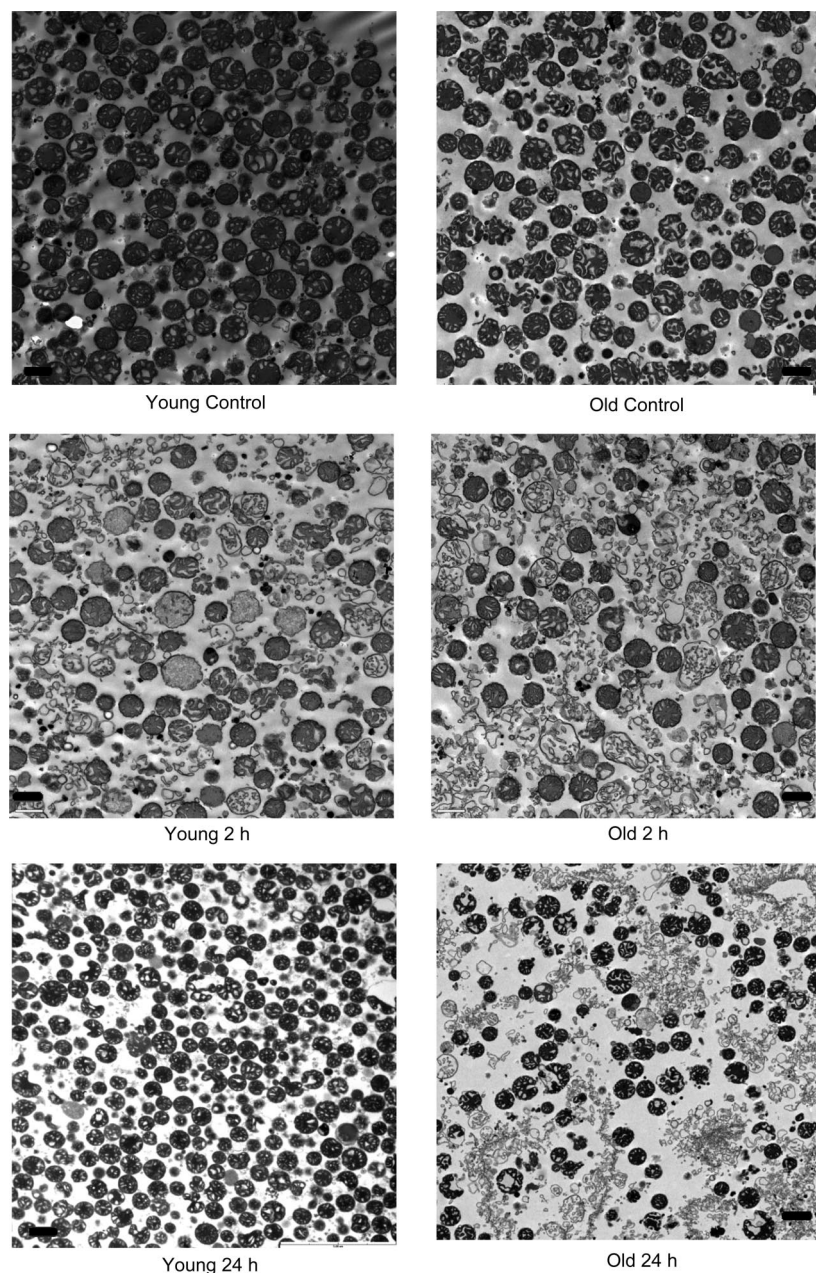


Fig. 1. Heat stress causes structural damage to mitochondria in young and, to a larger extent, old rat livers. Mitochondria were isolated from young and old control and heat-stressed rats and immediately fixed. After dehydration, embedding, and cutting, transmission electron microscopy was used to capture representative micrographs. These images revealed morphological damage in old control mitochondria, while young control mitochondria had normal morphology (*top*). Extensive damage was observed in isolated mitochondria (e.g., severe swelling, loss of cristae, disruption of membranes) from old and young animals after heating at the 2-h time point (*middle*) and in old animals only at the 24-h time point (*bottom*). Scale bar = 10 μ M.

Mitochondrial Damage Markers

4HNE-modified proteins. Damage to proteins resulting from the reaction of the lipid peroxidation byproduct, 4HNE, to form protein adducts was significantly increased in liver mitochondria with time after heat stress. Immunoblot density analysis revealed that young and old mitochondria showed significant increases above control in 4HNE at the 24-h time points during the recovery period after heat stress (Fig. 3).

Cytochrome *c*. Cytochrome *c* levels decreased in isolated hepatic mitochondria from old rats at the 2- and 24-h time points after heat stress compared with control values (Fig. 4, *top*). In young mitochondria, there were no changes in cytochrome *c* levels between controls and the heat-stressed groups. Since cytochrome *c* can leak from the mitochondria into the cytosol when mitochondria are damaged, immunoblot analyses

were performed on the supernatant (i.e., nonmitochondrial cytosolic fraction) collected during mitochondrial isolation. The cytosolic fraction from old mitochondria contained significantly elevated cytochrome *c* levels at both the 2- and 24-h time points after heat stress (Fig. 4, *bottom*).

Mitochondrial Heat Shock Proteins

Hsp60. To determine whether there was an age-related alteration in the stress response in liver mitochondria, immunoblots were performed for Hsp60, which is involved in refolding and transporting damaged mitochondrial proteins. Young heat-stressed mitochondria showed greater levels of Hsp60 protein than control at the 2-h recovery time point, and Hsp60 levels remained elevated at 24-h poststress (Fig. 5). Old mitochondria showed a delayed Hsp60 response, with no

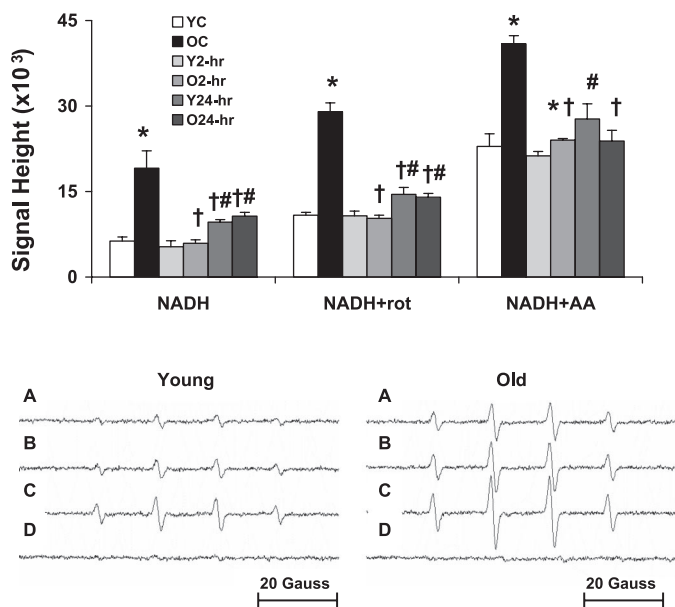


Fig. 2. Effects of age and heat stress on mitochondrial superoxide production. *Top*: height of the 5-dimethyl-1-pyrroline N-oxide (DMPO)-OH signal, a result of the reaction of superoxide with the spin-trap DMPO, was measured on an EPR spectrometer. Values represent means \pm SE of 4–6 animals in each group. * $P < 0.05$ compared with young at same time point. † $P < 0.05$ compared with nonheated age-matched control. # $P < 0.05$ compared with 2-h time point. *Bottom*: examples of EPR experiments from young and old rat mitochondria incubated with the Complex I substrate NADH alone (spectrum A); NADH + rotenone (spectrum B); and NADH + antimycin A (spectrum C). Spectrum D for young and old represent a repeat of trial C (NADH + antimycin A) in the presence of the superoxide scavenger SOD. All EPR signals were virtually eliminated with addition of SOD. Y, young; O, old; C, nonheated control.

increase in protein levels until 24 h after heating. However, even at 24 h, the amount of Hsp60 remained lower in old compared with young mitochondria.

Hsp10. Measured levels of Hsp10, a co-chaperone that works together with Hsp60, were not different between young and old mitochondria in control conditions. However, Hsp10 levels were higher in young vs. old mitochondria at the 2-h time point (Fig. 6). Protein levels of Hsp10 remained elevated 24 h after heat stress in young mitochondria but did not change at any time point in the aged mitochondria.

MtHsp70. MtHsp70, which is primarily involved in protein import and refolding, was expressed at higher levels in old compared with young mitochondria in control conditions (Fig. 7). Young mitochondria displayed a moderate, transient increase in mtHsp70 at the 2-h time point that was not present in old mitochondria. However, at 24-h after heat stress, old mitochondria showed no difference in mtHsp70 protein levels from their young counterparts. In addition, data from immunoblots for Hsp60 and mtHsp70 were also assessed. There was no evidence of mitochondrial stress proteins being present in the cytosol, suggesting that they were retained in the mitochondria during heat stress and the isolation process (data not shown).

DISCUSSION

Taken together, results from the current study support the notion that physiological stressors such as hyperthermic challenge can cause substantial damage to mitochondria, which can

subsequently have a direct impact on cellular integrity and function. Aging is associated with not only increased damage, but also an impaired ability to contain and repair the damage that is manifested via the stress response. Collectively, these two factors suggest that mitochondria in organisms exposed to environmental stressors that are widely and frequently encountered, such as hyperthermia, could sustain severe, compounding injury that leads to increased cellular oxidative damage via superoxide production and, possibly, stimulation of apoptotic and cell death process. This loss of cell viability *in vivo* could then effect a loss of tissue function and have negative consequences at the systemic level.

Strikingly, these data demonstrate that while heat stress causes oxidative damage to both young and old rat liver mitochondria, old mitochondria displayed a particular susceptibility to hyperthermic challenge along with the accumulation of 4HNE-modified proteins, indicating morphological damage related to lipid peroxidation was more severe in the aged animals. Furthermore, as it has been suggested that cytochrome *c* can peroxidize mitochondrial lipids (1, 20, 43), and substantially more cytochrome *c* was released from the mitochondria of old vs. young animals, the current results suggest that liver mitochondria from old animals are more susceptible to oxidative damage compared with liver mitochondria from their young counterparts. The transition of cytochrome *c* from the mitochondria to the cytoplasm along with the accompanying accumulation of hydroperoxides has been shown to lead to the release of proapoptotic factors that can certainly have severe consequences for the cell (24, 37, 55).

These data are also consistent with our recent observations in which rat hepatocytes from heated young and old animals were found to have mitochondria that displayed similar mor-

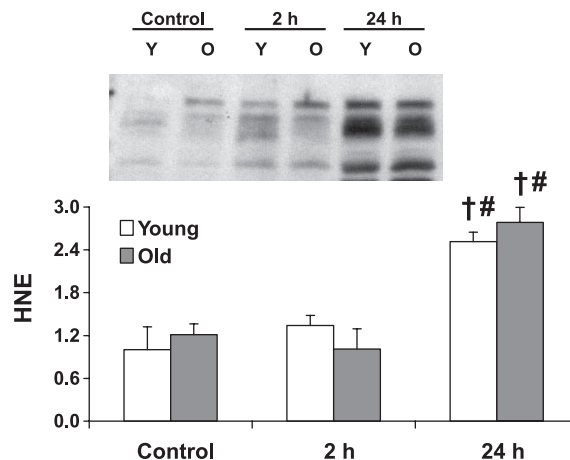


Fig. 3. Lipid peroxidation is increased in mitochondria after heat stress. Mitochondria were isolated from liver samples obtained from young and old rats in control conditions and at 2 and 24 h after heat stress. *Bottom*: densitometry values for immunoblot results are presented. Mitochondrial 4-hydroxynonenal (4HNE) levels were unchanged from control levels for young and old rats at the 2-h time point. At 24-h after heat stress, 4HNE was significantly higher in both young and old mitochondria compared with control and 2-h groups. Four to six rats were analyzed per age group at each time point. The density of each lane was normalized to the density of Gel Code blue-stained gel. Values are means \pm SE. † $P < 0.05$ compared with nonheated age-matched control. # $P < 0.05$ compared with 2-h time point. *Top*: representative 4HNE immunoblots for mitochondria from livers of young and old rats during a time course of recovery from a heat-stress protocol. Each lane represents a liver mitochondrial sample from a different rat with 30 μ g of protein loaded per lane.

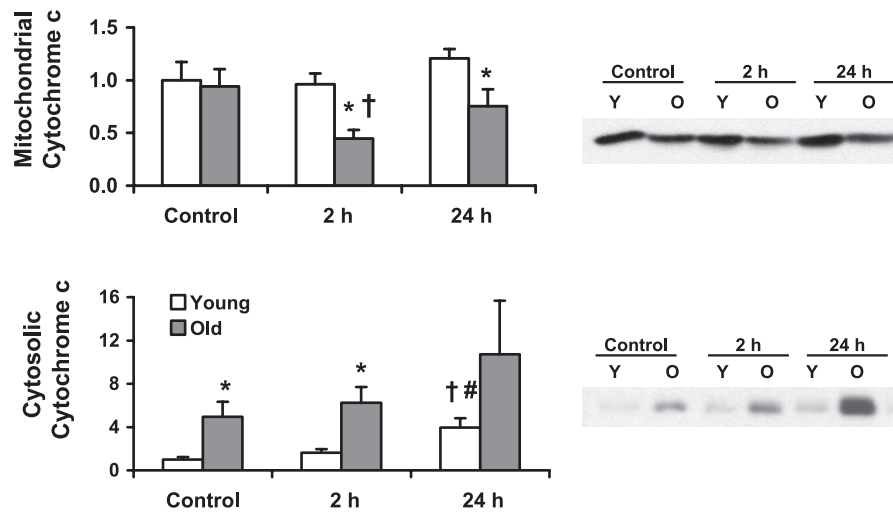


Fig. 4. Aging increases the loss of cytochrome *c* from mitochondria after heat stress. Mitochondria were isolated from liver samples obtained from young and old rats in control conditions and at 2 and 24 h after heat stress. *Upper left*: densitometry values for immunoblot results for mitochondrial cytochrome *c* levels were decreased in old vs. young rats at the 2- and 24-h time points. *Lower left*: densitometry values for immunoblot results for cytosolic cytochrome *c* levels were increased in old vs. young rats at the control and 2-h time points. Four to six rats were analyzed per age group at each time point. The density of each lane was normalized to the density of Gel Code blue-stained gel. Values are expressed as means \pm SE. * P < 0.05 compared with young at the same time point. † P < 0.05 compared with nonheated age-matched control. # P < 0.05 compared with 2-h time point. Representative cytochrome *c* immunoblots are presented for mitochondrial (*upper right*) and cytosolic (*lower right*) fractions from livers of young and old rats during a time course of recovery from a heat-stress protocol. Each lane represents a sample from a different rat with 30 μ g of protein loaded per lane.

phological damage to the isolated mitochondria from these experiments (36). Additionally, in the previous report, livers from heated old rats were observed to have higher levels of damage and increased autophagy compared with their young counterparts. In the current experiments, examination of iso-

lated mitochondria by electron microscopy at the structural level revealed severe morphological damage in mitochondria isolated from heat-stressed animals after 2 h of recovery. After 24 h of recovery, mitochondria isolated from young heat-stressed rats were morphologically similar to nonheated controls. However, old mitochondria continued to display severe morphological damage 24 h after heating, which is consistent

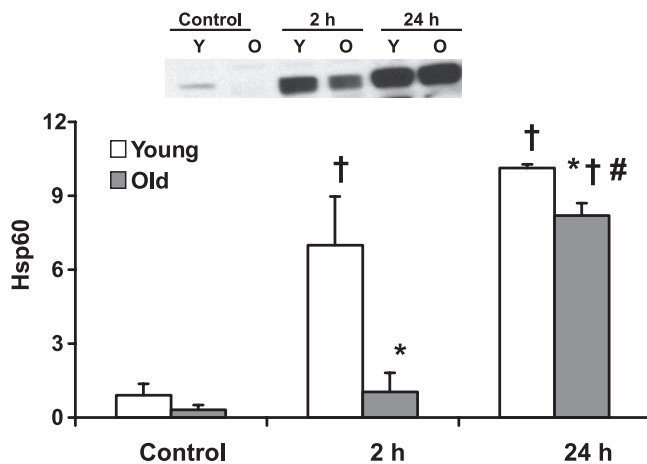


Fig. 5. The stress protein Hsp60 is increased in mitochondria after heat stress. Mitochondria were isolated from liver samples obtained from young and old rats in control conditions and at 2 and 24 h after heat stress. *Bottom*: densitometry values for immunoblot results are presented. Mitochondrial Hsp60 levels were elevated compared with control at 2 and 24 h after heat stress in young rats. Hsp60 levels in old rat mitochondria were unchanged from control and significantly lower than young at 2 h. At 24 h after heat stress, Hsp60 was significantly higher in both young and old mitochondria compared with control. Four to six rats were analyzed per age group at each time point. The density of each lane was normalized to the density of Gel Code blue-stained gel. Values are expressed as means \pm SE. * P < 0.05 compared with young at the same time point. † P < 0.05 compared with nonheated age-matched control. # P < 0.05 compared with 2-h time point. *Top*: representative Hsp60 immunoblots for mitochondria from livers of young and old rats during a time course of recovery from a heat-stress protocol. Each lane represents a liver mitochondrial sample from a different rat with 30 μ g of protein loaded per lane.

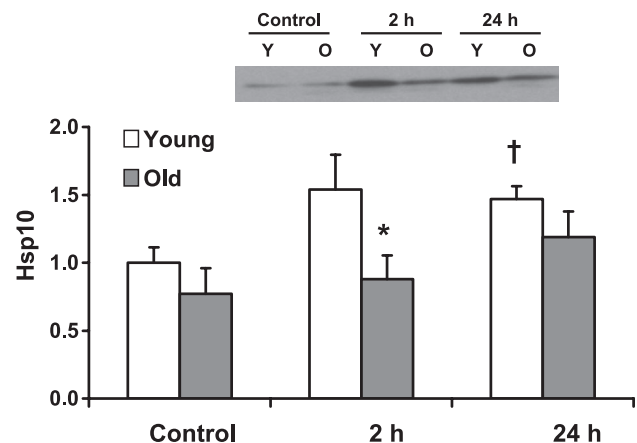


Fig. 6. Heat shock protein 10 (Hsp10) levels are elevated in young, but not old, mitochondria after heat stress. Mitochondria were isolated from liver samples obtained from young and old rats in control conditions and at 2 and 24 h after heat stress. *Bottom*: densitometry values for immunoblot results are presented. Mitochondrial Hsp10 levels in young rats were elevated compared with old at 2 h after heat stress and elevated compared with young control at 24 h. In old rat mitochondria, Hsp10 levels were unchanged from control at either time point after heat. The density of each lane was normalized to the density of Gel Code blue-stained gel. Values are expressed as means \pm SE. * P < 0.05 compared with young at same time point. † P < 0.05 compared with nonheated age-matched control. *Top*: representative Hsp10 immunoblots for mitochondria from livers of young and old rats during a time course of recovery from a heat-stress protocol. Each lane represents a liver mitochondrial sample from a different rat with 30 μ g of protein loaded per lane.

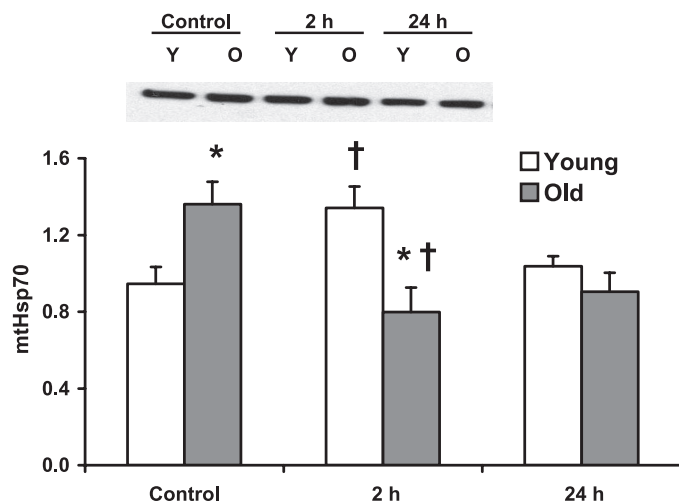


Fig. 7. Age-related changes in mitochondrial Hsp70 (mtHsp70) levels in mitochondria after heat stress. Mitochondria were isolated from liver samples obtained from young and old rats in control conditions and at 2 and 24 h after heat stress. *Bottom*: densitometry values for immunoblot results are presented. Mitochondrial mtHsp70 levels in old rats were elevated compared with young in the control condition. At 2 h after heat stress, young rats showed elevated mtHsp70 levels compared with young control and old 2-h rats. No differences were observed at the 24-h time point. The density of each lane was normalized to the density of Gel Code blue-stained gel. Values are expressed as means \pm SE. * $P < 0.05$ compared with young at the same time point. † $P < 0.05$ compared with nonheated age-matched control. *Top*: representative mtHsp70 immunoblots for mitochondria from livers of young and old rats during a time course of recovery from a heat-stress protocol. Each lane represents a liver mitochondrial sample from a different rat with 30 μ g of protein loaded per lane.

with the accumulation of 4HNE-modified proteins and suggests that these aged organelles have an impaired ability to repair stress-induced damage.

In addition to implicating mitochondrial damage as a key contributor to stress-induced cellular dysfunction, these data suggest that aged organisms cannot mount an appropriate stress response, at the level of the mitochondria, to damage that they incur as a result of a physiological stressor. While young mitochondria exhibit a rapid (i.e., within 2 h) increase in levels of the heat-inducible mitochondrial heat shock proteins, old mitochondria show either no induction or a delay in this protective process, suggesting that the stress response is altered with aging. This observation is in agreement with data demonstrating that protein degradation and import, two main functions of the mitochondrial stress proteins, are impaired with aging (4, 6, 40). In this scenario, an impaired stress response will allow oxidatively damaged proteins to accumulate in the mitochondria, which can ultimately lead to the loss of mitochondrial integrity.

Because we specifically focused on the effects of heat stress on age-related mitochondrial responses, the impact of these mitochondrial alterations on cellular death is outside the scope of the present study. However, it would be enlightening in future studies to investigate the impact of heat stress on cellular apoptotic processes in old vs. young animals. While hyperthermic challenge has been shown to induce apoptosis in young mice and rats (20, 41), the high levels of cytochrome *c* release observed in the current study suggest that old organisms would display a strong activation of the apoptotic caspase cascade. Additionally, the blunted Hsp60 levels in older mitochondria

may contribute to an apoptotic response after a challenge, as this mitochondrial stress protein has been reported to play a role in suppressing apoptosis (10). The release of cytochrome *c*, along with the decreased protein levels of Hsp60, may combine to promote apoptosis in aged animals after a stress-induced disruption of normal function.

Interestingly, mitochondria isolated from old control rats displayed increased superoxide production compared with their young counterparts as measured via EPR. The increased DMPO-OH signal observed in older animals is not present with the addition of the Complex II substrate succinate, implicating an alteration in Complex I with aging. This observation is consistent with other reports that identify Complex I as the main source of increased ROS with age (13, 35, 48). Additionally, a recent report in yeast points to Complex I as being a source of prooxidants during heat stress (18).

However, the increase in superoxide production that was apparent in old control mitochondria was not exhibited in mitochondria isolated from old heated animals, as there was no difference in measured superoxide production between young and old heated animals. A potential explanation for this observation lies in the hypothesis that mitochondria that have high superoxide production are likely dysfunctional. Therefore, adding the additional insult of hyperthermic stress could cause the already damaged and fragile mitochondria to cease functioning. Higher rates of superoxide production have been linked to damaged mitochondria (2), and aged mitochondria display decreased membrane potential and increased fragility (5, 12, 34). It is, therefore, possible that heat stress damages the mitochondria in aged animals in such a way that during the isolation process, severely damaged mitochondria are easily disrupted and discarded with the cellular debris. This would then skew the results of subsequent experiments toward the intact mitochondria remaining in the isolation preparation. In support of this hypothesis, electron micrographs show severely damaged old mitochondria with heat stress (Fig. 1). In addition, increased release of cytochrome *c* from the old mitochondria was revealed via immunoblot analysis (Fig. 4).

The current findings implicate mitochondria as targets of both oxidative and structural damage with heat stress. These observations are consistent with previous studies that have demonstrated increased mitochondrial ROS production and damage in yeast and bivalves with heat exposure (7, 18). Our results also support findings from a recent study in which a decrease in the ability of cardiomyocyte mitochondria to produce ATP was observed following heat stress in anesthetized rats (39). However, the current study is unique in that it examined oxidant production from mitochondria, as well as oxidative damage to mitochondria after a severe *in vivo* heat stress.

Perspectives and Significance

Loss of ability to respond to a stressor can have dire consequences for an organism. While aged mitochondria may be sufficiently functional in a quiescent state of homeostasis, this study demonstrates that perturbing the system, in this case via a physiologically relevant environmental challenge, may cause the manifestation of aging-related impairments. The introduction of a severe challenge to aged animals resulted in structural damage to mitochondria, loss of a critical component

of the electron transport chain, and oxidative injury. Mitochondria, which are responsible for a variety of functions integral to the cell, must be able to repair stress-induced damage to their macromolecular constituents to promote survival. While young mitochondria show the ability to increase the protein levels of stress proteins that can repair damage caused by a disturbance to the organism, a loss of ability of mitochondria from old animals to properly up-regulate Hsp60 and Hsp10 could potentially lead to the accumulation of misfolded and unfolded proteins, further compromising mitochondrial stability. In the wake of the results of this study, it appears that aging-related impairments of the mitochondrial stress response may have a broad negative influence on the ability of aged organisms to tolerate physiological stressors.

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