

# Cytochrome *c* release from mitochondria in the aging heart: a possible mechanism for apoptosis with age

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Received 24 May 2001; accepted in final form 18 October 2001

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Cytochrome *c* release from mitochondria in the aging heart: a possible mechanism for apoptosis with age. *Am J Physiol Regulatory Integrative Comp Physiol* 282: R423–R430, 2002; 10.1152/ajpregu.00296.2001.—There is a loss of myocytes in the aging heart due to necrosis and apoptosis. Oxidative stress, an apoptosis-inducing signal, may also increase in the aging heart. Cytosol and mitochondria isolated from the left and right ventricle of the hearts of 6-, 16-, and 24-mo-old male Fischer 344 rats were used to measure key markers of apoptosis and to assess oxidative stress. Cytosolic cytochrome *c* content was significantly elevated in the 16- and 24-mo-old animals compared with the 6-mo-old animals. Furthermore, Bcl-2, an antiapoptotic protein, showed a strong tendency to decrease with age, whereas Bax, a proapoptotic protein, remained unchanged. Apoptotic protease-activating factor 1 levels and caspase-3 activities were not different among the three age groups. Indicative of the chronic oxidative stress with age, heart mitochondria from old animals showed increases in manganese superoxide dismutase and glutathione peroxidase activity and increases in lipid peroxidation. This is the first study to report cytochrome *c* release from the mitochondria and alterations in Bcl-2 with age in vivo, providing a potential mechanism for the increase in apoptosis seen in the aging heart.

apoptosome; free radicals; myocyte; Bcl-2 family; programmed cell death

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AGING IS CHARACTERIZED BY a progressive deterioration in physiological functions and metabolic processes, ultimately leading to morbidity and mortality. In both humans and animals, the aging process in the heart has been associated with a decrease in the total number of myocytes and reactive hypertrophy of the remaining cells (2, 3, 28, 29). It is important to note that this cell loss occurs even in the absence of pathologies known to cause heart damage, such as atherosclerosis, diabetes, hypertension, and ischemic heart disease. For example, a healthy 70-yr-old male will have a 30% reduction in myocyte number strictly as a result of aging (28, 29). A reduction in the total number of myocytes may lead to an accelerated decline in cardiac functional capacity in the aged heart. In addition to cell

loss via necrosis, apoptosis may be a major factor contributing to the loss of cardiac myocytes with age.

Apoptosis is a highly regulated form of cell death that is characterized by specific morphological, biochemical, and molecular events (38, 41, 42). It is essential to normal development of multicellular organisms and is involved in cell turnover and remodeling in healthy tissue (11, 41, 42). Apoptosis also plays a critical role in removing unwanted and potentially dangerous cells such as tumor cells (43) and cells infected by viruses (39). Disorders such as cancer, acquired immunodeficiency syndrome, Alzheimer's disease, and rheumatoid arthritis are thought to be, at least partially, a result of aberrant regulation of apoptosis (11). It is unclear what the "natural" role of apoptosis is in postmitotic cells and whether cell loss in the heart is beneficial or detrimental for the entire organism.

Kajstura et al. (18) showed that apoptosis in the left ventricles increased by more than 200% in 24-mo-old male Fischer rats compared with 16-mo-old rats, with no change in necrotic cell death. This study strongly suggests that apoptosis may be more prevalent than necrosis in very old rats, and interventions to attenuate this loss could benefit cardiac health. Although this group has provided evidence that the incidence of apoptosis increases in cardiac myocytes with age, there has been little investigation into the possible mechanisms.

Recent evidence demonstrates that mitochondria play a key role in regulating apoptosis (15). There appear to be numerous death-inducing signals, such as reactive oxidant intermediates, decreases in mitochondrial redox status, and increases in intracellular calcium, which can trigger mitochondria to release caspase-activating proteins such as cytochrome *c* and apoptosis-inducing factor. The release of cytochrome *c* from the mitochondria into the cytosol can result in the activation of caspase-9, which is responsible for activating effector caspases, such as caspase-3, to induce cell death. Therefore, mitochondrial dysfunction with age due to factors such as oxidants and deregulation of redox homeostasis could be directly responsible for cardiomyocyte apoptosis.

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This is the first study to examine the role that mitochondria may play in influencing the apoptotic process in vivo in the aging heart. Key markers of apoptosis were measured in isolated cardiac mitochondria and cytosol to determine whether or not they changed with age. Our previous studies show that oxidative stress occurs in cardiac myocytes with age and that caloric restriction attenuated protein damage (22). Because oxidative stress can be an apoptosis-inducing signal, we also evaluated antioxidant enzyme status and oxidative stress. If the mechanisms underlying the increase in apoptosis seen with age can be identified, it could help to explain the loss of ventricular function with age and may lead to discoveries of specific therapeutic interventions that can attenuate this type of cell loss.

## METHODS

**Animals.** Eight 6-mo-, eight 16-mo-, and eight 24-mo-old male Fischer 344 rats (National Institutes of Aging colony, Harlan Sprague Dawley, Indianapolis, IN) were used in this study. The animals were housed two per cage in a temperature- (18–22°C) and light-controlled environment with a 12:12-h light-dark cycle and provided with food and water ad libitum. After a 1-wk acclimation period, the animals were killed on 4 consecutive days (6/day, 2 from each age group).

**Heart perfusion and removal.** Animals were anesthetized with pentobarbital sodium (5 mg/100 g body wt) given intraperitoneally. The chest was opened, the inferior vena cava was severed, and the right ventricle was perfused with 10 ml of ice-cold antioxidant buffer containing 100  $\mu$ M diethylenetriaminepentaacetic acid (DTPA), 1 mM butylated hydroxytoluene (BHT), 1% ethanol, 10 mM 3-aminotriazole, and 50 mM NaHPO<sub>4</sub> (pH 7.4) to remove blood from the heart. DTPA serves as a metal chelator, and BHT acts as a chain-breaking antioxidant to prevent lipid peroxidation *ex vivo*. 3-Aminotriazole was added to inhibit nitric oxide synthase, myeloperoxidase, and other heme proteins (21).

**Mitochondrial and cytosolic protein isolation.** The right and left ventricles were used to isolate mitochondria. A protease was not used during the isolation procedure to avoid the possibility that it could cleave and activate caspases. Therefore, the mitochondria obtained using this procedure are primarily subsarcolemmal rather than interfibrillar (see DISCUSSION on how this relates to aging). Tissue was weighed and minced in 10 vol of isolation buffer (0.225 M mannitol, 0.075 M sucrose, 0.2% fatty acid-free bovine serum albumin, 10  $\mu$ M BHT, 100  $\mu$ M DTPA, pH 7.4). The tissue was homogenized in a Potter-Elvehjem glass homogenizer and centrifuged for 7 min at 480 *g*. The resulting supernatant was stored on ice while the pellet was resuspended in 5 ml of isolation buffer and centrifuged for 7 min at 480 *g*. The combined supernatants were centrifuged for 7 min at 7,700 *g*. The supernatant (cytosolic fraction) was used as an aliquot and stored at –80°C until analyses were performed. The mitochondrial pellet was resuspended in 5 ml of wash buffer (0.225 M mannitol, 0.075 M sucrose, 0.001 M EGTA, 10  $\mu$ M BHT, 100  $\mu$ M DTPA, pH 7.4) using a cotton swab so as not to break the mitochondria and was centrifuged for 7 min at 7,700 *g*. The final mitochondrial pellet was resuspended in 1 ml of storage buffer (0.25 M sucrose, 0.002 M EDTA, 1 mM BHT, 100  $\mu$ M DTPA, pH 7.4), used as an aliquot, and stored at –80°C.

**Determination of mitochondrial membrane integrity.** We used two assays to evaluate mitochondrial membrane integ-

ity. First, cytosolic citrate synthase activity was measured according to Shepherd and Garland (34). Second, cytochrome *c* reduction in isolated intact mitochondria was determined immediately following the isolation procedure. The incubation buffer consisted of 6 mM succinate, 70 mM sucrose, 220 mM mannitol, 2 mM HEPES, 25 mM KH<sub>2</sub>PO<sub>4</sub>, 2.5 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 5  $\mu$ g/ml catalase, pH 7.4, and 40  $\mu$ M acetylated cytochrome *c*. The change in absorbance was measured at 550 nm at 37°C using a spectrophotometric plate reader from Molecular Devices (Sunnyvale, CA).

**Western blotting for Bcl-2, Bax, and apoptotic protease-activating factor 1.** Proteins were separated using 4–12% SDS-polyacrylamide gel under denaturing conditions and electrotransferred onto nitrocellulose. Bcl-2 and Bax were measured in mitochondria, whereas apoptotic protease-activating factor 1 (APAF-1) was measured in the cytosol. The following amounts of protein were loaded in each lane for the different blots: 12  $\mu$ g for Bcl-2, 6  $\mu$ g for Bax, and 45  $\mu$ g for APAF-1. After the transfer, membranes were blocked for 1 h at room temperature with 5% nonfat milk in PBS containing 0.05% Tween 20 (PBS-T/5% milk). Membranes were then incubated with primary antibodies (Bcl-2 or Bax were obtained from Santa Cruz Biotechnology, Santa Cruz, CA and APAF-1 was obtained from BioVision, Palo Alto, CA) at a 1:100 concentration (for Bcl-2 and Bax) and a 1:500 concentration (for APAF-1) in PBS-T/5% milk overnight. After being washed (2  $\times$  5 min with H<sub>2</sub>O), membranes were incubated for 1.5 h at room temperature with secondary anti-rabbit-Ig horseradish-linked whole antibody (Amersham, Piscataway, NJ) at a 1:1,000 concentration in PBS-T/5% milk. Blots were developed using ECL Western blotting detection reagents (Amersham) and analyzed using ImageJ (National Institutes of Health).

**Biochemical analyses.** Manganese superoxide dismutase (MnSOD) activity was assayed according to Oyanagui (31) with slight modification. One unit of MnSOD activity is defined as the concentration of enzyme that inhibits nitrite formation from hydroxylamine in the presence of xanthine oxidase by 50%. Selenium-dependent glutathione peroxidase (GPX) activity was assayed at 37°C according to Flohe and Gunzler (13), with H<sub>2</sub>O<sub>2</sub> as the substrate. Glutathione reductase (GR) activity was measured at 30°C according to Carlberg and Mannervik (7). Catalase activity was measured at 20°C according to Aebi (1), with slight modification. Lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARs) according to Ohkawa et al. (27), with some modification. Cytochrome *c* was measured using an immunoassay obtained from R&D Systems (Minneapolis, MN). Caspase activity was measured using the synthetic peptide *N*-acetyl-DEVD-7-amino-4-methylcoumarin (AMC) (BD PharMingen, San Diego, CA). This assay detects activated caspase-3 as well as caspases-6, -7, and -8. Active caspases will cleave the AMC from the peptide, and the free AMC will fluoresce. Briefly, 1 ml of assay buffer (20 mM HEPES, 10% glycerol, 1 M dithiothreitol, and 14  $\mu$ l of Ac-DEVD-AMC/ml of buffer) and 50  $\mu$ l of sample were added to a microfuge tube and protected from the light. Ten- and 100-ng standards of active caspase-3 were also prepared. Samples were incubated at 37°C for 60 min after which fluorescence was measured on a spectrofluorometer with an excitation wavelength of 380 nm and an emission wavelength of 440 nm. In addition, caspase-3 activity was measured using a colorimetric assay kit, which is specific for caspase-3, from CalBiochem (San Diego, CA). Protein content was analyzed using the Bradford method. Biochemical analysis was performed in triplicate, and the mean was used for statistical analysis.

*Statistical analysis.* Multiple comparisons were performed using a one-way analysis of variance. When appropriate, Tukey's post hoc analysis was performed. The independent *t*-test was used when only two groups were compared. A *P* value of <0.05 was considered significant.

**RESULTS**

*Heart weight and body weight of animals.* Table 1 shows that both the heart and body weights of the 16- and 24-mo-old animals were significantly higher (*P* < 0.001) compared with those of the 6-mo-old animals. The 24-mo-old animals also had a significantly higher heart weight-to-body weight ratio than the 16-mo-old animals (*P* < 0.05). There was no difference in mitochondrial and cytosolic protein levels between groups.

*Membrane integrity was not different due to mitochondrial isolation procedures.* To ensure that mitochondria isolated from young and old animals showed no differences in damage during the isolation procedure, we determined membrane integrity with two independent assays. First, membrane integrity was assessed immediately after mitochondrial isolation by measuring cytochrome *c* reduction in intact mitochondria (Table 2). An increase in mitochondrial membrane damage would allow more cytochrome *c* to enter the mitochondria and become reduced by superoxide produced by the inner membrane. After 10 min, no significant difference in cytochrome *c* reduction was found between the young and old animals. Membrane integrity was also determined by measuring citrate synthase activity in the cytosol (Table 2). Citrate synthase activity levels in the cytosol of the old animals were decreased compared with the young animals, suggesting a decrease in the activity of this enzyme with age. Taken together, these results indicate that there was no significant increase in mitochondrial membrane damage in the old animals.

*Cytosolic cytochrome c is significantly increased with age in the left and right ventricles of the heart.* In cell culture, the release of cytochrome *c* from the mitochondria is a key apoptotic event, and therefore, we measured levels of cytochrome *c* in the cytosol and the mitochondria of the three different ages of animals (Fig. 1). Cytosolic and mitochondrial samples were diluted 1:500 and 1:5,000, respectively. Cytosolic cytochrome *c* levels were significantly higher in the 16- and 24-mo-old animals compared with the 6-mo-old ani-

Table 2. Mitochondrial membrane integrity as determined by cytosolic citrate synthase activity and mitochondrial cytochrome *c* reduction in hearts of male F344 rats

	6 Months (n = 8)	24 Months (n = 8)
Cytochrome <i>c</i> reduction	0.14 ± 0.01	0.16 ± 0.01
Cytosolic citrate synthase	0.36 ± 0.02	0.26 ± 0.01*

All values are presented as means ± SE; \**P* = 0.0007. Citrate synthase activity (μmol·min<sup>-1</sup>·mg protein<sup>-1</sup>) was measured in the cytosol, and cytochrome *c* reduction (absorbance at 550 nm) was measured in intact isolated mitochondria. An unpaired, 2-tailed *t*-test was used for statistical analysis.

mals. Importantly, there was no change in mitochondrial cytochrome *c* levels among the three age groups. Therefore, these data strongly suggest that mitochondria from the left and right ventricles of the heart release detectable levels of cytochrome *c*. This is very significant because the study by Kajstura et al. (18) indicates that with age, apoptosis is primarily confined to the left ventricle (see DISCUSSION).

*Mitochondrial Bcl-2 tended to decrease with age, and no major changes in Bax or APAF-1 protein were found.* We evaluated the levels of mitochondrial Bcl-2 and Bax, proteins that are proposed to control cytochrome *c* release, and APAF-1, a critical protein implicated in the formation of the apoptosome. This is the first time that Bcl-2 and Bax have been investigated in the mitochondria of aging animals. Mitochondrial Bcl-2 levels tended to decrease with age (Fig. 2) (*P* = 0.0565, *n* = 7 in each group), whereas the levels of Bax (Fig. 3) and

Table 1. Body weights, heart weights, and protein levels in 6-, 16-, and 24-mo-old male F344 rats

	6 Months (n = 8)	16 Months (n = 8)	24 Months (n = 8)
Body weight, g	329.5 ± 9.9	425.3 ± 4.3*	415.8 ± 8.2*
Heart weight, g	0.77 ± 0.02	0.95 ± 0.03*	1.04 ± 0.03*
Heart/body weight, g/kg	2.3 ± 0.03	2.2 ± 0.06	2.5 ± 0.11†
Cytosolic protein, mg/ml	4.0 ± 0.11	4.0 ± 0.15	4.3 ± 0.12
Mitochondrial protein, mg/ml	5.6 ± 0.49	5.5 ± 0.25	5.4 ± 0.3

Values are means ± SE. \**P* < 0.001 vs. 6 mo. †*P* < 0.05 vs. 16 mo. F344, Fischer 344.

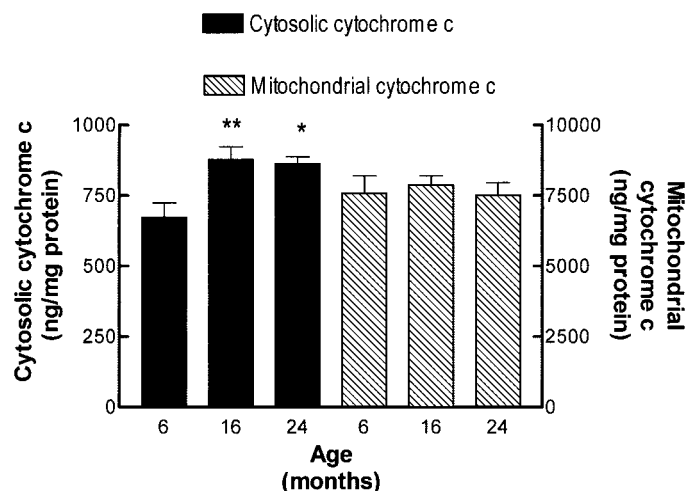


Fig. 1. Effect of age on cytochrome *c* levels in heart cytosol and mitochondria of 6-, 16-, and 24-mo-old male Fischer 344 (F344) rats (*n* = 8 for each group). Cytosolic cytochrome *c* values are presented (means ± SE) as solid bars. \*\**P* < 0.01, 16- vs. 6-mo-old animal; \**P* < 0.05, 24- vs. 6-mo-old animal. Mitochondrial cytochrome *c* values are presented (means ± SE) as hatched bars. There were no differences in mitochondrial cytochrome *c* content among the 3 age groups. Cytosolic and mitochondrial samples were diluted 1:500 and 1:5,000, respectively. Cytochrome *c* was measured using an immunoassay obtained from R&D Systems (see METHODS).

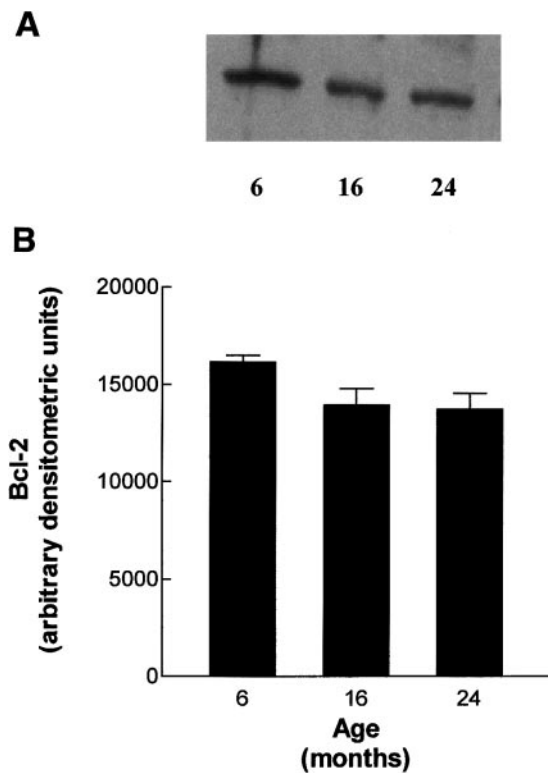


Fig. 2. Effect of age on Bcl-2 levels in isolated heart mitochondria of male F344 rats. A: representative Western blot of Bcl-2 protein (see METHODS) in a 6-, 16-, and 24-mo-old animal. B: densitometric graph of Bcl-2 levels in the 3 ages of animals ( $n = 7$  for each group). Values are means  $\pm$  SE;  $P = 0.0565$ .

APAF-1 (Fig. 4) did not show any changes with age. A decrease in Bcl-2, without any change in Bax, may leave the cell more vulnerable to apoptosis, because it is the ratio of these proteins, rather than the absolute levels, that influences cytochrome *c* release and apoptosis.

*Caspase activity was not different in old animals compared with young animals using two different assays.* Programmed cell death is a transient process that occurs in minutes to hours. Despite this, we were interested in seeing if caspase activity was elevated in the old animals. With the use of the synthetic peptide *N*-acetyl-DEVD-AMC, no detectable difference in caspase activities (caspases-3, -6, -7, and -8) was found between the young and old animals, despite the fact that there was significantly more cytochrome *c* in the cytosol of the older animals. The means and standard errors for caspase activities (expressed as arbitrary fluorescent intensity units) were  $244 \pm 8.6$  (6 mo),  $250 \pm 3.2$  (16 mo), and  $262 \pm 9.5$  (24 mo). We used an additional colorimetric assay specific for caspase-3 activity that also showed no change in caspase activity with age. Means and standard errors expressed as picomoles per minute were  $2.4 \pm 0.17$  (6 mo),  $2.0 \pm 0.14$  (16 mo), and  $2.5 \pm 0.12$  (24 mo). Adaptive factors, such as age-dependent increases in caspase inhibitors, could possibly explain why caspase activity was not elevated in the old animals.

*Major antioxidant enzymes MnSOD and GPX were elevated due to the chronic oxidative stress in the aging heart.* Antioxidant enzyme activities in the mitochondria of the three different ages of animals are shown in Table 3. Compared with the 6-mo-old animals, MnSOD activity was significantly elevated (+50%) in the 16- and 24-mo-old animals. GPX activity was higher in the mitochondria of the 24-mo-old animals (+25%) compared with the 6-mo-old animals. However, there were no age-related differences in GR activity or catalase activity.

*Malondialdehyde was elevated in the aging heart.* The levels of TBARS in the cytosol were increased twofold in the 24-mo-old rats compared with the 6-mo-old rats (Fig. 5). This supports the findings of others that the aging heart accumulates markers of oxidative DNA, lipid, and protein damage (4, 8, 20, 22, 45).

## DISCUSSION

There are no studies to date that have attempted to identify the molecular mechanisms underlying the loss of cardiomyocytes with age in an acute in vivo animal model. Although it has been shown that there is an increase in cardiomyocyte apoptosis with age (18, 26), the reasons for this increase remain largely unknown. This study was designed to evaluate markers of apoptosis to determine whether or not they change with age and become active players in cell death. Because oxi-

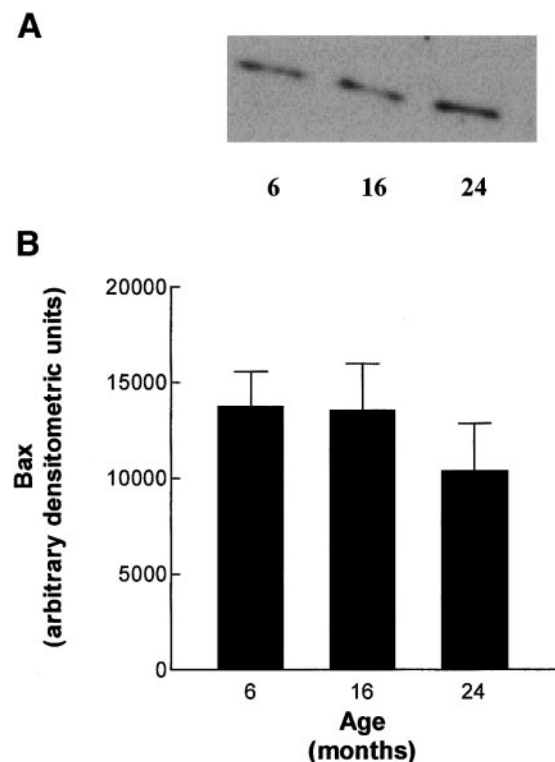


Fig. 3. Effect of age on Bax levels in isolated heart mitochondria of male F344 rats. A: representative Western blot of Bax protein (see METHODS) in a 6-, 16-, and 24-mo-old animal. B: densitometric graph of Bax levels in the 3 ages of animals ( $n = 7$  for each group). Values are means  $\pm$  SE;  $P = 0.5135$ .

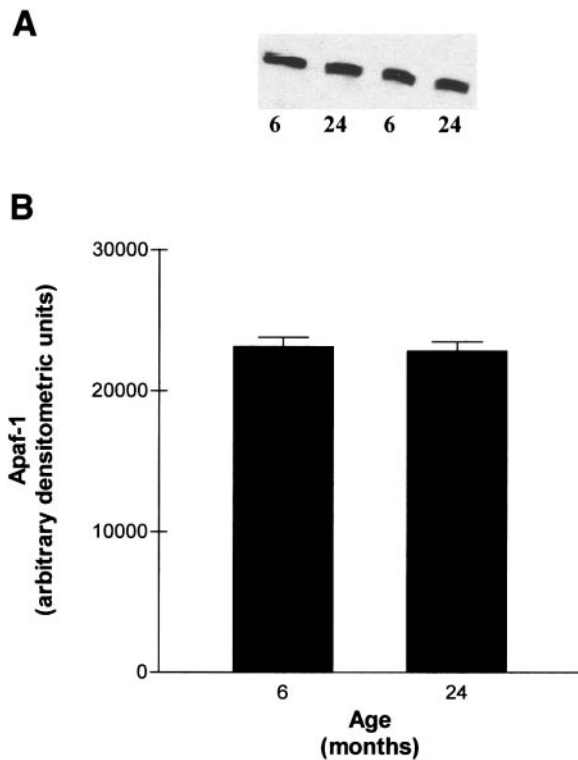


Fig. 4. Effect of age on apoptotic protease-activating factor 1 (APAF-1) levels in heart cytosol of male F344 rats. A: representative Western blot of APAF-1 protein (see METHODS) in 6- and 24-mo-old animals. B: densitometric graph of APAF-1 levels in the 2 ages of animals ( $n = 5$  for each group). Values are means  $\pm$  SE;  $P = 0.7416$ .

ductive stress is one of the many factors that can induce apoptosis, activities of major mitochondrial antioxidant enzymes and heart lipid peroxidation were also assessed.

Although apoptosis can occur via cytochrome *c*-independent mechanisms, it is well established that in most cell types, once cytochrome *c* is released into the cytosol it interacts with APAF-1 and procaspase-9, leading to the generation of active caspase-9, which is capable of proteolytically activating caspase-3. Active caspase-3 can then initiate the apoptotic degradation phase followed by the death of the cell (14). Cytochrome *c* is normally located in the intermembrane

Table 3. Activities of antioxidant enzymes in cardiac mitochondria of 6-, 16-, and 24-mo-old male F344 rats

Age	<i>n</i>	MnSOD	GPX	GR	Cat
6 Mo	8	99.1 $\pm$ 5.2	33.8 $\pm$ 2.3	5.3 $\pm$ 0.7	0.031 $\pm$ 0.004
16 Mo	8	139.9 $\pm$ 10.1*	33.9 $\pm$ 1.8	4.1 $\pm$ 0.5	0.026 $\pm$ 0.002
24 Mo	8	146.8 $\pm$ 2.5*	42.5 $\pm$ 3.3†	5.6 $\pm$ 0.5	0.025 $\pm$ 0.004

Values are means  $\pm$  SE;  $n =$  number of rats per group. MnSOD, manganese superoxide dismutase (U/mg protein); GPX, glutathione peroxidase ( $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ ); GR, glutathione reductase ( $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ ); Cat, catalase (K/mg protein). \* $P < 0.001$  vs. 6 mo; † $P < 0.05$  vs. 6 and 16 mo. Assays for MnSOD, GPX, GR, and Cat are described in METHODS.

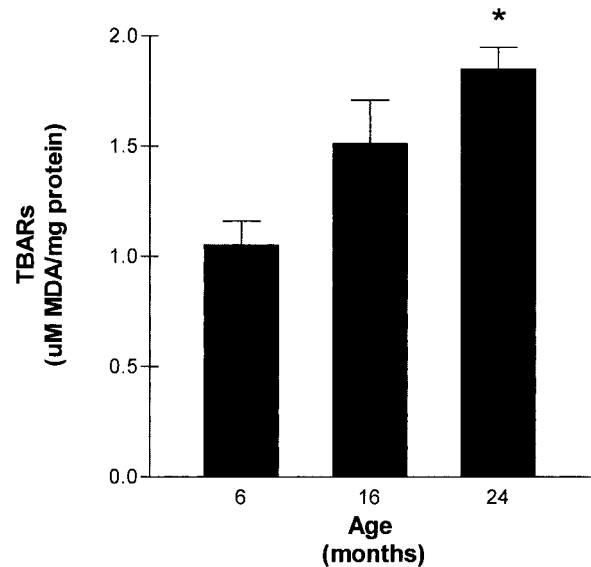


Fig. 5. Effect of age on lipid peroxidation in heart cytosol of male F344 rats ( $n = 8$  for each group). Values are means  $\pm$  SE; \* $P < 0.01$  vs. 6-mo-old animal. TBARs, thiobarbituric acid reactive substances.

space of the mitochondrion, loosely bound to the inner membrane.

In this study, using subsarcolemmal mitochondria obtained from the left and right ventricles, cytochrome *c* levels in the cytosol of the 16- and 24-mo-old animals were significantly higher compared with those in the 6-mo-old animals. The study by Kajstura et al. (18) clearly indicates that with age, apoptosis is primarily confined to the left ventricle. We used both the left and right ventricles to obtain enough mitochondria to perform all planned analyses. Therefore, it can be hypothesized that if we were able to use only the left ventricle, where apoptotic processes appeared to be confined, we would have provided a set of data leading to more clear-cut conclusions. However, our findings are very significant, considering that the quantified differences in cytosolic cytochrome *c* between age groups represent an average release of cytochrome *c* from all myocytes. In the future, we plan to isolate mitochondria solely from the left ventricle and focus on apoptotic adaptations and signaling events in this location. Furthermore, Fannin et al. (12) have shown that two populations of cardiac mitochondria exist, subsarcolemmal mitochondria that reside beneath the plasma membrane and interfibrillar mitochondria located between the myofibrils. They showed that interfibrillar mitochondria isolated from 6-, 24-, and 28-mo-old Fischer 344 rat hearts have a greater reduction in oxidative metabolism compared with submitochondrial mitochondria. Therefore, in future studies, we plan to add proteases during the isolation procedure to isolate the intermyofibrillar mitochondria and study their adaptive responses to oxidative stress and apoptotic signaling events.

Mitochondrial Bcl-2 levels showed a very strong tendency to decrease with age, whereas no significant changes were found in mitochondrial Bax levels. The

mechanisms by which the Bcl-2 family proteins regulate apoptosis have not yet been clearly defined (30, 33). Many of the Bcl-2 family proteins are located in the outer mitochondrial membrane, although Bax is primarily located in the cytosol and translocates to the outer membrane upon receiving an apoptotic signal (44). A widely accepted hypothesis is that these proteins form channels or pores in the outer mitochondrial membrane and thereby directly regulate the permeability of the outer membrane to cytochrome *c* (33).

Although it appears that both Bcl-2 and Bax can regulate apoptosis independently, there also seems to be an *in vivo* competition that exists between the two. Homodimers of Bax (Bax/Bax) create large pores in the outer membrane and promote apoptosis by facilitating the release of cytochrome *c*, whereas heterodimers of Bcl-2/Bax prevent pore formation and inhibit apoptosis (30). Therefore, the trend for Bcl-2 to decrease in the mitochondria of the aged animals, while Bax remained unchanged, increases the probability that there will be fewer Bcl-2/Bax heterodimers and more Bax homodimers. This could possibly explain why elevated levels of cytochrome *c* were observed in the cytosol of the 16- and 24-mo-old animals compared with the 6-mo-old animals.

Caspase activation in mammalian cells can occur in several ways (5). For example, tumor necrosis factor cytokine receptors (death domains) can proteolytically activate caspase-8, which then directly or indirectly activates caspases-3, -6, and -7. Alternatively, caspases can be activated by the release of cytochrome *c* from the mitochondria into the cytosol. On the basis of the increased levels of cytochrome *c* in the older animals, it was expected that caspase activity would also be elevated. However, using two independent assays, no change in caspase activity was observed in the three different ages of animals. There are several hypotheses that could help explain these findings. The release of cytochrome *c* from the mitochondria into the cytosol has been shown to be one of the earliest apoptotic events (19, 23, 46). Cytochrome *c* release occurs before mitochondrial depolarization, caspase activation, and DNA fragmentation (46). Because this was an acute study, it is possible that at the time the animals were killed, the cells that were undergoing the early phases of apoptosis (i.e., release of cytochrome *c*) were not yet able to activate caspases.

Another possibility to explain our findings is that there could be a threshold level of the amount of cytochrome *c* required to activate caspases. All ages of animals had detectable levels of cytochrome *c* in the cytosol, and it is possible that cells have evolved to become unresponsive to small amounts of cytosolic cytochrome *c* to prevent excessive apoptosis in healthy cells. Moreover, it has been found that there are endogenous inhibitors of caspases, also known as inhibitors of apoptosis or IAPs (9, 10). IAPs were initially discovered in baculoviruses where they function to suppress the cell death response of the host cell, thereby allowing the virus to survive and replicate. Mammalian IAPs have the ability to bind pro-

caspase-9, consequently preventing further caspase activation. Additionally, they can directly bind to and inhibit caspase-3. Cells that contain large amounts of endogenous caspase inhibitors may not be susceptible to cytochrome *c*-induced apoptosis and may instead die as a result of necrosis (15). Future studies will determine if these inhibitors are indeed present and if they increase in the cytosol with advanced age in the hearts of these animals, thereby preventing apoptosis.

As mentioned earlier, cytochrome *c* is not the only factor required to activate caspases. APAF-1 and ATP/dATP must also be present in sufficient amounts in the cytosol in order for the complex to bind and activate procaspase-9, which then activates downstream caspases. We were able to detect significant levels of APAF-1 in cardiac myocytes, and this is significant because Burgess et al. (6) showed that human skeletal muscle lacked APAF-1. However, we found no difference in APAF-1 levels between the 6- and 24-mo-old animals, and therefore, this was most likely not a limiting factor for caspase activation.

Numerous signaling pathways could have been responsible for the activation of apoptosis, such as decreased levels of mitochondrial reducing equivalents (ATP, NADPH, and GSH), increases in cytosolic and mitochondrial calcium levels, and increased production of reactive oxygen species. We, therefore, investigated if there was oxidative stress in the heart and if there were adaptive responses to the increase in oxidant production with age. In addition, the literature reports conflicting results as to how they change with age in the heart. In this study, the activity of MnSOD was significantly elevated in heart mitochondria of the 16- and 24-mo-old rats, whereas GPX activity was significantly elevated only in the 24-mo-old animals. These data are consistent with those reported previously (16, 17), and they indicate that upregulation of cardiac antioxidant enzymes may be due to chronic life-long exposure to oxidants. There was no age-dependent change in GR activity, possibly because the heart contains sufficient levels of GR to handle the increase in oxidized glutathione and therefore its synthesis is not induced. Evidence for this can be found in the fact that in skeletal muscle, which has lower levels of GR than the heart, GR only increased half as much as GPX as a function of age (40). Not too long ago, it was believed that catalase was found mainly in the peroxisomes and that mitochondria did not contain significant levels of this antioxidant enzyme. However, very low levels of catalase activity have been detected in cardiac mitochondria (32). Our study is in agreement with this finding, although we did not see any changes in activity with age. This suggests that catalase plays only a minor role in scavenging H<sub>2</sub>O<sub>2</sub> in heart mitochondria with age compared with GPX (24).

Lipid peroxidation was measured to see if oxidative damage to the heart increases as a function of age. Despite an increase in MnSOD and GPX activities, there was an increase in lipid peroxidation in the cytosol of the old animals compared with the young and middle-age groups. This increase in oxidative damage

corresponds with the findings of Ji et al. (17). In addition, others show that protein oxidation and DNA damage are also significantly increased in old animals compared with young animals (4, 22, 35, 36). Products of lipid peroxidation, protein oxidation, and DNA oxidation could form specific adducts, i.e., lipid-DNA adducts, and possibly induce apoptosis. Further investigation into the possibility that secondary products of oxidation could affect apoptotic pathways is of current interest.

### Perspectives

This is the first study to demonstrate in vivo that there are elevated levels of cytosolic cytochrome *c* in the aging heart. The levels of the antiapoptotic protein Bcl-2 tended to decrease with age, and this may be one explanation for the age-related changes observed in cytochrome *c*. It is also possible that other Bcl-2 family proteins (i.e., Bcl-x1 and Bak) may change with age and influence the release of cytochrome *c* from the mitochondria. To study all of the interactive possibilities of apoptosis with aging in vivo would be an immense task. However, understanding age-associated alterations in the key proteins regulating apoptosis may directly lead to targeted interventions. Additionally, mice with specific gene deletions or mice overexpressing MnSOD may provide additional avenues to investigate aging, apoptosis, and oxidative stress. We found that mitochondrial MnSOD and GPX activities were significantly elevated in the old animals, possibly suggesting that they are upregulated in response to increases in superoxide radicals and hydrogen peroxide (25, 37). Therefore, there is a strong possibility that oxidative stress in the hearts of the old animals may partly be responsible for myocyte apoptosis. Future studies using interfibrillar mitochondria from the left ventricle will attempt to determine if this subpopulation of mitochondria is mainly responsible for oxidative stress and myocyte apoptosis in vivo.

We thank A. Dirks, A. Childs, and T. Phillips for critical reading of the manuscript.

This research was supported by grants from the Society of Geriatric Cardiology, National Institutes of Health, and National Institute on Aging Grant AG-17994-01.

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