LEcT ure 7
Apoptosis in Aging, Disease and Exercise
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Simplified Signaling Pathways of Apoptosis

Chronic Oxidative Stress and Apoptosis
Myocardial Aging, Sarcopenia, Neurodegeneration

- Decrease in total number of skeletal and heart myocytes as well as neurons with age
- May lead to accelerated decline in cardiac functional capacity, sarcopenia, neurodegenerative diseases
- Oxidative Stress and Apoptosis may be one major factor

Mitochondria and Apoptosis

- Aging \rightarrow progressive deterioration in physiological functions and metabolic processes, i.e., Mitochondrial Dysfunction
- Radical-mediated mitochondrial damage accumulates over time and may account for age-related induction of apoptosis.

Mitochondrial/Free Radical Theory of Aging

- Oxidative stress
- mt-DNA damage
- mt-DNA deletions
- Oxidized proteins
- Lipid peroxidation
- Lipid-adduct formation
- Decrease in repair systems

ACUTE ACTIVATION OF MITOCHONDRIAL MEDIATED APOPTOSIS

- Doxorubicin treatment
- Cytochrome c release
- Caspase-3 activation
- Apoptotic index
In disease conditions there is strong evidence that apoptosis occurs and is partly mitochondrial-mediated.

Is there evidence in normal aging?

Apoptosis in heart failure: Release of cytochrome c from mitochondria and activation of caspase-3 in human cardiomyopathy

Necrotic and apoptotic myocyte cell death in the aging heart of Fischer 344 rats (Kajstura et al., Am. J. Phys., 1996)

- Apoptosis and necrosis ↑ with age in hearts of male F344 rats
- Apoptosis = 140 Cells at 3 months vs. 874 Cells at 24 months LV
- Did not look at mechanisms underlying this phenomenon!

Left and Right Ventricular Function (Kajstura et al., Am. J. Phys., 1996). Other ages 3,7,12 months

<table>
<thead>
<tr>
<th></th>
<th>16 Months</th>
<th>24 Months</th>
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<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart Rate</td>
<td>409±33</td>
<td>380±35</td>
</tr>
<tr>
<td>Systolic BP</td>
<td>108±9</td>
<td>96±12*†‡</td>
</tr>
<tr>
<td>MBP</td>
<td>92±8</td>
<td>79±13*†‡</td>
</tr>
<tr>
<td>LVSP</td>
<td>108±8</td>
<td>96±12*†‡</td>
</tr>
<tr>
<td>RVSP</td>
<td>24±2.9</td>
<td>23.5±2.6*</td>
</tr>
<tr>
<td>LV +dP/dtmax</td>
<td>8,937±1,051</td>
<td>6524±1,157†‡§</td>
</tr>
<tr>
<td>LV -dP/dtmax</td>
<td>8,714±891</td>
<td>5,053±995 ‡§</td>
</tr>
</tbody>
</table>
Increased cytosolic cytochrome c in the heart of 24-month old rats

![Graph showing increased cytosolic cytochrome c with age]

What happens with age with the pro- and anti-apoptotic proteins in the mitochondrial outer membrane?

Apoptosis Regulators: Bcl-2 family

- **Bcl-2**: A protein that inhibits cell suicide
  - Originally thought to promote cancer
  - Over-expression leads to accumulation of cells
- **Bax**: A protein which promotes PTP.
  - Promotes cytochrome c release and cell death

![Diagram of apoptosis regulation]

**The Bcl-2 Family**

Fig. 5. Bcl-2 gene family. An expanded family of proteins homologous to Bcl-2 are most highly conserved within Bcl-2 homology. BH1 and BH2 domains. Identical amino acids are in black background and conserved proteins in gray background.

![Graph showing cytochrome c release from mitochondria in the aging heart]

Since skeletal muscle is a multi-nucleated cell..

- Reported data on the occurrence of skeletal muscle apoptotic nuclei in human or animal disease models range between 0.03% and 2.1%. To enter the discussion about significance and relevance of apoptosis in skeletal muscle, it is important to solve the question of how long is an apoptotic nucleus detectable and what would be the relevance of such a small loss of nuclei for myocyte integrity and function?
- Is this phenomena important for muscle dysfunction?
- There could be a substantial loss of muscle mass over a longer time period, decreasing skeletal and heart muscle function?

Occurrence of apoptosis in mature skeletal myocytes

- Duchenne muscular dystrophy (DMD)
- Motor neuron disorders or unloading
- Denervation
- Ischemia-Reperfusion
- Chronic heart failure (CHF)
Is there skeletal muscle fiber loss with age in humans and animals?

**HUMANS**


**ANIMALS**


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**Characteristics of investigated individuals**

<table>
<thead>
<tr>
<th>Group</th>
<th>Case</th>
<th>Age (Years)</th>
<th>Cause of Death</th>
<th>Chronic Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>55</td>
<td>Male</td>
<td>Ventricular septal defect</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>65</td>
<td>Male</td>
<td>Sudden infant death syndrome</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>70</td>
<td>Female</td>
<td>Posttraumatic brain injury</td>
<td>None</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
<td>Female</td>
<td>Posttraumatic soft tissue injury</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>90</td>
<td>Male</td>
<td>Posttraumatic soft tissue injury</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>95</td>
<td>Male</td>
<td>Posttraumatic soft tissue injury</td>
<td>None</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
<td>Male</td>
<td>Posttraumatic soft tissue injury</td>
<td>None</td>
</tr>
<tr>
<td>8</td>
<td>110</td>
<td>Male</td>
<td>Hysterical abortion</td>
<td>Abruptio placentae</td>
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<tr>
<td>9</td>
<td>120</td>
<td>Male</td>
<td>Abruptio placentae</td>
<td>Lower uterine segment</td>
</tr>
<tr>
<td>10</td>
<td>130</td>
<td>Male</td>
<td>Abruptio placentae</td>
<td>Lower uterine segment</td>
</tr>
<tr>
<td>11</td>
<td>140</td>
<td>Male</td>
<td>Abruptio placentae</td>
<td>Lower uterine segment</td>
</tr>
<tr>
<td>12</td>
<td>150</td>
<td>Male</td>
<td>Abruptio placentae</td>
<td>Lower uterine segment</td>
</tr>
<tr>
<td>13</td>
<td>160</td>
<td>Male</td>
<td>Abruptio placentae</td>
<td>Lower uterine segment</td>
</tr>
<tr>
<td>14</td>
<td>170</td>
<td>Male</td>
<td>Abruptio placentae</td>
<td>Lower uterine segment</td>
</tr>
<tr>
<td>15</td>
<td>180</td>
<td>Male</td>
<td>Abruptio placentae</td>
<td>Lower uterine segment</td>
</tr>
<tr>
<td>16</td>
<td>190</td>
<td>Male</td>
<td>Abruptio placentae</td>
<td>Lower uterine segment</td>
</tr>
<tr>
<td>17</td>
<td>200</td>
<td>Male</td>
<td>Abruptio placentae</td>
<td>Lower uterine segment</td>
</tr>
</tbody>
</table>

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**Apoptosis in Skeletal Muscle of Aging Rats**

- **Gastrocnemius**

  - **6-Months**
  - **24-Months**

  This change could merely indicated a loss in nuclei, since skeletal muscle is multi-nucleated. Therefore, this is not evidence for apoptosis in end-differentiated skeletal myocytes.

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**No gold standard for the specific detection of apoptosis is available**

- Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling reaction (TUNEL). This method labels the free 3'-ends of DNA by terminal transferase (TNT), and the label is then visualized by immunohistochemical techniques.
- Nevertheless, the TUNEL reaction seems to be prone to false positive or negative findings and several improvements of the methods have been proposed.
- The staining is very dependent on 1) fixation time of the tissue samples, 2) proteolytic pretreatment of the section and 3) the concentration of the nucleotides and terminal transferase used for labeling.

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**Other Methods to Detect Apoptosis**

- **DNA laddering** – DNA is cleaved at sites located between nucleosomal units, thereby generating DNA mono- and oligonucleosomal fragments (180bp multimers), which may be visualized on agarose gels. Difficult to quantify!!
- **ELISA can quantify** – detects and quantifies cytosolic mononucleosomes and oligonucleosomes.
Poly ADP-Ribose Polymerase

- Highly conserved nuclear enzyme present in higher eukaryotes.
- Recognizes DNA strand breaks and is implicated in DNA repair and in the apoptotic response of cells – marker of apoptosis.
- Correlates well with chromatin condensation.
- During apoptosis a major fragment of 89kDa is observed.

**Scientific American**

Does caloric restriction attenuates age related alterations in apoptosis signaling?

- Are caspases effected?
- Inhibitors of caspases?
- Other key pro- and anti-apoptotic proteins influencing the apoptotic potential?
Caspase-3; cleaved caspase-3; and X-linked inhibitor-of-apoptosis (XIAP) protein content as well as the enzymatic activity of caspase-3 in the gastrocnemius muscle.

**Question?** This change could merely indicated a loss in some nuclei, since skeletal muscle is multi-nucleated. Therefore, is this evidence for apoptosis in end-differentiated skeletal myocytes?

Caspase-12

- Intracellular calcium levels increase with age. Several studies have suggested that intracellular calcium handling is drastically improved following periods of caloric restriction.
- A ~350% increase in the expression of caspase-12 (caspase located at the sarcoplasmic reticulum) with age, CR reduced this age-associated rise.
- These data suggest that the caspase-12-mediated pathway of apoptosis may play a key role in sarcopenia and is attenuated by CR.

Apoptosis Inducing Factor (AIF) is a Caspase-independent Apoptosis

AIF is a cytochrome c independent pathway. AIF = apoptosis inducing factor (independent from cytochrome c pathways.
Causes large scale DNA fragmentation.

Skeletal Muscle is Multi-Nucleated

- The exact time-course in which an apoptotic nucleus is degraded is unclear, but will certainly affect atrophy in specific fibers and specific regions of muscle fibers.
- This modulation of myonuclear number to maintain a constant nuclear to cytoplasmic ratio appears central to muscle remodeling in response to injury, adaptation, and aging.

Total and nuclear apoptosis inducing factor (AIF) in the plantaris muscle (Type II)

- Total tissue levels of apoptosis inducing factor (AIF) in the plantaris muscle increased with age and was reduced by CR.
- There were no significant changes of this pro-apoptotic protein in the isolated nuclei.
Apoptosis Inducing Factor

- Total protein level of apoptosis inducing factor (AIF) in the *plantaris* muscle increased with age and was reduced by CR.
- There were no significant changes of this pro-apoptotic protein in the isolated nuclei.

### Table 1. Overview of changes ($↑$ increase, $↓$ decrease, $→$ no change) in apoptosis and apoptotic regulatory proteins in skeletal muscle with aging and calorie restriction (12AD v 26AD and 26CR v 26AD).

<table>
<thead>
<tr>
<th>Muscle Function with Age and Caloric Restriction</th>
</tr>
</thead>
<tbody>
<tr>
<td>This study suggests multiple beneficial effects of life-long calorie restriction on muscle function.</td>
</tr>
<tr>
<td>Calorie restriction was able to attenuate the age-associated decline in muscle mass-to-body mass ratio, strength-to-body mass ratio, muscle specific force, and reduce the extracellular space in the fast <em>extensor digitorum longus</em> muscle.</td>
</tr>
<tr>
<td>Only some of these beneficial effects of CR were observed in the slow twitch <em>soleus</em> muscle.</td>
</tr>
</tbody>
</table>

**Summary**

- We found that DNA fragmentation (mono- and oligo-nucleosomes) increased with age in the *gastrocnemius* muscle and CR attenuated this increase significantly.
- Pro- and cleaved caspase-3 levels increased significantly with age and CR suppressed this age-associated rise.
- The levels of the X-linked inhibitor-of-apoptosis (XIAP), particularly an inhibitor of caspase-3, increased with age and was reduced by CR.
However, a significant correlation between levels of cytosolic cytochrome c and caspase-3 activity. Cytosolic cytochrome c levels (A), caspase-3 activity (B), and correlation between cytosolic cytochrome c and caspase-3 activity (C) in gastrocnemius muscle in 6- and 24-month old rats. 6-Months old rats ($r = 0.79$); 24-month old rats ($r = 0.62$).

**Significant correlation between caspase-3 activity and the apoptotic index in old rats**

Caspases = Cysteine Proteases

↓

CAD = Caspase Activated Dnase (nucleus)

↓

Mono- Oligo-nucleosomal fragmentation (Apoptosis)

**Other Studies on CR and Brain Aging**

- CR can protect neurons against degeneration in animal models of AD, PD, HD and stroke.

- Can stimulate the production of new neurons from stem cells and can enhance synaptic plasticity \( \Rightarrow \) resist aging, restore function following injury.

- Beneficial effects of CR \( \Rightarrow \) result of cellular stress response \( \Rightarrow \) stimulates the production of proteins that enhance neuronal plasticity and resistance to oxidative and metabolic insults \( \Rightarrow \) BDNF, NGF, HSP’s, mitoUCP’s.

**Brain and Cell Loss**

- The frontal cortex and parts of the hippocampus system are regions important for learning and memory, appear especially affected by age. Previous studies on neuronal loss with aging reported disparate results.

- Some studies suggest that most neocortical areas and certain hippocampal subfields lose 25 to 50% of their neurons with age in humans.

- Studies often measured neuron density in a given structure instead of total neuron number.

- Age-related loss does occur in the hilus of the dentate gyrus and the subiculum, but loss in neocortical regions in humans remains controversial.

- Specific regions of the brain.
Is function improved in the brain?

- Longevity, preserved strength, coordination, spontaneous alternation behavior, and improved responses to enclosed alleys evaluated by tests of motor coordination (rotorod) and learning (complex maze) and improvements in word recall performance have been demonstrated in humans at the end of short term caloric restriction.

What are the mechanisms?

- The translocation of ARC may explain the rise in cytosolic cytochrome c levels observed with age and CR.

### Neuron loss in the brain with age and improved brain function with CR

- Improved responses to enclosed alleys
- Previous studies on neuronal loss with aging reported disparate results.
- Studies often measured neuron density in a given structure instead of total neuron number.
- Some studies suggest that most neocortical areas and certain hippocampal subfields lose 25 to 50% of their neurons with age in humans.
- Specific regions of the brain appear to be affected (hippocampus, thalamus, the dentate gyrus and the subiculum).

### Summary (Muscle and brain)

- CR is able to attenuate the age-associated increase in apoptosis in skeletal muscle and neurons by altering several key apoptotic proteins towards cellular survival, thereby reducing the potential for sarcopenia and neurodegenerative diseases.
- A diminished activation of mitochondrial-mediated pathways of cell death with life-long caloric restriction could have a profound affect on apoptosis and the susceptibility to apoptosis as well as on muscle function.
- The information obtained from these studies could potentially permit the development of physiological or genetic interventions that may attenuate the loss of skeletal muscle myocytes (sarcopenia) and neurodegeneration indicative of advancing age.
In vitro experiments

Pro-caspase-9 in muscle may be associated with an inhibitor, which blocks its activation by cytochrome-c.

In vitro experiment in skeletal muscle homogenate

Cyt c → Apaf-1 → Caspase-9

Cyt c & ATP → Procaspase-9 → Caspase-3

SMAC → Inhibitors? → IAPs (XIAP) → Caspase-3

CAD = caspase activated DNase

In vitro experiments to determine which pro-apoptotic proteins are released from heart and skeletal muscle mitochondria and do they activate caspase-9, and caspase-3

Apoptosis and Exercise

Evidence for Apoptosis During Exercise?

Atrophy
• Apoptosis may be involved in disuse atrophy
  – After 14 days of rat hindlimb suspension, double-stranded DNA fragmentation (an indicator of apoptosis) was substantially increased
  – Combined treatment with growth hormone, insulin-like growth factor 1, and resistance exercise ameliorated accumulation of apoptotic markers in unweighted limbs (Allen et al. 1998)

Exercise, Apoptosis, and getting sick often!!!

- DNA fragmentation occurs in human lymphocytes after exhaustive exercise
- Apoptosis in lymphocytes also occurs after exhaustive exercise and persists for 24 hours

**RESULTS**

**Table 2**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Pre-test (%)</th>
<th>Post-test (%)</th>
<th>24 hours (%)</th>
<th>48 hours (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.7</td>
<td>57.0</td>
<td>57.9</td>
<td>58.2</td>
</tr>
<tr>
<td>2</td>
<td>0.9</td>
<td>38.9</td>
<td>22.9</td>
<td>11.5</td>
</tr>
<tr>
<td>3</td>
<td>64.3</td>
<td>95.4</td>
<td>87.9</td>
<td>5.1</td>
</tr>
</tbody>
</table>

Note: n = 3 subjects.

**Transgenic mice models**

- **LONG-LIVED p66^shc^-**
  - Genetically engineered mutant with extended longevity phenotype – 30% extended life span (Migliaccio et al., 1999).
  - P66^shc: adaptor protein important in the signaling response to ROS (Luzi et al., 2000).
  - Cell lacking this protein have enhanced resistance to apoptosis by UV light or H2O2 treatment and mice lacking this gene have an increased resistance to paraquat (Migliaccio et al., 1999).

- **SHORT-LIVED POLG exo-mice**
  - Accumulates mitochondrial deletions and degenerate by middle age with progeroid syndrome (Tom Prolla, University of Wisconsin)

**Future and Current Studies**

- **Caloric Restriction, Aging, Oxidative Stress, and Apoptosis**
  - CR started at 14 weeks of age (10% restriction), increased to 25% restriction at 15 weeks, and maintained at 40% restriction from 16 weeks throughout the animal’s life.

**Future Directions**

- *In vivo* studies needed to elucidate the adaptations and mechanisms of programmed cell death during aging
- Use long-lived and short lived mice.
- Interventions which may attenuate apoptotic potential; Exercise Training and Caloric Restriction
- *In vitro and in vivo* experiments to test the susceptibility for apoptosis with aging and following exercise training
  - AIF = apoptosis inducing factor (independent from cytochrome c pathways). Causes large scale DNA fragmentation.
  - PARP (DNA repair) = (poly-ADP-ribose polymerase) after activation of caspases PARP becomes cleaved and inactivated.
  - CAD = caspase activated DNase
  - SMAC=(Second Mitochondria-derived Activator of Caspase)