Chapter 6: Discussion

Comparison of physiological changes and transcriptional changes induced by AHL, AHL associated with mtDNA, and CR are shown in Table 6.1. The ABR results from the B6 mouse study revealed that CR retarded the progression of AHL. The histology results revealed that CR prevented degeneration of the organ of Corti in the cochlea from the CR B6 mouse, supporting the ABR finding. The gene expression profile results from the B6 mouse study revealed that AHL-related changes in the gene expression were remarkably reversed by CR. The gene expression profile data also revealed that CR induced hearing-related genes. This result was also consistent with that of the ABR findings, and suggests that hearing function was preserved at the molecular level.

CR induced the increased expression of genes involved in energy metabolism such as *Sirt1*. CR extends lifespan in numerous species (Lee *et al.*, 2002; Lee *et al.*, 1999; Lee *et al.*, 1999; Prolla, 2002). In yeast, the *SIR2* gene mediates the life-extending effects of CR (Lin *et al.*, 2002; Vaziri *et al.*, 2001; Tissenbaum *et al.*, 2001; Gasser *et al.*, 2001; Lin *et al.*, 2000). Mutations that inactivate SIR2 protein shorten the lifespan, and overexpression of SIR2 protein extends the lifespan (Lin *et al.*, 2002; Lin *et al.*, 2000). SIR2, a member of sirtuin family of NAD^+-dependent protein

Table 6-1. Comparison of physiological changes and transcriptional change induced by AHL, AHL associated with mtDNA mutations, and CR. This table lists selected classes of physiological changes and transcriptional changes induced by AHL, AHL induced by mtDNA mutations, and CR.

Methods	Results		
	Aged DBA	D257A	CR B6
ABR analysis:			
Hearing function	Severe hearing loss	Moderate hearing loss	Good hearing
Histological analysis:			
Organ of Corti	Severe degeneration	Mild degeneration	Normal
Hair cells	Severe loss	Mild loss	Normal
Spiral Ganglion cells	Severe loss	Severe loss	Normal
Gene expression analysis:			
Hearing	↓	↓	↑
Energy metabolism	↓	↓	↑
Neurotransmission	↓	↓	↑
Apoptosis	↑	↑	↓
Stress Response	↑	↑	↓
Inflammatory Response	↑	↑	↓

deacetylase, require NAD^+ for its function (Lin *et al.*, 2000; Dutnall and Pillus, 2001; Gasser and Cockell, 2001; Vaziri *et al.*, 2001). CR can activate SIR2 protein in yeast by increasing energy metabolism (Lin *et al.*, 2002). When glucose levels in the media are lowered (calorie-restricted), yeast cells respond by shunting more of the carbon to the TCA cycle to generate ATP by respiration (Lin *et al.*, 2000; Kubova and Guarente, 2003). This shift toward respiration is necessary and the activation of respiration converts more NADA to NAD^+ (Kubova and Guarente, 2003). The resulting increase in the NAD^+/NADH ratio may activate SIR2 protein (Kubova and Guarente, 2003). Thus, the gene expression results suggest that CR induces increased energy metabolism, which in turn activates Sirt1 gene.

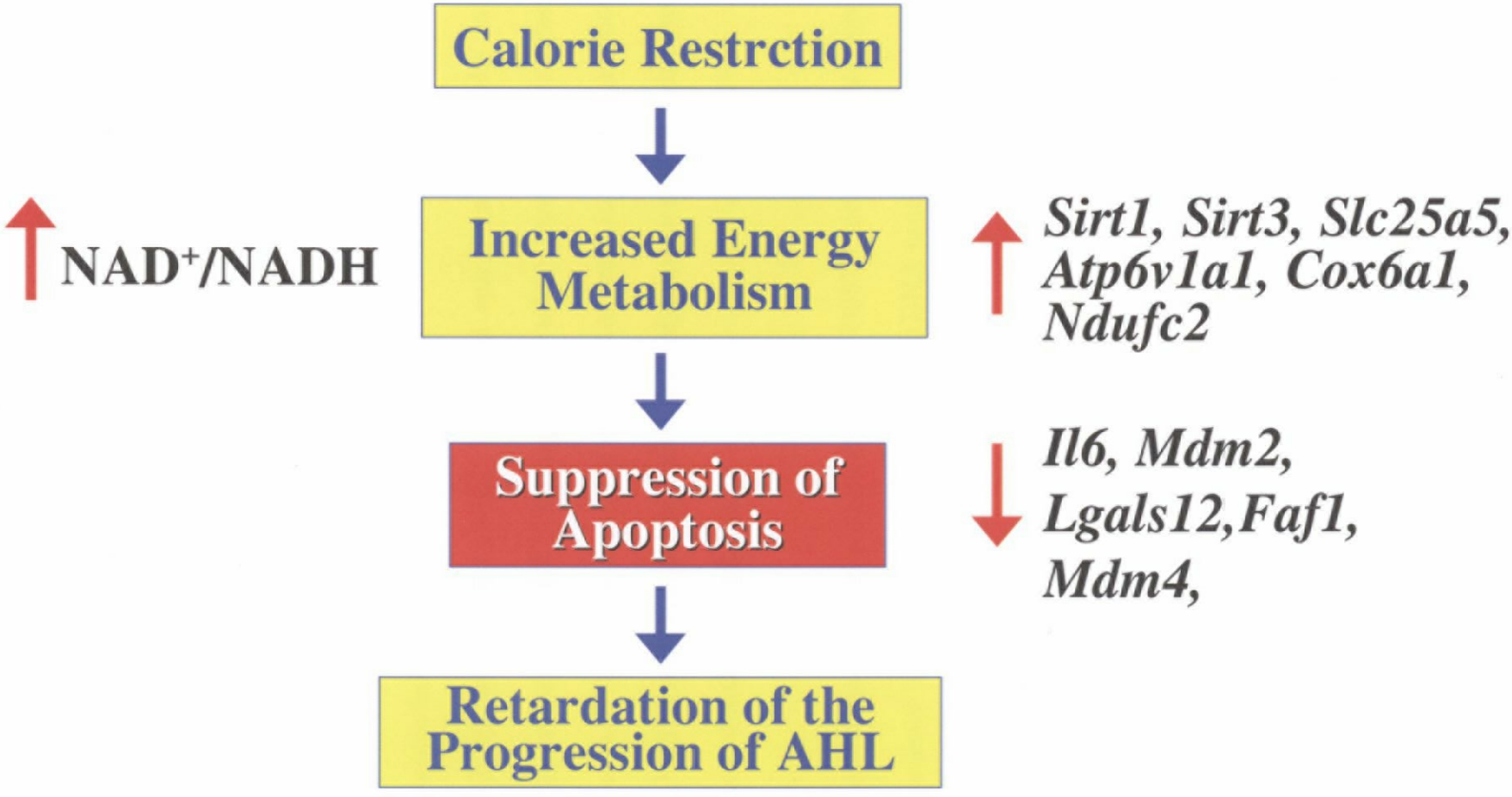
CR also lowered the gene expression of apoptosis-related gene, *Mdm2*, which mediates p53-dependent apoptosis, and stress response. Mdm2 is an oncoprotein that controls apoptosis through p53-dependent mechanisms (Prives and Hall, 1999; Vogelstein *et al.*, 2000). Mdm2 protein binds to p53, inactivates the ability of p53 to function as a transcription factor, and induces apoptosis (Prives and Hall, 1999; Vogelstein *et al.*, 2000). In contrast, Mdm2 mRNA level is transcriptinally regulated by p53 in response to stress, such as DNA damage and oxidative stress, and Mdm2 mRNA level is dependent of p53 (Prives and Hall, 1999; Chang *et al.*, 2004). Thus, reduced p53 activity should lead to reduced Mdm2 mRNA level (Chang *et al.*, 2004). Therefore, the gene expression profile results suggest that CR downregulated p53-dependent apoptosis by suppressing Mdm2 gene. The

mammalian *SIR2* orthologue, *Sirt1* also mediates cell survival in mammals (Koubova *et al.*, 2003; Luo *et al.*, 2001; Picard *et al.*, 2004). p53 is a short-lived protein whose activity is maintained at low levels in normal cell (Prives and Hall, 1999; Vogelstein *et al.*, 2000). The activation of p53 by cellular stress is thought to involve posttranslational modification of p53, such as phosphorylation and acetylation (Prives and Hall, 1999; Vogelstein *et al.*, 2000). Sirt1, NAD^+-dependent protein deacetylase, inactivates p53 by deacetylating p53, thereby repressing p53-dependent apoptosis in response to DNA damage and oxidative stress in mammals (Koubova *et al.*, 2003; Luo *et al.*, 2001). Thus, the gene expression profile results suggest that CR retards the progression of AHL by induction of energy metabolism-related genes, such as *Sirt1*, which in turn represses p53-dependent apoptosis, and hence slows down the progression of AHL.

Based on these findings, I propose a model of how CR retards the progression of AHL (Figure 6.1): CR causes a metabolic shift toward increased energy metabolism, perhaps by increasing the NAD^+/NADH ratio. This metabolic shift induces energy metabolism-related genes such as *Sirt1*, which in turn represses p53-dependent apoptosis by interacting with p53. This repression leads to preventing the cochlea from loss of hair cells and spiral ganglion cells, and hence to slowing down the progression of AHL.

Physiological changes and transcriptional changes induced by AHL associated with mtDNA mutations of the D257A mouse study were

Figure 6-1. Model of how CR slows the progression of AHL. CR causes a metabolic shift toward increased energy metabolism, which may lead to an increased NAD/NADH ratio. This metabolic shift induces energy metabolism-related genes, such as *Sirt1*, which in turn represses p53-dependent apoptosis by interacting with p53. This repression leads to suppression of apoptosis-related genes, such as *Mdm2* and *Il6*, and to preventing the cochlea from loss of hair cells and spiral ganglion cells, and hence to slowing down the progression of AHL.


Calorie Restrction
NAD+/NADH
Increased Energy Metabolism
Sirt1, Sirt3, Slc25a5, Atp6v1a1, Cox6a1, Ndufc2
Suppression of Apoptosis
Il6, Mdm2, Lgals12,Faf1, Mdm4,
Retardation of the Progression of AHL

remarkably similar to the changes induced by AHL of the DBA mouse study (Table 6.1). ABR results from the D257A mouse study revealed that old D257A mice exhibited moderate hearing loss, and ABR results from the DBA mouse study also revealed that aged DBA mice exhibited sever hearing loss. The histology results from the D257A mouse study revealed that old D257A mouse exhibited severe loss of spiral ganglion cells in the cochlea, and the histology results from the DBA mouse study also revealed that the aged DBA mouse exhibited severe loss of hair cells and spiral ganglion cells in the cochlea, supporting the ABR findings. Thus, taken together, these results suggest that mtDNA mutations play a causative role in the progression of AHL since D257A mice exhibit mitochondrial dysfunction with aging due to accumulation of mtDNA mutation.

The gene expression profile results from the D257A mouse study revealed that AHL-related changes, associated with mtDNA mutations, in the gene expression were remarkably similar to the AHL-related changes in the gene expression of the DBA mouse study. These data from the D257A mouse study revealed that AHL induced by mtDNA mutations resulted in suppression of hearing-related genes. This result was consisted with that of the DBA mouse study, and these results suggest that hearing function decreased with aging at the molecular level. AHL induced by mtDNA mutations also resulted in decreased energy metabolism for the D257A mouse study, and this result was also consistent with that of the DBA mouse study. These results, the decreased energy metabolism, correlated well with the fact that D257A mice

exhibit mitochondrial dysfunction with aging due to accumulation of mtDNA mutation.

AHL, from the DBA mouse study induced apoptosis-related genes, such as *Bak1* and *Scotin*, which mediate p53-dpendent apoptosis (Leu *et al.*, 2004; Degenhardt *et al.*, 2002; Bourdon *et al.*, 2002), and increased stress response. As to the D257A mouse study, AHL associated with mtDNA mutations also induced apoptosis-related genes, such as *Chek1*, which mediates p53-dependent apoptosis (Vogelstein *et al.*, 2000; Prives and Hall, 1999). p53 activates Bak1 gene, which in turn causes release of cytochrome c from mitochondria, and hence to induction of apoptosis (Leu *et al.*, 2004; Degenhardt *et al.*, 2002). The Scotin protein is localized to the ER and the nuclear membrane (Bourdon *et al.*, 2002). The p53 also activates Scotin gene, which in turn induce apoptosis (Bourdon *et al.*, 2002). p53 activation is initiated by DNA damage and oxidative stress (Vogelstein *et al.*, 2000; Prives and Hall, 1999). This damage is sensed by checkpoint proteins, such as Chek1 (Vogelstein *et al.*, 2000; Prives and Hall, 1999). The kinase, Chek1 activates p53 by phosphorylating at amino-terminal sites that are close to the Mdm2-binding region of the protein, thereby blocking its interaction with Mdm2, and leading to activation of p53 (Vogelstein *et al.*, 2000; Prives and Hall, 1999). Fischel-Ghodsian examined the spiral ganglion and membranous labyrinth from archival temporal bones of five patients with AHL for mutations within the mitochondrially encoded cytochrome c oxidase II genes, and showed that at least a proportion of people with AHL have a

significant load of mtDNA mutations in the auditory tissue (Fischel-Ghodsian, 1999). The B6 mouse study results suggest that CR retards the progression of AHL by repressing p53-dependent apoptosis through induction of increased energy metabolism. The TUNEL result from the POLG mouse study also suggests that apoptosis plays a role in the progression of AHL. Thus, taken together, those suggest that AHL may develop by induction of p53-dependet apoptosis.

Mitochondrial DNA damage or oxidative stress can stimulate DNA-dependent kinases, such as Chek1, that modify p53 by phosphorylation (Vogelstein *et al.*, 2000; Prives and Hall, 1999). This results in increased levels of activated p53 protein (Vogelstein *et al.*, 2000; Prives and Hall, 1999). The expression of target genes such as *Bark1* and *Scotin* are then activated by binding of the activated p53 to their regulatory regions (Vogelstein *et al.*, 2000; Prives and Hall, 1999; Leu *et al.*, 2004; Degenhardt *et al.*, 2002; Bourdon *et al.*, 2002). Thus, taken together, these suggest that AHL develops by mechanisms that may be related to induction of p53-dependent apoptosis by accumulation of mtDNA mutations, which in turn lead to hearing loss during aging.

Although AHL may be enhanced by many factors such as accumulated exposure to noise, diet, and genetics (Gacek and Schuknecht, 1969; NIDCD, 2004), the molecular mechanisms of how AHL develops remain unknown. Based on our findings, I propose a model of how AHL

develops in mammals (Figure 6-2): oxidative DNA damage initiates mutations in mtDNA in the cochlea. The mtDNA mutations accumulate in the cochlea, and lead to mitochondrial dysfunction. This dysfunction results in altered energy status and DNA damage, resulting in the induction of apoptosis-related genes, such as *Chek1, Bak, Scotin*, which in turn induce p53-dependent apoptosis by interacting with p53. This leads to loss of hair cells and spiral ganglion cells in the cochlea. AHL develops when loss of these cells reaches a critical level.

CR extends lifespan in yeast (Lin et al., 2002; Lee *et al.*, 2002; Prolla, 2002), and this effect requires Sir2 protein (Lin *et al.*, 2002; Vaziri *et al.*, 2001; Tissenbaum *et al.*, 2001; Gasser *et al.*, 2001; Lin *et al.*, 2000). The resveratrol, a polyphenol found in red wine, mimics CR by activating Sir2, and extends lifespan in yeast (Howltz *et al.*, 2003; Wood *et al.*, 2004). Thus, polyphenols which activate Sirt1 in mammals could be good candidates for a new preventive intervention or new functional supplement which could slow down the progression of AHL in humans (Howltz *et al.*, 2003; Wood *et al.*, 2004). Our laboratory identified several candidate polyphenols from Japanese persimmons and Chinese white teas (Suzuki *et al.*, 2005; Hu *et al.*, 2005). Currently, we are planning to test effects of these polyphenols on the progression of AHL.

Figure 6-2. Model of how AHL develops. Oxidative DNA damage initiates mutations in mtDNA in the cochlea. The mtDNA mutations accumulate in the cochlea, and lead to mitochondrial dysfunction. This dysfunction results in altered energy status and DNA damage, resulting in induction of apoptosis-related genes, such as *Chek1, Bak, Scotin*, which in turn induce p53-dependent apoptosis by interacting with p53. This leads to loss of hair cells and spiral ganglion cells in the cochlea. AHL develops when loss of these cells reaches a critical level.

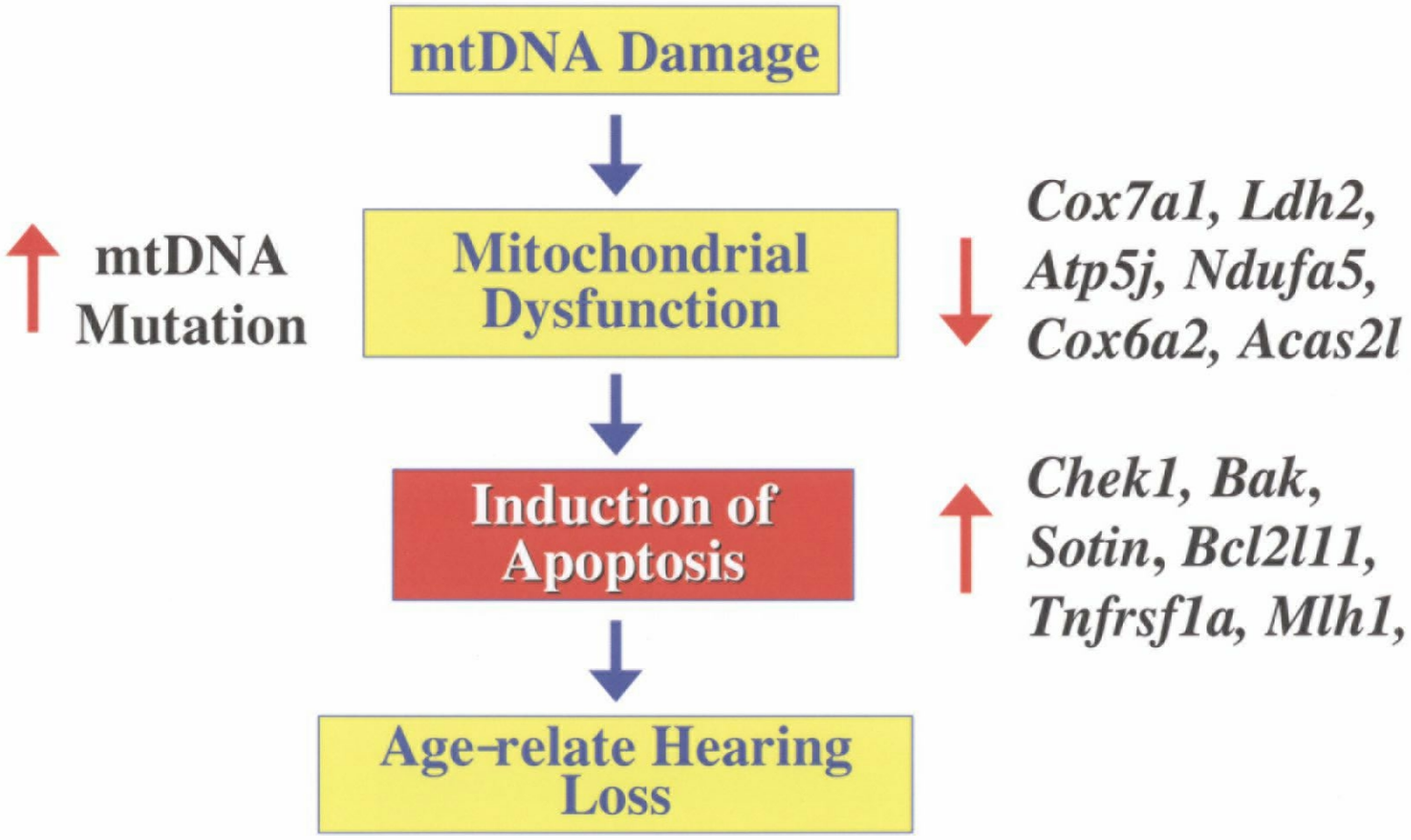

mtDNA Damage
mtDNA Mutation
Mitochondrial Dysfunction
Cox7a1, Ldh2, Atp5j, Ndufa5, Cox6a2, Acas2l
Induction of Apoptosis
Chek1, Bak, Sotin, Bcl2l11, Tnfrsf1a, Mlh1,
Age-relate Hearing Loss

Figure 6-3. Future Study. The polyphenols which activate Sirt1 protein in mammals could be good candidates for a new preventive intervention or new functional supplement which could slow down the progression of AHL in humans because resveratrol, a polyphenol found in red wine, mimics CR by activating Sir2 protein, and extends lifespan in yeast.

Polyphenols
Sirt1
Suppression of Apoptosis
Retardation of the Progression of AHL

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