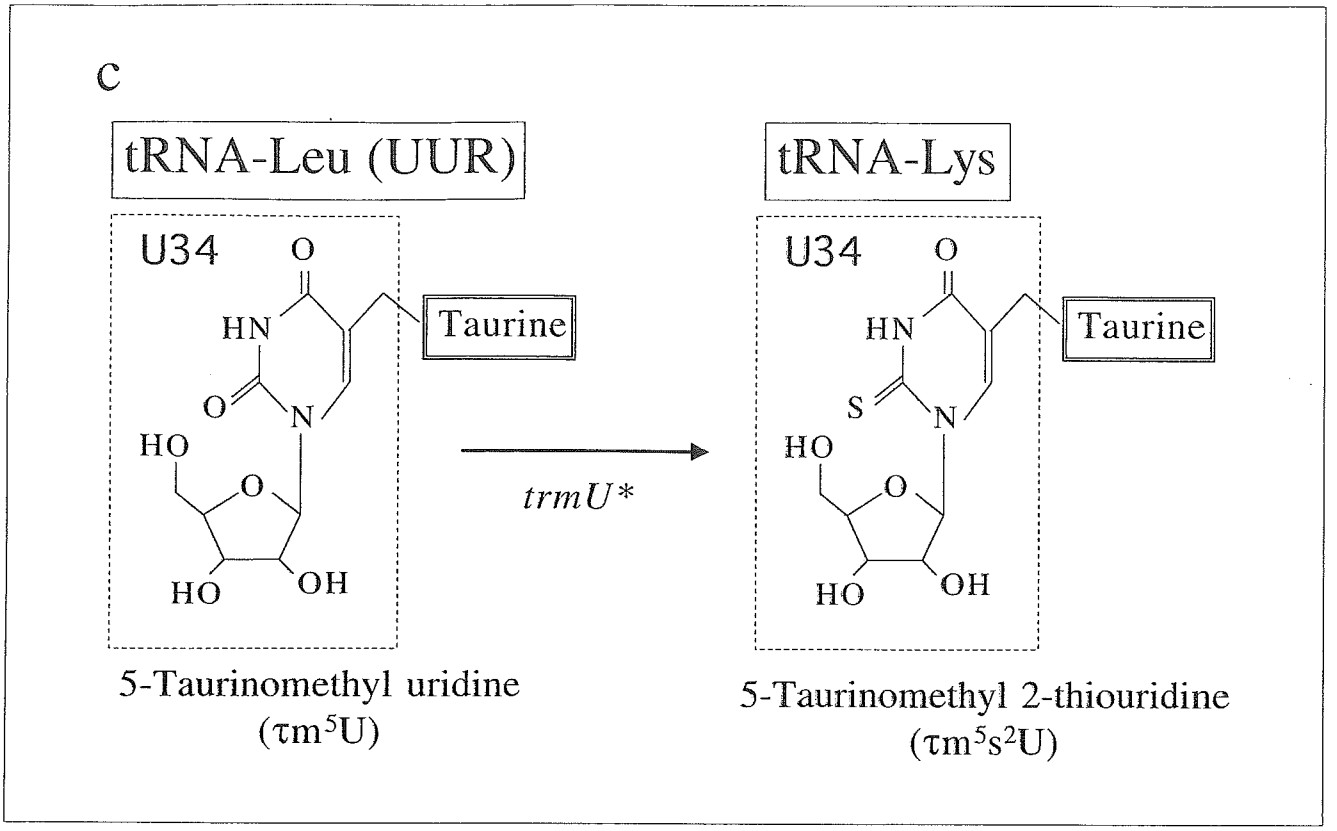

Fig. 3

な要因であるので，たいへん興味深い．

b．アンチコドン修飾異常

MEMの代表的な点変異である3243変異，3271変異とMEMの代表的な変異である8344変異は，それぞれロイシン（UUR）転移RNAとリジン転移RNA内に存在する（**Fig. 3a**）．UUR転移RNAはコドンUUAとUUGを認識し，リジン転移RNAはコドンAAAとAAGを認識する．東京大学の安川大学院生，鈴木講師，渡辺教授，日本医科大学の大田教授らは，コドン3文字目がAかGの場合に認識できるこれらの転移RNAにおいて，正常ではアンチコドンの1文字目（U34）がタウリン修飾を受けているのに，患者由来の変異を持つ転移RNAではその修飾が欠損していることを見出した[26~28]（**Fig. 3c**）．そして，実際の転移RNA活性を調べ，対応するアミノ酸の翻訳効率が落ちること，コドン-アンチコドンの対合自体が弱まることを証明した．これらの実験結果は，変異をもつ転移RNAそのものが，翻訳反応レベルで異常をきたしうることを直接的に証明したことになる．

2．組織・臓器レベルの病態に関する新知見

組織・臓器レベルのMEM患者の研究も不可欠である．細胞レベルでの基礎研究が進んでも，その知見が直ちに組織・臓器レベル，すなわち臨床レベルでの情報とはなりにくい．特に，MEMにおいて，再生が活発な骨格筋や肝臓細胞と再生の乏しい心筋や神経細胞とでは，その病態が異なることは明らかである．MEMにおいてもいくつかの疾患モデルが作製されてきたが，mtDNA変異を有するモデル動物は実在しなかった．しかし，2000年にわれわれと筑波大学，国立感染症研究所との共同研究で，ヒトの病気と同様な欠失mtDNAを有するマウス（Mito-Mice）の作出に成功した[29, 30]．

欠失mtDNAを80％以上で有するMito-Miceは生後約2月くらいから体重増加不良を認め，おおよそ6月で死亡する．死因は腎不全で，病理学的には高度の糸球体硬化像を認める．また，網膜変性，心伝導障害，高乳酸血症を認め，筋病理所見も，ヒトの所見と酷似している．したがって，Mito-Miceは，ヒトのKearns-Sayre症候群（KSS）

の疾患モデルといえる．KSSでは，大脳白質の変性所見や糖尿病の合併なども認められるので，その点の確認などが今後の課題である．

Mito-Miceとヒトの病気の間のもっとも大きな相違点は，遺伝形式である．KSSは通常遺伝性がなく，突然変異で起こると考えられている．ただし，ヒトの場合でも欠失と同時に重複が存在するときは，母系遺伝したと考えられる症例が報告されている．Mito-Miceを調べたところ，骨格筋を含むいくつかの臓器で，重複mtDNAの存在が確認されている[29]．重複mtDNAの存在と母系遺伝との関係について，Mito-Miceの卵細胞を詳細に検討することで，新しい知見が得られる可能性がある．

III. 治療に関する新しい知見

1. エネルギー代謝賦活薬

酵素異常の確定した症例の蓄積とともに，理論的な代謝賦活薬の投与が試み始められている（**Fig. 1**）．特に，電子伝達系酵素異常症で比較的残存活性が残っている症例に対して，ピルビン酸脱水素酵素活性を高めるdichloroacetateとビタミンB_1の併用療法が効果的であるという報告がいくつかの施設からでている[31, 32]．特に，複合体I欠損症（Leigh脳症，MEM）に効果的であるという印象が強いが，その厳密な効果判定にはケースコントロール研究が必要である．本薬剤は試薬であり，使用に際しては各病院の倫理審査委員会での承認を必要とする．また，過量投与で，意識障害，肝機能障害，末梢神経障害，低カルシウム血症などが報告されているので，血中濃度のモニタリングが重要である．

米国では，uridineの治験が始まっており，また欧米ではクレアチン療法を行っているところもある．クレアチンは，酸化ストレスの原因である活性酸素を除去する働きもあることから，今後さらにその使用が増加する可能性がある．MEMに対するこの2者の効果については，いまのところ定かではない．

また，従来から用いられてきたコエンザイムQやカルニチンは，おそらくそれぞれの欠乏状態が存在する患者においては劇的に効く可能性がある．実際に，コエンザイムQ欠乏やカルニチン欠乏を骨格筋を用いて測定する検査がルーチン化されていない現状では，今後も頻繁に臨床的に使用されるのはやむを得ない．

2. 病態から考え出された新しい治療法

MEMの脳卒中様発作がどのような機序で起こるかについては，脳細胞（神経細胞やグリア細胞）の代謝異常と脳内小動脈の機能異常の両者の要因があると考えられている．久留米大学の古賀博士らは，急性期にL-アルギニンを投与することで血管拡張をうながし，症状を軽減できたと報じている[33]．さらに，アルギニンの経口薬により，脳卒中発作の予防効果があるという予備的な成果が出てきている．早急に治験を行いその有効性を確認すべきである．

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The Prospects for Pathogenesis and New Therapy of Mitochondrial Encephalomyopathy

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Recent advances in research on mitochondrial encephalomyopathy are described based on etiology, pathomechanism and therapy. First, nuclear pathogenic genes have been identified such as structural genes for complex I and II, assemble factors for complex III and IV, and several factors associating with mtDNA replication. Second, the pathogenesis of the disease has been extensively studied using culture cells and mouse models. Inter-mitochondrial compensation of the mtDNA genome having different pathogenic point mutations, wobble modification defect of 3243 and 8344 mutations, and the newly established mouse with large-scale deletion of mtDNA are reviewed. Third, topics of the therapeutic research, such as dichroloacetate and L-arginine as new drugs are also described in this review.

今月の主題　ミトコンドリア病

ミトコンドリア病の臨床検査

ミトコンドリア病の組織診断
—ゴモリ染色，活性染色，免疫染色

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ミトコンドリア病の組織診断—ゴモリ染色，活性染色，免疫染色

後藤雄一[1)]

〔SUMMARY〕 ミトコンドリア病の診断において，組織診断は有力な情報を提供する．典型的な病理所見である，赤色ぼろ線維(ragged-red fiber)，高SDH活性血管(strongly SDH-reactive blood vessel)，シトクロームc酸化酵素欠損線維のいずれか，もしくはその組み合わせを検出することが肝要である．〔臨床検査　49：45-49，2005〕

〔KEYWORDS〕 RRF，SSV，シトクロームc酸化酵素欠損線維

はじめに

ミトコンドリア病の診断においては，特徴的な臨床症状をとらえることに加え，ミトコンドリア異常の存在を証明する必要がある．その基本となる検査手段は，病理学検査，生化学検査，分子遺伝学的検査であり，その中でも病理学的検査は，歴史的にミトコンドリア病の疾患概念を確立する際に重要な役割を果たしたばかりでなく，現在においても確定診断には不可欠な検査である．

ミトコンドリア病の罹患臓器は全身の臓器に及び，その病理学的検査の対象は多種多様になるはずである．しかし，臨床診断という意味ではその対象は骨格筋に絞られるといってよい．その理由は，骨格筋は生検が比較的容易な臓器であり，これまでの研究による知見が蓄積されていること，筋線維は細胞が一列に連なって形成された多核の細胞であり，ミトコンドリア異常があっても筋線維全体が失われることなく(細胞死になりにくい)その異常がそのまま病理学的にとらえられやすいことが挙げられる．他の臓器・組織では，ミトコンドリア異常によって細胞死が起こり，病理変化をとらえようにもそもそも細胞が失われている可能性が高い．

ゴモリ染色

ゴモリ染色とは，Gomoriトリクローム変法染色のことである．英文ではmodified Gomori trichrome stainingと記載される．この染色では，細胞小器官としては小胞体とミトコンドリアが赤く染まり，それ以外の成分は青緑色に染まる．ミトコンドリア病の患者生検筋において，赤色ぼろ線維(ragged-red fiber；RRF)がこの染色法で観察できる(図1)．筋線維内部が点状に赤く染色されるが，筋鞘膜直下が特に強く赤く染色される場合が多い．これは，電子顕微鏡像で明らかなように，その部位でミトコンドリアが異常に増殖していることを反映した像である．さらに電子顕微鏡像を詳細にみると，病理学的には3つの変化が起きている．数の増加，大きさの変化(巨大化)，内部構造の変化(クリスタの変形，封入体の出現)である．

これらの変化は機能が低下したミトコンドリアが，数を増すことでその機能を代償させようとする反応であると考えられている．しかし，どのような機能低下がこのようなミトコンドリアの形態変化を促進させるのかの詳細はわかっていない．現象としては，ミトコンドリア病の原因のうち，

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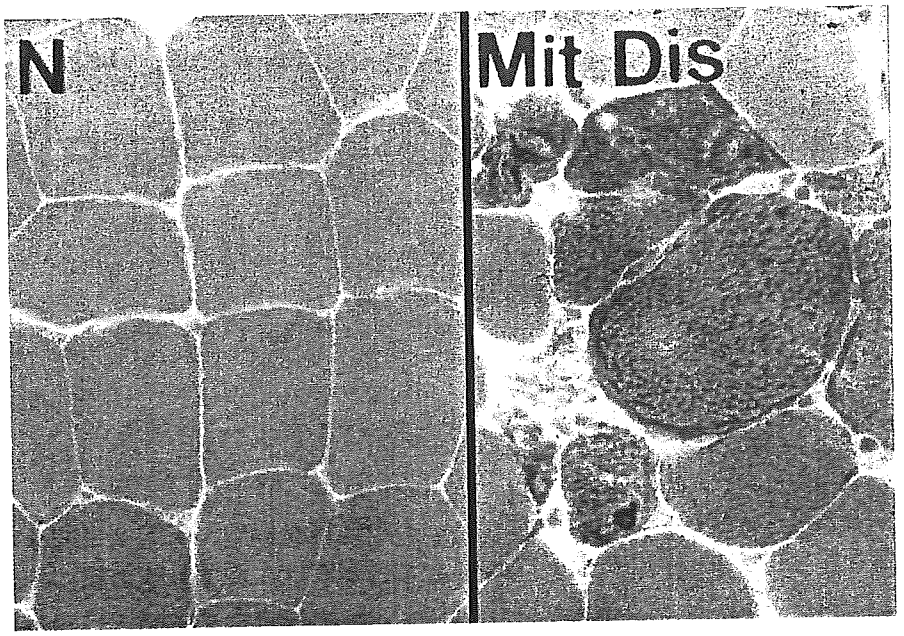

図1　赤色ぼろ線維(Ragged-red fiber；RRF)のゴモリ染色像
右図中央にみえるいくつかの線維は，ミトコンドリアが増殖したRRFである．特に，筋鞘膜直下の赤色が著明であるが，内部の赤色染色性も増加している．左上の2つの線維は，すでの筋線維の構造が崩壊しつつあるのがみえる．

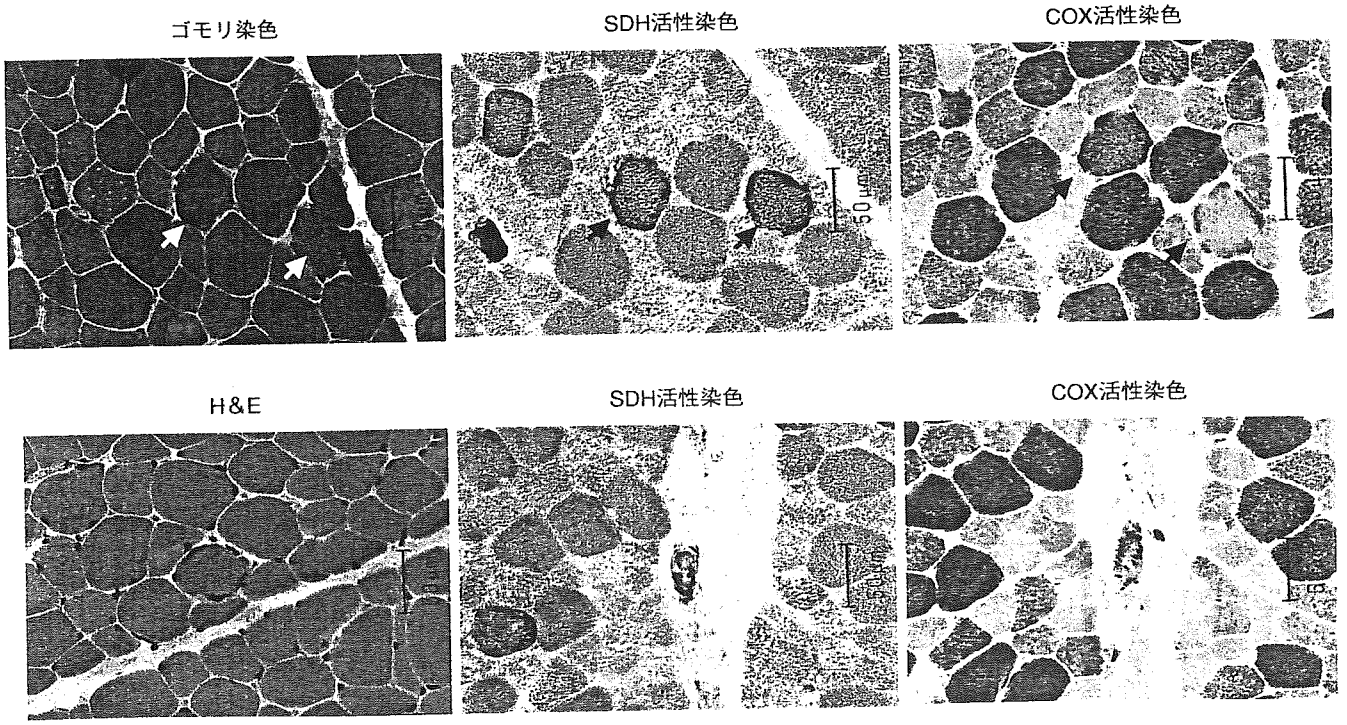

図2　赤色ぼろ線維，高SDH活性血管(SSV)の光顕像
上段はRRFを，下段はSSVの光顕像である．RRFはSDHで濃染し，COX染色では，濃染するもの(高活性)と淡染するもの(活性低下)がある．SSVもSDHで濃染する．

mtDNA異常があり，点変異がtRNA領域に存在するときや大きな欠失があるときにRRFを認める場合が多い．一方，同じmtDNA異常でも，点変異が蛋白コード領域の場合や核DNA上に存在する原因遺伝子変異の場合は，RRFは比較的まれである．しかしこれにも例外があって，核DNA上の遺伝子の異常が予想されている乳児致死型，乳児良性型シトクロームc酸化酵素欠損症では，多数のRRFが認められる．

RRFの存在比率と臨床症状の軽重とは直接には結びつかない．筋力低下をもたらすのは筋線維数の減少であり，病理変化の強い筋線維が多数あ

ることと筋線維数は直接には関係ないからである．またRRFは年齢の高い人の骨格筋ではときどき認められることがある．40歳以上の患者筋において，生検筋全体で(検体の大きさによるが)，例えば1,000個の筋細胞になかに1，2個存在するRRFが，病的意味をもつかどうかの判断は難しい．赤筋(タイプ1)の大小不同，筋線維タイプの分布異常，小角化線維の出現などの他の病理所見と合わせた総合的な判断が必要になる．場合によっては，生化学検査や分子遺伝学的検査の結果に頼らざるを得ない．

活性染色

ミトコンドリア病の組織化学染色でよく使用させる2つの活性染色を紹介する．活性染色は，構造そのものを正確にとらえているのではなく，あくまで生化学的な反応をみているだけであり，加える基質や発色物質の量，反応時間などに大きく影響を受ける．したがって，常にコントロールを同じプレパラート上において染色することが不可欠である．また，正確な活性値の測定は，生化学的な方法によらなくてはならない．

1．コハク酸脱水素酵素活性染色

コハク酸脱水素酵素(succinate dehydrogenase；SDH)は，TCA回路の構成酵素であるとともに，電子伝達系の複合体II(コハク酸—ユビキノン酸化還元酵素)の部分反応にもなっている．すなわち，TCA回路と電子伝達系を結びつける重要な役割を果たしている．複合体IIは4つのサブユニットからなり，すべて核DNA上にコードされているが，そのうち最も大きなフラボプロテインサブユニット(Fp)がSDH反応を担っている．

SDHはミトコンドリア内に限局して存在している．またmtDNAにコードされたサブユニットをもたないので，mtDNAの異常が存在するかどうかにあまり影響を受けない．RRFはSDH染色で濃染する(図2)．それは，たとえmtDNAに変異があろうとも，単純にミトコンドリアの数(内膜面積)が増加することがSDH活性の上昇としてとらえられるからと考えられる．

脳卒中様発作を特徴とするミトコンドリア病であるMELASにおいては，骨格筋や脳(おそらく全身)の小動脈にも病変が認められる．SDH染色では，その活性がホルマザン顆粒の大きさとして表現されるので，小動脈の横断面をみると全周性ではなくところどころに粗大な顆粒が存在しているように観察できる．このような血管を，高SDH活性血管(strongly SDH-reactive blood vessel；SSV)といい，MELAS患者の80%に存在する(図2)．SSVを電子顕微鏡でみると，血管平滑筋細胞内にミトコンドリアが集簇している像が確認できる．

また，ミトコンドリア病の一病型にSDH欠損症がある．この場合はSDH染色で骨格筋(および血管など)が染まらないことが診断の一助になる．

2．シトクロームc酸化酵素活性染色

シトクロームc酸化酵素(cytochrome c oxidase；COX)は，電子伝達系の複合体IVと同義である．13個のサブユニットからなる複合体で，そのうち3個はmtDNAにコードされている．したがって，mtDNA変異でも，核DNA上の遺伝子変異でもCOX欠損症が起きる．これまで診断されたミトコンドリア病で最も多いのがCOX欠損症であり，かつ最も研究が進んでいる．その理由は，COX活性染色で酵素欠損が容易に確認できるからである．

COX欠損は，病理学的に部分欠損とびまん性欠損に大別できる．ここでいう部分欠損というのは，英語でfocal deficiencyの意味である．典型的なCOX欠損線維はmtDNAに大きな欠失がある患者筋で認められ，正常活性の筋線維のなかに散在しているCOX活性の全くない筋線維が確認できる．その多くはRRFであるが，ゴモリ染色で一見正常にみえる筋線維でCOX欠損を示しているものもある．

RRFはいつもCOX欠損であるかというと必ずしもそうではない．多くはCOX欠損であるが，MELAS患者骨格筋ではときに正常もしくは高活性のRRFが認められる(図2)．この現象は以下のように考えると理解しやすい．個々の筋線維は異なる比率で3243位や3271位の変異mtDNAを有している．通常このようなヘテロプラスミーの場合，変異率がある値(閾値)以上にな

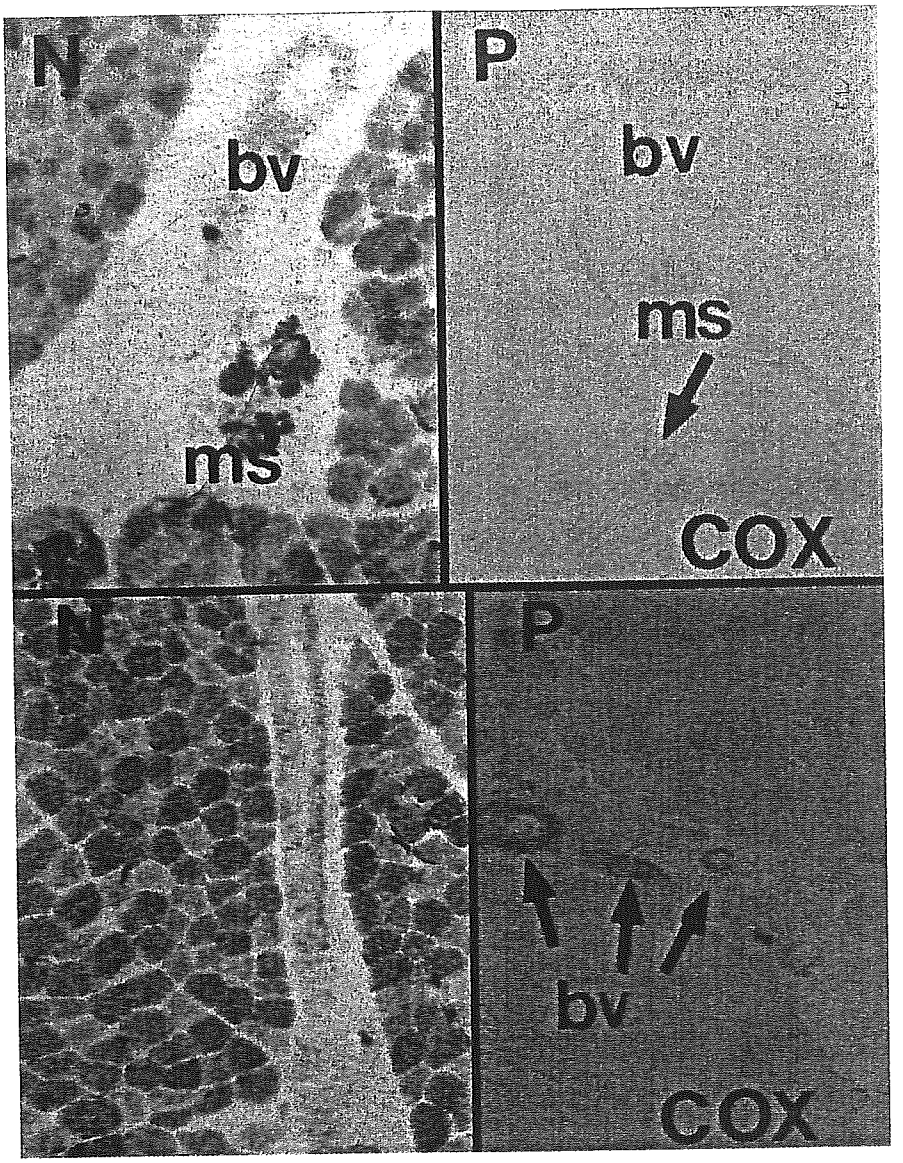

図3　びまん性シトクローム c 酸化酵素欠損
上段は非特異性びまん性 COX 欠損，下段は筋特異性びまん性 COX 欠損の例である．非特異的の場合は，筋細胞以外の，筋紡錘(ms)，血管(bv)も COX が欠損している．一方，筋特異的の場合は，筋紡錘，血管の COX 活性が保たれている．N：健常者筋，P：患者筋

ると機能障害を起こす．実はこれら MELAS の変異の場合は，複合体 I 活性低下の閾値が COX 活性低下の閾値よりも低く，この両者の閾値の間の比率をもつ細胞は，ミトコンドリアが増殖した RRF にはなるが増加したミトコンドリアは COX 活性が維持されていると考えられる．したがって，COX 活性が正常だったり，高かったりするものと考えられる．同じ転移 RNA の変異でも，8344 などの MERRF の変異の場合は，両者の閾値にほとんど差がなく，RRF はすべて COX 欠損線維となっている．このように，RRF の COX 活性の有無によって MELAS タイプと MERRF タイプが分けられ，これが責任遺伝子変異の検索の有力な情報になる．また，RRF が全筋線維数の 5%でも病理学的には優位な所見であるが，同じ検体を生化学的に検査すると COX 活性の低下は優位になることはない．多くの正常活性をもつ筋線維が一部の活性低下をマスクするからである．このように，病理組織検査は生化学検査より鋭敏に異常をとらえることができる．

びまん性 COX 欠損の場合は，全筋線維の活性が低下していることから，生化学的にも検出が可能である．骨格筋のびまん性 COX 欠損には 2 種類あり，筋細胞とともに，筋紡錘内の筋細胞(核鎖線維，核袋線維)，間質に存在する血管細胞，線維芽細胞などのすべてが COX 欠損を示す非特

異的COX欠損の場合と，筋細胞だけがCOX欠損を示す筋特異的COX欠損の場合がある(図3)．前者の病理像で，Leigh脳症の臨床病型である場合，多くはSURF 1遺伝子変異の場合の可能性がある．一方，筋特異的COX欠損の場合は，いまだ責任遺伝子が同定されてない．

COX欠損は遺伝的な病的状態ばかりでなく，他の要因でも起きることがある．皮膚筋炎や多発性筋炎など障害の強い部位の筋線維は欠損を示すことがある．また，長期臥床のために四肢を動かしていないとCOX欠損が認められる．ラットを2週間程度低重力状態にしただけでもCOX欠損が生じることが知られている．したがって，病気が重篤で長期にベッドで臥床している患者の生検筋においては，COX欠損が基礎疾患に由来するのか，長期臥床に由来するのかを見極める必要がある．

免疫染色

ミトコンドリア病の原因となる遺伝子産物が存在するかどうかについて抗体を用いて検査することが理論的に可能である．臨床現場ではあまり普及していないが，ミトコンドリア病の種々の病因が明らかになるにつれて，今後はその重要性が増すことが予想される．

1. SURF 1

COX欠損を示すLeigh脳症のなかで，SURF 1遺伝子の変異によるものがある．抗SURF 1抗体による免疫組織化学染色では，あきらかに染色性が落ちることで欠損の判断が可能である．

2. COXサブユニット，複合体Iサブユニット

COXの各サブユニットに対する抗体は市販されており容易に手に入る．しかしながら，各サブユニットの抗体を用いた免疫組織化学染色を診断に利用する段階まで達していない．その理由は，遺伝子変異が存在するサブユニットが特異的に欠損するのではなく，1つの遺伝子産物の異常でその後のアッセンブリーが障害され多数の遺伝子産物の欠損となることが挙げられる．

同様なことは複合体Iサブユニットにもいえる．

おわりに

ミトコンドリア病の病理検査は情報量が多い，有意義な検査である．しかしながら，この検査だけでも確定診断に至らない場合もあることを事前に被験者に十分理解していただく必要がある．

また，病理検査の試料となる生検筋は，生化学検査や分子遺伝学的検査にも利用されるものであり，また線維芽細胞や筋芽細胞などの樹立の出発点にもなる．他の検査方法での結果を合わせた総合的な診断が不可欠であるという意味でも，この病理検査の意義は大きいと考える．

最後に，骨格筋以外の生検組織を用いてミトコンドリア病を診断しようとする場合は，正常組織と比較検討することを忘れてはならない．ミトコンドリア異常による心筋症を診断する場合に，患者心筋でミトコンドリアが増殖していることを根拠にミトコンドリア心筋症とする例が文献的に散見する．正常心筋でも比較的ミトコンドリアが集積して存在する像が得られることから，その解釈は慎重であるべきである．

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Ebselen prevents noise-induced excitotoxicity and temporary threshold shift

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Abstract

This investigation tested the hypothesis that a noise-induced temporary threshold shift (TTS) can be attenuated by a peroxynitrite scavenger, ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one). Guinea pigs received an oral dose of the vehicle or 10 mg/kg ebselen 1 h before exposure to 115 dB SPL 4-kHz octave band noise for 3 h. In controls, auditory brainstem response (ABR) thresholds increased by 25–45 dB immediately after noise and returned to pre-exposure baseline thresholds 7 days later. Ebselen eliminated this ABR threshold shift following noise exposure. In controls, swelling of the afferent dendrites beneath the inner hair cells was evident immediately after noise, whereas ebselen significantly reduced this pathology. These findings suggest that scavenging peroxynitrite can attenuate noise-induced excitotoxicity and, thereby, TTS.

Keywords: Guinea pig; Noise-induced hearing loss; Nitric oxide; Peroxynitrite

After exposure to intense sound, auditory thresholds can be elevated permanently, or temporarily for minutes, hours or days, depending on the parameters of acoustic overstimulation. These two phenomena, permanent (PTS) and temporary threshold shift (TTS), are dependent on mechanisms not fully understood [1,9,18]. At least two different mechanisms have been proposed for PTS: a direct mechanical trauma and a metabolic overstimulation of the cellular elements of the organ of Corti (OC). Direct mechanical trauma may damage the delicate stereocilia of the sensory hair cells or, with higher stimulation intensity, the structural integrity of the OC and basilar membrane. Intense noise exposure may also overdrive the pathways that are responsible for maintaining OC homeostasis, thus leading to metabolic changes that compromise the system. Metabolic overstimulation may be associated with biochemical traumatic processes, most notably the generation of reactive oxygen species (ROS), which may serve as triggers for necrosis or apoptosis [4,10].

Several mechanisms proposed for TTS include synaptic fatigue, metabolic fatigue of either stria vascularis or the hair cells, and changes in cochlear blood flow. Histopathological changes reported in TTS include disarrayed, splayed, fused, collapsed, or floppy stereocilia of the hair cells, buckling of the pillar bodies, and swelling of the afferent nerve terminals [9,16,17]. Postsynaptic damage in the afferent dendrites beneath the inner hair cells (IHCs) is an important component of noise-induced hearing loss [15–17], and synaptic repair mechanisms are thought to be involved in restoring function after acoustic trauma [15,17].

It has been shown that the IHCs release glutamate as a neurotransmitter activating the afferent dendrites [11] and that excess release of glutamate due to intense noise exposure causes excitotoxicity in the afferent dendrites [14]. Excess synthesis of nitric oxide (NO), which can react with $O_2^{\bullet -}$ to form highly aggressive peroxynitrite ($ONOO^-$) radicals, is known to play an important role in glutamate excitotoxicity [3,6]. Glutamate and NO can act independently or sequentially to cause excitotoxicity. NO may induce glutamate release by neurons, which then stimulates NMDA receptors and triggers excitotoxicity [8]. Conversely, when NMDA receptors are activated, Ca^{2+} influx stimulates NO production through calcium- and calmodulin-dependent neuronal nitric oxide synthase, potentially leading to neuronal death [3]. The

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toxic effects originally attributed to NO are now considered to be mediated mostly by the compound peroxynitrite [2]. Thus, in the present study, we tested the hypothesis that ebselen, a glutathione peroxidase mimic and a scavenger of peroxynitrite, attenuates noise-induced excitotoxicity and TTS.

Twenty-four male albino guinea pigs (250–350 g), with normal auditory brainstem response (ABR) thresholds at 2, 4, 8 and 16 kHz, were randomly divided into vehicle-treated ($n=14$) and ebselen-treated experimental groups ($n=10$). These animals received an oral dose of 0.25 ml of 99% chloroform solution alone (vehicle) or containing 10 mg/kg ebselen one hour before exposure to noise. Ten mg/kg of ebselen was selected because this dose most effectively attenuates PTS in guinea pigs [13]. The animals were subjected to a 3-h noise exposure (115 dB SPL, 4-kHz octave band noise) generated within a single-walled, sound-deadened chamber. Two separately caged animals were tested at one time and allowed to move freely during exposure. The sound chamber was fitted with speakers driven by a noise generator and power amplifier. A 0.5-in. Bruel and Kjaer condenser microphone and a fast Fourier transform analyzer were used to measure and calibrate the sound level at various locations within the chamber to ensure stimulus uniformity within ±1 dB.

Five animals in each group underwent ABR measurements immediately and 1, 3, 7, and 14 days after noise exposure to assess the effect of ebselen on TTS. The method of ABR measurement has been described previously [13]. In brief, animals were anesthetized with a mixture of xylazine hydrochloride (10 mg/kg, i.m.) and ketamine hydrochloride (40 mg/kg, i.m.), and needle electrodes were placed subcutaneously at the vertex (active electrode), beneath the pinna of the measured ear (reference electrode), and beneath the opposite ear (ground). The stimulus duration was 15 ms; the presentation rate, 11/s; and the rise/fall time, 1 ms. Responses of 1024 sweeps were averaged at each intensity level (5 dB steps) to assess threshold. Threshold was defined as the lowest intensity level at which a clear reproducible waveform was visible in the trace.

Four animals given vehicle alone were euthanized under deep anesthesia with xylazine hydrochloride and ketamine hydrochloride 7 days after noise exposure to assess hair cell damage. Two animals unexposed to noise served as controls. The cochleae were perfused intrascalarly with 4% paraformaldehyde and immersed in the same fixative overnight. The surface of the organ of Corti was stained with rhodamine phalloidin and each turn of the cochlea was observed under fluorescent microscope as previously described [13]. The influence of ebselen on noise-induced morphological changes in the cochlea was examined in the remaining animals ($n=5$ in each group). These animals were deeply anesthetized with a mixture of xylazine hydrochloride and ketamine hydrochloride immediately after the termination of noise exposure. The left bulla was then exposed and the perilymphatic spaces perfused for 10 min with 2% paraformaldehyde in 2.5% glutaraldehyde. The animals were then sacrificed and the left cochlea quickly removed, immersed in the same fixative for 24 h, and decalcified in 10% ethylenediaminetetraacetic acid for 14 days. The specimens were post-fixed in 1% osmium tetroxide for 2 h, dehydrated in a graded series of ethanol, and embedded in epoxy resin. Ultra-thin sections were obtained from the very upper part of the basal turn corresponding to the region most responsive to frequencies in the range of 4–6 kHz and observed under transmission electron microscope (TEM). We focused on the histological changes in the stria vascularis, the hair cells and supporting cells, and the afferent dendrites beneath the IHCs. Because the extent of swelling of the afferent dendrites was obviously different between vehicle- and ebselen-treated animals, we compared the area (μm^2) of the swollen dendrites in the two groups. In each animal, a total of 10 sections that contained the nucleus of different IHC were collected. From these, five sections were chosen randomly for measuring and statistical analysis by a blinded technician who did not know the aim of the current study. The images of these sections were captured using a scanner, the area of the swollen dendrites measured using Adobe Photoshop image analysis software, and the mean of the areas calculated. Student's *t*-test was used for statistical analysis.

The experimental protocol was approved by the University Committee for the Use and Care of Animals at the University of Tokyo and conforms to the National Institute of Health Guide for the Care and Use of Laboratory Animals.

ABR thresholds before noise exposure were essentially equivalent among all animals. In vehicle-treated controls, ABR thresholds were moderately increased immediately after noise; the threshold shifts were approximately 25 dB at 2 kHz and 45 dB at 4, 8, and 16 kHz. The ABR thresholds then showed gradual recovery, returning to pre-exposure baseline thresholds 7 days later, indicating that the noise exposure induced a TTS. In contrast, ebselen-treated animals showed virtually no ABR threshold shifts after noise (Fig. 1). The ABR thresholds shifts immediately after noise were significantly different ($p<0.001$) at all frequencies measured between control and ebselen-treated animals. The IHCs and outer hair cells (OHCs) were well preserved in both vehicle-treated noise-exposed animals and unexposed controls; the number of missing OHCs in the lower two turns was 6.4 ± 2.5 in the former and 4.9 ± 4.4 in the latter, suggestive that the noise used did not cause hair cell death.

In controls that were euthanized immediately after noise, the OC in the upper basal turn showed numerous swollen dendrites beneath the IHCs. In contrast, pathological changes were quite limited in ebselen-treated animals (Fig. 2). The mean area of swollen dendrites was significantly smaller ($p<0.01$) in ebselen-treated animals compared to controls. In neither controls or ebselen-treated animals sacrificed immediately after noise exposure was there obvious evidence of other pathological changes reported to be correlated to TTS, such as edema or vasoconstriction in the stria vascularis, buckling of the pillar bodies, or fusion or loss of stereocilia.

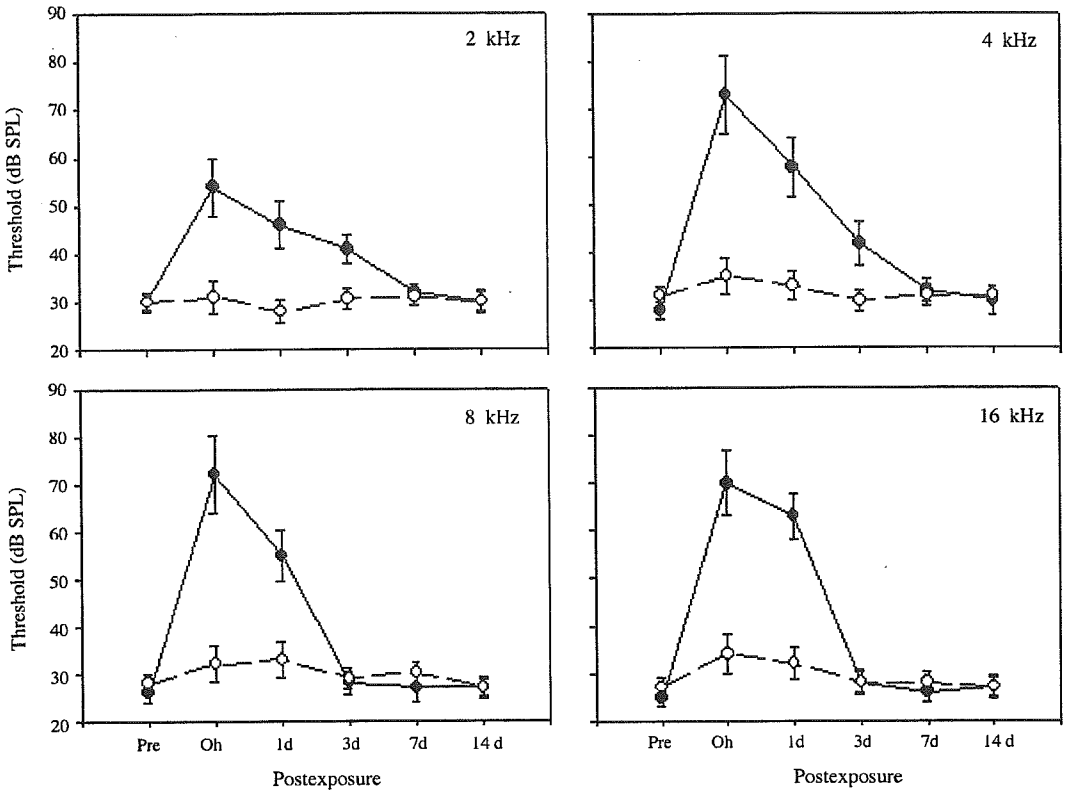

Fig. 1. Thresholds of auditory brainstem response (mean ± S.D.) measured before and immediately and 1, 3, 7, and 14 days following noise exposure in vehicle-treated controls (●) and ebselen-treated animals (○).

The current study shows that ebselen virtually prevents noise-induced TTS under conditions where vehicle-treated controls developed a TTS of approximately 25–45 dB. TEM observation revealed that immediately after noise, the afferent dendrites beneath the IHCs were severely swollen in control animals, whereas such pathological changes were significantly reduced in ebselen-treated animals. Other pathological changes were not evident in either controls or ebselen-treated animals. Because we did not examine otoacoustic emissions or use scanning electron microscopy, it is possible that functional and/or subtle anatomical changes in the regions other than the afferent dendrite, especially the OHCs, were missed. However, the above findings indicate that ebselen can attenuate noise-induced changes at least in the afferent dendrites beneath the IHCs and thereby TTS.

Using fluorescent dye 4,5-diaminofluorescein diacetate, it has been shown that in the normal guinea pig cochlea, NO is present in the afferent nerves and their putative endings near the IHCs, putative efferent nerve endings near the OHCs, the IHCs and OHCs, and blood vessels [20], and that inducible NO synthase (iNOS) is expressed in cochlear nerve fibers, as well as in hair cell stereocilia, Hensen's cells, and the stria vascularis [19]. It has also been reported that, when exposed to broadband noise (3 h/day at 110 or 120 dB SPL) for three consecutive days, NO concentration is increased in the perilymph, NO fluorescence becomes more intense in the IHCs and OHCs [21], and iNOS fluorescence signals becomes more intense in cochlear tissues compared to unexposed controls [19]. In addition, it has been reported that cochlear perfusion with kainic acid (KA), a conformationally restricted analog of glutamate known to have excitotoxic effects on spiral ganglion cells, causes significant elevation of thresholds of the cochlear nerve compound action potential and that this threshold shift is significantly reduced by pretreatment with nitroindazole, a competitive inhibitor of neuronal NOS [6]. The toxic effects originally attributed to NO are now regarded to be mediated mostly by the compound peroxynitrite [2]. Considering these findings, it is likely that ebselen attenuated noise-induced excitotoxicity and thereby TTS, by scavenging peroxynitrite formed by the reaction between noise-induced $O_2^{\bullet -}$ and NO.

It may also be possible that ebselen attenuated TTS because of its antioxidant property. It has been shown, however, that topical application of R-PIA, which increases endogenous antioxidant levels, to the chinchilla cochlea facilitates the recovery of hearing function 4 days after noise exposure but does not attenuate initial noise-induced threshold shifts [5]. Ohinata et al. [10] have shown that lipid peroxidation (8-isoprostane formation) in the OC induced by intense noise is significantly attenuated by *N*-methyl-D-aspartate (NMDA) receptor antagonists and anti-oxidant *N*-acetylcysteine, but not by NOS inhibitor, L-*N* (omega)-nitroarginine methyl

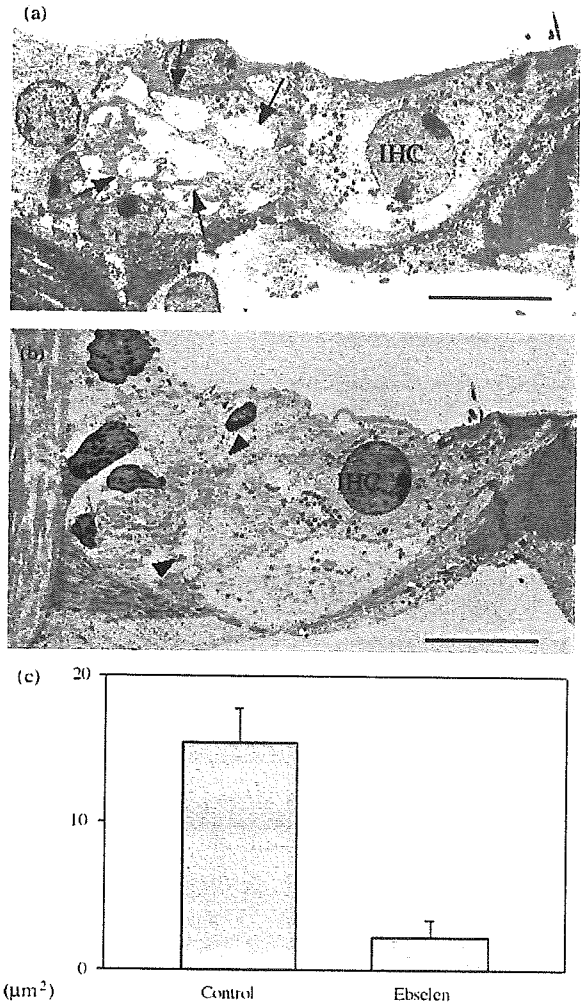

Fig. 2. Typical transmission electron microscopic findings in the medial side of the organ of Corti immediately following noise exposure in vehicle-treated controls (a) and ebselen-treated animals (b). The afferent dendrites are markedly swollen in vehicle-treated controls (arrows) but relatively normal in ebselen-treated animals (arrowheads). The bar indicates 10 μm; IHC: inner hair cell. (c) The mean ± S.D. of areas (μm^2) of the swollen afferent dendrites in the vehicle-treated control and ebselen-treated animals.

ester. Therefore, it is unlikely that ebselen's protective effect against TTS is provided chiefly by scavenging ROS or reducing ROS formation in the OC.

In conclusion, the current study shows that ebselen can prevent noise-induced excitotoxicity and thereby TTS. This prevention likely reflects ebselen's scavenging of peroxynitrite, thus supporting the view that peroxynitrite, at least in part, mediates excitotoxicity induced by intense noise. It has previously been shown that ebselen can also attenuate PTS in guinea pigs [13] and rats [7]. Since ebselen has already been used in humans clinically to treat ischemic stroke with few or no side effects [12], our findings reinforce the potential clinical utility of ebselen to prevent and/or treat noise-induced hearing loss in humans.

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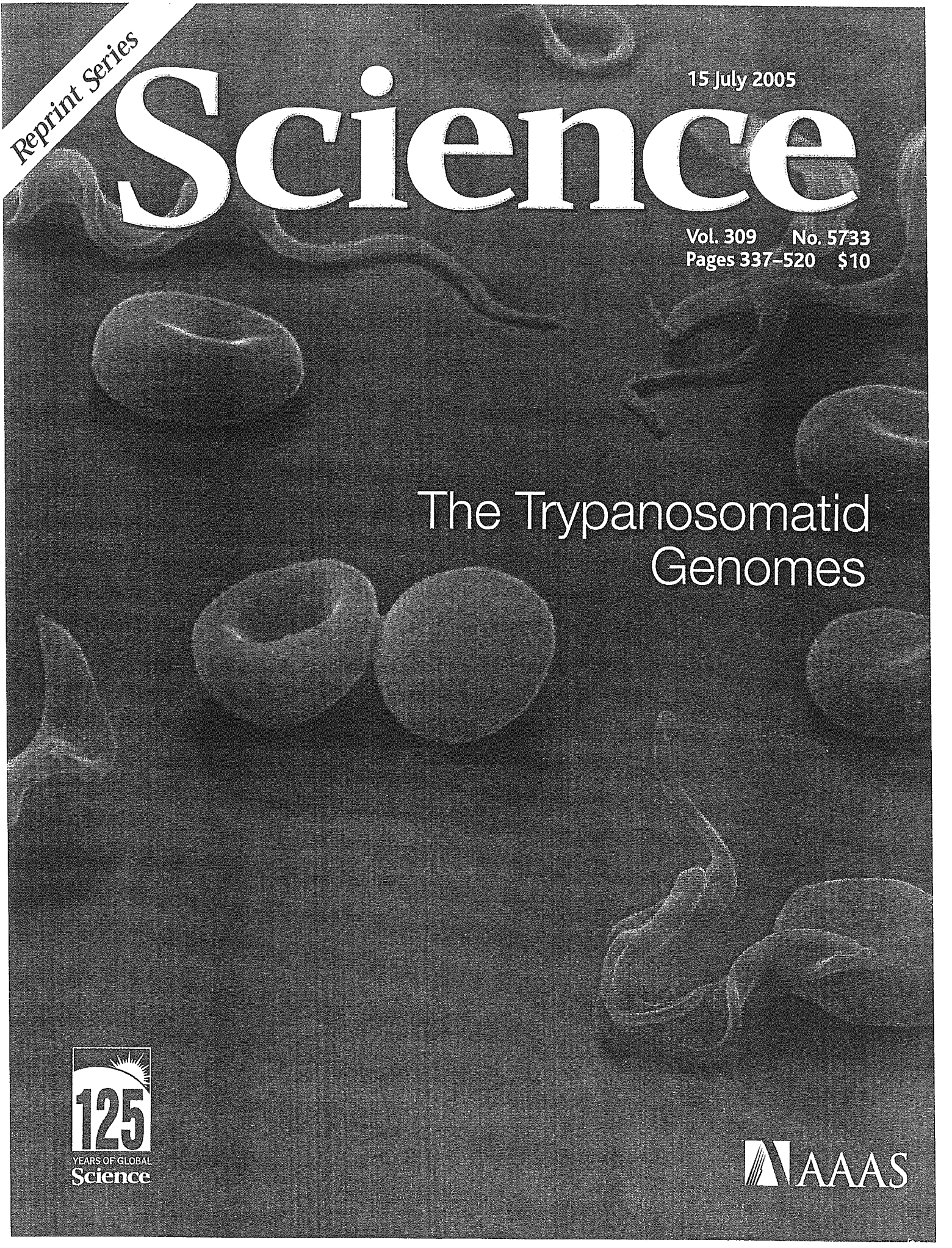
The Trypanosomatid Genomes



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Mitochondrial DNA Mutations, Oxidative Stress, and Apoptosis in Mammalian Aging

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Mutations in mitochondrial DNA (mtDNA) accumulate in tissues of mammalian species and have been hypothesized to contribute to aging. We show that mice expressing a proofreading-deficient version of the mitochondrial DNA polymerase γ (POLG) accumulate mtDNA mutations and display features of accelerated aging. Accumulation of mtDNA mutations was not associated with increased markers of oxidative stress or a defect in cellular proliferation, but was correlated with the induction of apoptotic markers, particularly in tissues characterized by rapid cellular turnover. The levels of apoptotic markers were also found to increase during aging in normal mice. Thus, accumulation of mtDNA mutations that promote apoptosis may be a central mechanism driving mammalian aging.

Mitochondria are the main source of cellular adenosine triphosphate and play a central role in a variety of cellular processes. These include fatty acid β-oxidation, phospholipid biosynthesis, calcium signaling, reactive oxygen species (ROS) generation, and apoptosis. Because the mitochondrion contains its own ~16-kilobase circular DNA, a central role for mtDNA mutations in aging has been postulated (*1*, *2*). Indeed, mtDNA mutations have been shown to accumulate with aging in several tissues of various species (*3*–*7*). The causal role of mtDNA mutations in mammalian aging is supported by a recent study demonstrating that mice with a mitochondrial mutator phenotype develop several aging phenotypes (*8*). Here, we used similar mice to investigate the cellular mechanisms by which mtDNA mutations contribute to aging.

We cloned the mouse POLG locus, *PolgA*, and used gene targeting in embryonic stem cells to introduce an AC → CT two-base substitution that corresponds to positions 1054 and 1055 of the *PolgA* cDNA (fig. S1) (*9*). This mutation results in a critical residue substitution in the conserved exonuclease domain of POLG, impairing its proofreading ability (*8*). Germline transmission of the mutation produced $PolgA^{D257A/+}$ mice, which were intercrossed to generate homozygous $PolgA^{D257A/D257A}$ mice, hereafter denoted D257A. Young D257A mice were indistinguishable from wild-type littermates, but long-term follow-up revealed a striking premature aging phenotype beginning at ~9 months of age, consisting of hair loss, graying, and kyphosis (Fig. 1, A and B). The mutant mice had a reduced life span (for

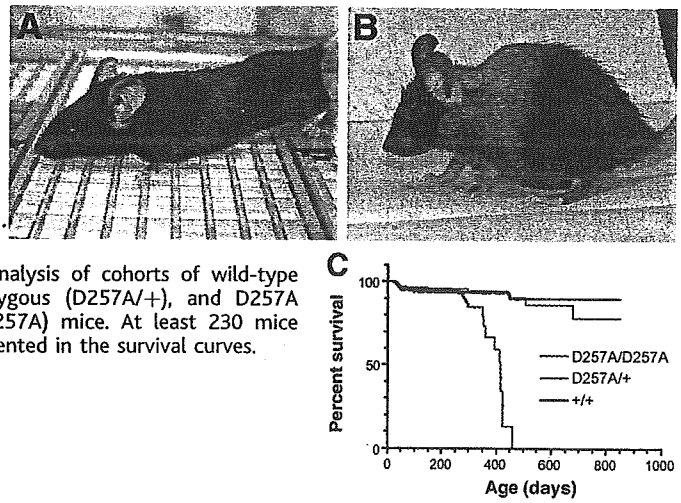

Fig. 1. D257A mice display a premature aging phenotype. Shown are wild-type (A) and D257A mice (B) at ~13 months of age. Progeroid features, including hair loss, graying, and kyphosis, become apparent at ~9 months of age. (C) Kaplan-Meier survival analysis of cohorts of wild-type (+/+), D257A heterozygous (D257A/+), and D257A homozygous (D257A/D257A) mice. At least 230 mice per genotype are represented in the survival curves.

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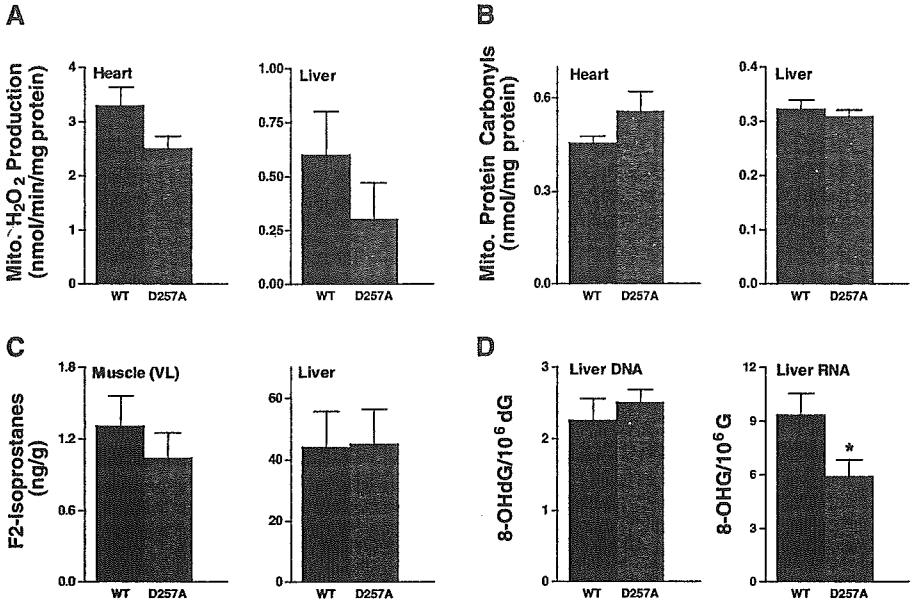

Fig. 2. Oxidative stress markers in isolated mitochondria and tissues from D257A mice. (**A**) Hydrogen peroxide production was measured by a sensitive fluorometric assay in mitochondria from wild-type (WT) and D257A mice at 9 months of age ($N \geq 8$). (**B**) Protein carbonyl levels, a marker of protein oxidation, were measured by an enzyme immunoassay in isolated mitochondria from WT and D257A mice at 9 months of age ($N \geq 7$). (**C**) F2-isoprostanes were measured by gas chromatography–negative ion chemical ionization mass spectrometry in liver and skeletal muscle (vastus lateralis) tissues from 6-month-old WT and D257A mice ($N = 6$). (**D**) Oxidative damage to DNA (8-OHdG) and RNA (8-OHG) was measured by high-performance liquid chromatography in liver tissue of 9-month-old WT and D257A mice ($N = 9$). *$P < 0.05$; error bars represent SEM.

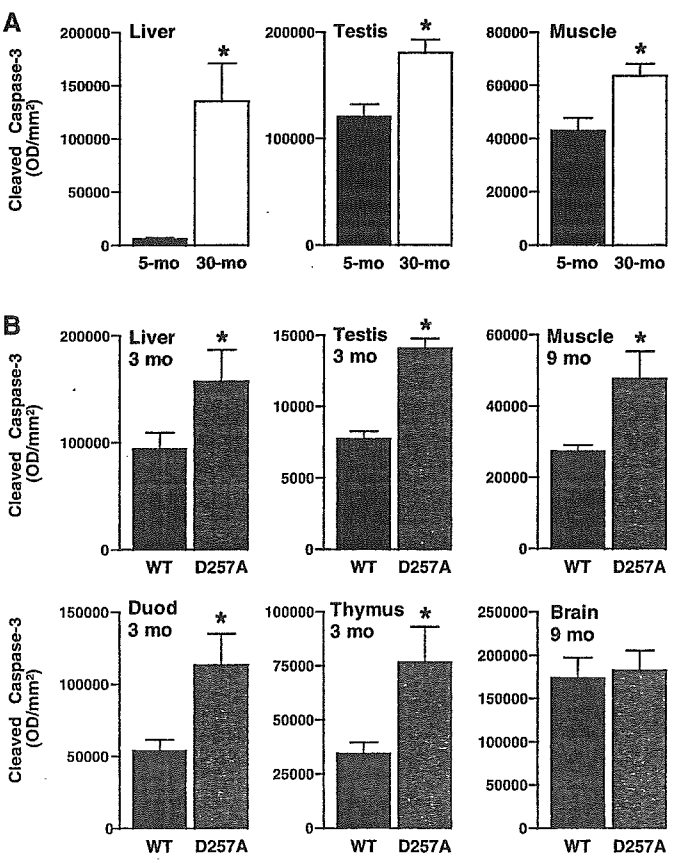

Fig. 3. Caspase activation in D257A mice and in normal aging. (**A**) Comparison of young (5 month) versus old (30 month) wild-type tissues. (**B**) Comparison of wild-type (WT) and D257A tissues at 3 months and 9 months of age. Activation of caspase-3 requires proteolytic processing of its inactive zymogen into activated fragments. The antibody used detects endogenous levels of the large fragment (17 to 19 kD) of activated caspase-3 resulting from cleavage adjacent to Asp^{175}. Extracts of tissues from WT and D257A mice of the indicated ages were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) and probed with a rabbit monoclonal antibody to cleaved caspase-3 (5A1, Cell Signaling, Beverly, MA). Units are arbitrary OD units. For all tissues shown except brain, the difference between WT and D257A mice was significant ($N = 6$ to 8, *$P < 0.05$); error bars represent SEM. Duod, duodenum; Muscle, gastrocnemius.

D257A mice, maximum survival 460 days, median survival 416 days; for wild-type littermates, maximum and median survival >850 days; $P < 0.0001$; Fig. 1C) and displayed several overt phenotypes in tissues undergoing rapid cellular turnover. These phenotypes were age-related and included thymic involution, testicular atrophy associated with the depletion of spermatogonia, loss of bone mass, loss of intestinal crypts, progressive decrease in circulating red blood cells, and weight loss (figs. S2 to S4).

Age-related hearing loss (presbycusis) is a hallmark of aging in multiple species, including mice (*10*) and humans (*11*), and has been correlated with the age-related accumulation of mtDNA mutations in auditory tissue. Hearing loss can be monitored by an elevation in auditory-evoked brainstem response (ABR). We conducted ABR analysis and observed no difference in auditory function between wild-type and D257A mice at 2 months of age (fig. S5G), but we found marked elevation of ABR thresholds at 4, 8, and 16 kHz ($P < 0.0001$) in D257A mice by 9 months of age, indicating severe hearing loss (fig. S5H). Histological analysis revealed age-related loss of spiral ganglion neurons (fig. S5), a feature of presbycusis (*12*). Thus, accumulation of mtDNA mutations can have a causal role in presbycusis.

Aging in rodents (*13*) and humans (*14*) is also characterized by loss of muscle mass (sarcopenia). Consistent with a causal role for mtDNA mutations in sarcopenia, the D257A mice displayed age-related loss of skeletal muscle. At 3 months of age, muscle weight in the D257A mice was similar to that of wild-type mice (fig. S6A); at 9 months of age, however, the mutant mice showed a significant reduction in the weight of both gastrocnemius ($P < 0.002$, ~10% decrease) and quadriceps ($P < 0.005$, ~10% decrease) muscles (fig. S6B). Therefore, age-related accumulation of mtDNA mutations is likely to contribute to sarcopenia.

To determine whether mtDNA mutations accumulated to varying extents in different tissues of the D257A mice, we sequenced a 525–base pair (bp) region of mtDNA that spans the control region, as well as a 487-bp region of the *COX1* gene. Sequencing revealed that the frequency of mtDNA mutations in the mutant mice was ~3 to 8 times that in wild-type mice for most tissues examined (fig. S7). Surprisingly, the frequency of mtDNA mutations in 5-month-old wild-type mice was as high as 2.1×10^{-4} in the *COX1* gene and 5.9×10^{-4} in the control region. This corresponds to average mutation frequencies of ~4 and ~10 mutations per mitochondrial genome, respectively.

A fraction of the oxygen consumed by cells results in the production of superoxide ($O_2^{\bullet-}$) in mitochondria, which is dismutated to hydrogen peroxide (H_2O_2) by superoxide dismutase. The main tenet of the free radical theory

of aging (*15*) is that aging is due to the progressive accrual of ROS-inflicted damage, including mtDNA mutations, the accumulation of which has been postulated to lead to a "vicious cycle" of further mitochondrial ROS generation and mitochondrial dysfunction (*1*, *2*). To test this hypothesis, we measured H_2O_2 produced by mitochondria isolated from the heart and liver of young and old (3 months versus 9 months) D257A and wild-type mice. Levels of H_2O_2 were not significantly different between genotypes in either young or old heart or liver mitochondria (Fig. 2A) (fig. S8A). We also assayed protein carbonyls, a marker of oxidative damage to proteins, and found no significant differences between 9-month-old D257A and wild-type mice in mitochondrial (Fig. 2B) or cytosolic fractions (fig. S8B) of either heart or liver. Thus, despite increased mutational load, mitochondria from D257A mice do not show increased oxidative stress.

We next examined additional markers of ROS-induced damage in tissues of D257A and wild-type mice. We measured F2-isoprostanes, a marker of lipid peroxidation (*16*), in liver, skeletal muscle (Fig. 2C), and heart (fig. S8C), and observed no significant differences between 6-month-old D257A mice and wild-type controls. We also examined oxidative damage to RNA and DNA in 9-month-old D257A and wild-type mice. Liver DNA from wild-type and mutant mice had similar levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) (Fig. 2D). Interestingly, liver RNA from the D257A mice had lower steady-state levels of 8-hydroxyguanosine (8-OHG) relative to samples from wild-type mice (Fig. 2D, $P < 0.05$). Thus, our observations do not support the idea that mtDNA mutations contribute to increased ROS production and oxidative stress in mitochondria with age.

One possible mechanism for the phenotypes in mitotic tissues of D257A mice is a defect in cellular proliferation. We derived mouse embryonic fibroblast lines (MEFs) from D257A and wild-type littermates and measured the number of cell doublings before senescence. At 20% oxygen, both wild-type and D257A MEFs underwent rapid replicative senescence, whereas neither underwent senescence or reduced growth after 40 days of culture at 2% oxygen (fig. S9). Thus, accelerated aging in D257A mice is not likely to be due to an intrinsic defect in cellular proliferation, or to accelerated cellular senescence.

In the mitochondrial pathway of apoptosis, mitochondrial dysfunction can lead to mitochondrial outer membrane permeabilization, the release of cytochrome c into the cytosol, and the activation of a key effector protease, caspase-3, by proteolytic cleavage (*17*, *18*). To determine whether an increased level of cleaved caspase-3 is a feature of normal aging, we examined tissues of 5-month-old and 30-month-old wild-type mice by immunoblotting. Cleaved caspase-3 levels increased with aging in liver, skeletal muscle, testis (Fig. 3A), and heart (fig. S10A) of wild-type mice by ~50% or greater. We also monitored the extent of apoptosis in tissues of D257A and wild-type mice. Levels of cleaved caspase-3 were significantly elevated in the cytosolic fractions of duodenum, liver, testis, and thymus of 3-month-old D257A mice relative to wild-type controls (Fig. 3B), and this induction preceded phenotypes in most tissues. Levels of cleaved caspase-3 were not altered in 3-month-old D257A skeletal muscle and brain relative to wild-type samples (fig. S10B), which suggests that postmitotic tissues may be more resistant to the induction of apoptosis mediated by mtDNA mutations. Cleaved caspase-3 levels were increased, however, in D257A skeletal muscle at 9 months of age relative to controls (Fig. 3B), a time at which mutant animals displayed loss of muscle mass. Thus, normal aging is associated with the activation of a caspase-3–mediated apoptotic pathway in several tissues, and D257A mice display an early onset of this phenotype.

Apoptosis is also associated with nuclear DNA fragmentation. Because the intestinal epithelium, thymus, and testis were severely affected in D257A mice, we examined these tissues with the TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling) assay, which detects apoptotic cells in situ. The 3-month-old D257A mice showed significantly more TUNEL positive cells relative to wild-type mice in all tissues examined (Fig. 4). Together, these findings strongly suggest that loss of critical, irreplaceable cells through apoptosis is a central mechanism of tissue dysfunction associated with the accumulation of mtDNA mutations.

We have demonstrated that accelerated development of aging phenotypes through mtDNA mutations can occur in the absence of increased ROS production or oxidative stress, and that tissue dysfunction is likely to arise through increased apoptosis. Moreover, we have shown that increased caspase-3 activation occurs in multiple tissues with normal aging. Tissues that are composed of mitotic cells display early caspase-3 activation in D257A mice, whereas skeletal muscle displays a later increase in cleaved caspase-3 and associated tissue degeneration. It is also clear that some cell types, such as spiral ganglion neurons, are exquisitely sensitive to the effects of age-related accumulation of mtDNA mutations. Because D257A mice display high levels of mtDNA mutations, it is possible that some of the phenotypes in these animals may be due to

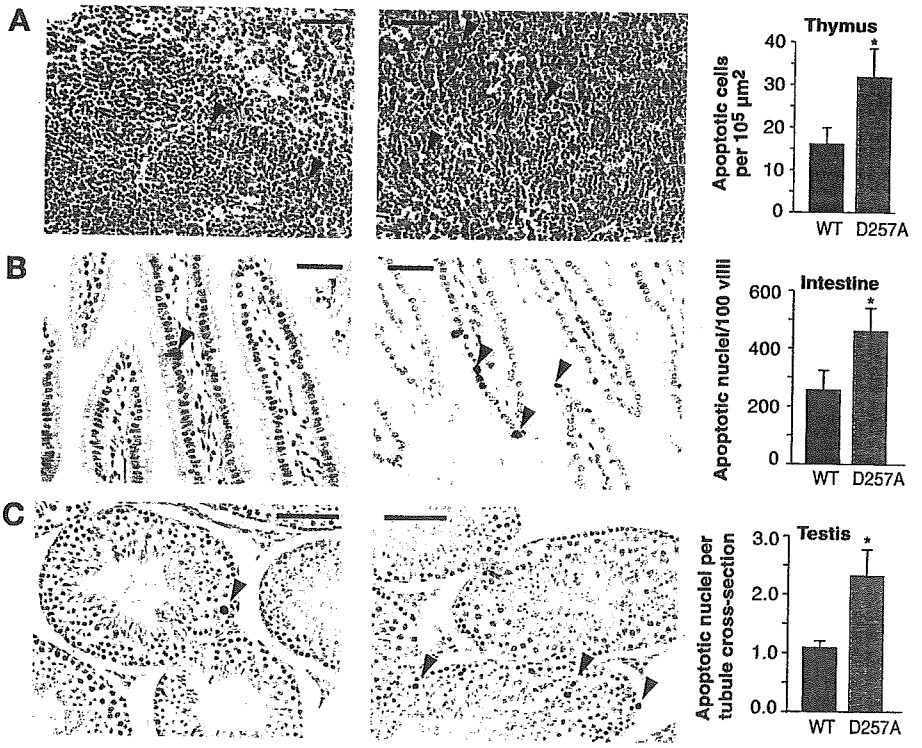

Fig. 4. Quantification of apoptosis by TUNEL in thymus (A), small intestine (B), and testis (C) of 3-month-old WT (left panels) and D257A (center panels) mice. Arrowheads indicate TUNEL-positive apoptotic nuclei. Numbers of apoptotic nuclei per 10^5 µm² section (thymus), per 100 villi (intestine), and per seminiferous tubule cross section (testis) were counted in hematoxylin-counterstained sections from the indicated genotypes. Each bar represents apoptotic nuclei from intestinal, thymus, and testis sections of at least four mice per genotype. *$P < 0.05$; error bars represent SEM. Scale bar, 100 µm.

complete exhaustion of tissue regenerative capacity, a process that may not be as severe in normal aging. However, the wide tissue distribution of phenotypes in D257A mice suggests that the age-related accumulation of mtDNA mutations reported in several species (*3*–*7*) contributes to physiological decline.

The concept that DNA damage contributes to aging is supported by the finding that humans and mice carrying mutations in several genes involved in DNA repair, including *Ercc2* (*Xpd*) (*19*), *Xrcc5* (*Ku86*) (*20*), and *Wrn* (*21*), display premature aging syndromes. It is likely that several types of DNA damage contribute to the aging process, and our findings suggest that apoptosis and subsequent loss of irreplaceable cells may be an important mechanism of aging in mammals. In agreement with this hypothesis, caloric restriction, the only nutritional intervention that retards aging, delays the accumulation of mtDNA mutations (*22*) and reduces mitochondria-mediated apoptotic pathways (*23*, *24*).

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Supporting Online Material

www.sciencemag.org/cgi/content/full/309/5733/481/DC1
Materials and Methods
Figs. S1 to S10

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