The mitochondrial free radical theory of aging is seriously challenged by the finding that mutant mtDNA never becomes abundant in vivo, a result disputed only in experiments using novel PCR variants whose quantitative accuracy is widely doubted. However, evidence continues to mount that mitochondria are the crucial site of free radical damage in vivo, most notably that mice lacking the nonmitochondrial isoforms of superoxide dismutase are healthy. It is thus important to determine whether a low level of mutant mtDNA could have serious systemic effects. This possibility exists because of the observed mosaic distribution of mutant mtDNA: some cells (or muscle fiber segments) lack any aerobic respiration. Such cells are presumed to satisfy their ATP needs by glycolysis. In vitro, however, NADH recycling by transmembrane pyruvate/lactate exchange does not suffice: cells only survive if they can up-regulate the plasma membrane oxidoreductase (PMOR). The PMOR’s physiological electron acceptor is unknown. It was proposed recently (de Grey, A. D. N. J. (1998) J. Anti-Aging Med. 1(1), 53–66) that a prominent in vivo acceptor from these mitochondrially mutant cells may be oxygen, forming extracellular superoxide. The mosaic (“hotspot”) distribution of this superoxide would limit its dismutation by extracellular superoxide dismutase; it may thus reduce transition metals leading to oxidation of circulating material, such as LDL. This would raise systemic oxidative stress, greatly amplifying the damage done by the originating mitochondrially mutant cells. This model, now known as the “reductive hotspot hypothesis,” has recently gained much indirect experimental support; several direct tests of it are also feasible.© 2000 Academic Press

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only two years after its publication, the highly respected gerontologist Comfort published an extremely forceful objection to it (9), which was not rebutted until 1993 (10). The objection is based on the discovery in 1961 (11), which was confirmed by very thorough later studies (12, 13), that all cells—even postmitotic ones—constantly renew their mitochondrial population by replication and destruction. Comfort reasoned that this turnover would prevent any accumulation of damaged mitochondria because they would be naturally destroyed and replaced by replication of undamaged ones.

The answer to this objection was provided by Müller-Höcker in 1993 (10). He showed, by in situ hybridization to the mtDNA, that the mosaic distribution of cytochrome c oxidase (COX) activity in skeletal muscle [reported by his group over the previous decade (14, 15)] was due to clonal expansion of a single mutation in each COX-negative fiber. In other words, the process of mitochondrial turnover was indeed affecting the accumulation of mitochondrial DNA, but in the opposite way to that which Comfort had imagined: turnover in fact amplifies the mutation at the expense of wild-type mtDNA. (Note: However, that turnover must indeed prevent accumulation of damage to mitochondrial components other than the mtDNA, since mitochondrial division dilutes out preexisting damage; this can fail only for a component that is synthesized by replication.) This phenomenon has recently been confirmed by other groups, some of them using different methods and in different cell types (16–18). Among other things it disproves the “vicious cycle” hypothesis for the accumulation of mtDNA mutations (19), which proposed that they occur at an increasing rate with age because prior mutations cause higher free radical production and therefore predicted a spectrum of mutations in each cell, rather than just one. The mechanism that gives the mutant mtDNA a selective advantage is still unknown, though one has been proposed (20) that appears consistent with all evidence so far available.

**CLONAL EXPANSION CHALLENGES THE “TIP OF THE ICEBERG” HYPOTHESIS**

The clonal expansion of mutant mtDNA thus removes a serious weakness of the mitochondrial free radical theory of aging. Unfortunately, it strengthens another, equally serious one.

Müller-Höcker’s earlier work (14, 15) showed that under 1% of cells or fibers in any tissue studied actually become COX-negative even by very old age. It was known that cells have a substantial surplus of bioenergetic capacity (21), so it seemed that these rare cells would easily be supported, with no systemic consequences, by glycolysis and export of lactate (which would be taken up by nearby, mitochondrially healthy cells). Thus, systemic consequences of accumulating mtDNA mutations could only occur if other cells—those which are histochemically COX-positive, so carry some functional mtDNA—also carry a large burden of mutant mtDNA, so have little overcapacity with which to support nearby COX-negative cells. (It is legitimate to make the approximation that COX activity exactly represents oxidative phosphorylation activity, because most mtDNA mutations observed in vivo either delete or mutate a tRNA gene (22), thus affecting COX subunit synthesis; mutations that affect only other enzymes are comparatively rare.) This “tip of the iceberg” model (23) is indeed supported by quantification of particular mtDNA mutations: base-pair substitutions were measured at levels which, if repeated for all possible substitutions, would give about 50 per mtDNA molecule (24), and very high levels of mtDNA deletions in the elderly have also been reported recently (25, 26).

However, the presence of such high levels of mutant mtDNA seems incompatible with the observed phenomenon of clonal expansion, because it requires that mutations be present stably (and at high levels) in the same cell as nonmutants. One must thus postulate that occasional mutations are donally expanded (leading to the observed rare COX-negativity) but most are not.

In fact, however, many different mutations are found to be amplified (17, 18), including ones which affect nonoverlapping regions of the mtDNA; this indicates that clonal expansion is not based directly on modulation of the DNA replication process [such as loss of a replicative cis-inhibitory region (27)]. The only apparent alternative is that expansion derives from the loss of oxidative phosphorylation (which is the process in which all mtDNA-coded proteins participate)—but this predicts that all mutants should be amplified, contrary to the requirement of the “tip of the iceberg” model described above. The studies mentioned previously (24–26) have also been criticised on methodological grounds, because they employ PCR variants whose quantitative accuracy is not generally accepted (28) and more direct quantitation has given contrary results (29, 30).

**THE REDUCTIVE HOTSPOT HYPOTHESIS: A WAY OUT?**

Thus, paradoxically, clonal expansion of mutant mtDNA challenges the mitochondrial free radical theory of aging. Only one hypothesis has so far been put forward to reconcile them (31). It was proposed that COX-positive cells do indeed have negligible levels of mutant mtDNA, but that COX-negative cells survive in the body not by lactate exchange but by export of electrons to extracellular acceptors via the plasma membrane oxidoreductase (PMOR). These acceptors may include molecular oxygen, so causing superoxide
formation in the medium surrounding these cells. Classical transition metal-dependent free radical chemistry might ensue, oxidizing circulating lipoproteins, which might later be imported by mitochondrially healthy cells, thereby increasing those cells' oxidative stress. Since the recipient cells can include any in the body, this process has the potential to amplify the toxicity of the originating mtDNA mutations far above what could result directly from their lack of bioenergetic function. This mechanism incorporates two distinctive proposals: (a) that the main source of systemic oxidative damage is reductive (32), rather than oxidative (33), stress imposed by cells on the extracellular medium, and (b) a highly focal distribution of that stress. Accordingly it has become known as the "reductive hotspot hypothesis," hereafter abbreviated RHH. The model is depicted in Fig. 1; see Ref. 31 for further details. This article surveys recent experimental and theoretical work relevant to RHH.

RECENT EXPERIMENTAL DATA PERTINENT TO RHH

The strongest evidence yet in favor of the mitochondrial free radical theory has come from the creation of knockout mice deficient for each of the three forms of superoxide dismutase (SOD). Superoxide is presumed to be the originating toxic radical, in whose absence little oxidative damage occurs, so if such damage is important in aging then these knockouts should show a severe phenotype. Indeed, homozygous knockouts of the mitochondrial SOD die a few days after birth (34, 35). Remarkably, however, homozygous knockouts of the other forms (cytosolic (36) and extracellular (37)) were healthy. They showed reduced tolerance to oxidative stress, but when treated well they showed no phenotype whatever and their lifespan was not appreciably reduced (though rigorous lifespan studies have yet to be reported). This indicates that oxidative damage elsewhere than the mitochondrion plays no part in mouse aging. It is surprising that the authors of both these startling studies (36, 37) placed less emphasis on the lack of effect on lifespan than on the wholly unstartling findings that the mice were less resilient to stresses such as hyperoxia. The above findings, taken together, imply that either mitochondrial oxidative damage is a major determinant of the rate of aging, or else oxidative damage in general is unimportant in aging. The latter option cannot be conclusively rejected at this stage, but it is powerfully challenged by all manner of data, such as interspecies correlations between lifespan and both superoxide production (38, 39) and membrane oxidizability (40, 41).

It was originally considered unlikely that the PMOR would use oxygen as an electron acceptor forming superoxide, because \( \rho^0 \) cells (cells whose mtDNA has been eliminated) that are induced to up-regulate the PMOR do not generate increased superoxide (42). But this tells us only that oxygen is not as high-affinity an acceptor as others (such as pyruvate) which are present extracellularly in vitro but not in vivo. Three groups have since identified conditions under which extracellular superoxide can indeed be formed by cells in conjunction with NADH dehydrogenation (43–45). In one case (43) the NADH was extracellular, perhaps suggesting a different enzyme, but it must be borne in mind that ubiquinone plays a crucial role in the PMOR system (46); ubiquinone may be reducible by NADH dehydrogenases on either side of the membrane but reoxidized by just one (superoxide-generating) enzyme on the outside. However, none of these reports shows directly that anaerobic cells form extracellular superoxide in vivo.

One paradox which RHH seeks to resolve is that low-density lipoprotein (LDL) is oxidized in vivo despite the presence, in blood, of levels of antioxidants which totally abolish its oxidation by endothelial cells in vitro (47). A proposal with widespread support is that oxidation occurs in the arterial intima—the space between the endothelial cells that form the inner surface of the artery wall and the surrounding smooth
The vicinity of anaerobic cells in the interstitium is a candidate for such a location.

RHH makes a strong prediction, however, with regard to circulating LDL: that its oxidisability should be correlated with lifespan (both across species and between individuals of the same species), because its contamination by oxidised lipids is proposed to be the major source of oxidative stress in most cells. Thus, dietary supplements which reduced that oxidisability should be life-extending. The major antioxidant in LDL is vitamin E, present at 6–12 molecules per LDL particle; this is raised by high dietary intake of vitamin E (49). But vitamin E is now established to function as an antioxidant much less well in the core of a lipoprotein particle than in membranes—in fact, without a sufficient supply of electrons from other antioxidants, vitamin E is actually pro-oxidant (50, 51). Therefore, the lack of life-extending effect of high vitamin E intake (52) can no longer be seen as challenging RHH.

Another way to render LDL less oxidisable is to reduce its levels of highly oxidizable material, and here there is positive evidence supporting RHH. A well-known conundrum in gerontology is the “French paradox,” the exceptional longevity of a population in which consumption of both alcohol and high-fat foods is unusually high. It was recently shown that the level of plasma hydroperoxides is markedly elevated after meals and that wine consumed with the meal dramatically suppresses this elevation (53). Demographic considerations suggest that French longevity cannot be ascribed purely to low susceptibility to cardiovascular disease: French mortality from this cause is still considerable (54), but mean lifespan in France is about three years more than the average of industrialized countries (54), which would require complete abolition of mortality from cardiovascular disease if other risk factors were unaltered (55). Genetic factors have also been ruled out by migrant studies (56). Thus, either there are other ways in which the French lifestyle extends life or (as proposed by RHH) the level of plasma hydroperoxides affects age-related pathologies beyond the vasculature.

A major requirement of RHH is that transition metals be occasionally present in LDL when it is in the vicinity of an anaerobic cell, in order to turn the reductive stress of superoxide into the oxidative stress necessary to drive peroxidation. LDL associated with redox-active metals has never been convincingly isolated from plasma, and strong doubts have been expressed regarding whether that binding ever occurs (57); there is a view that aggregation of LDL, which is the trigger for its endocytosis by macrophages, may not in fact require prior oxidation (58). But oxidation remains the only process that is known both to occur in vivo and to cause LDL aggregation, so its causal role remains attractive. Evidence that LDL does not associate with metals in vivo is restricted to the composition of LDL that is already sequestered in macrophages (59); no evidence exists regarding the situation in the vasculature, much less the interstitium. One source of redox-active iron, haemin, was proposed (31) as the most plausible in this context, since it binds LDL when it is still suspended in the erythrocyte membrane (60); this binding is transient, since albumin and haemopexin have much higher affinity for haematin, which makes it very hard to establish whether such binding occurs in vivo. But recent work (61) reinforces suspicions regarding haemin, showing that the half-life of haemin-LDL binding in the presence of albumin and haemopexin was more than 20 seconds—probably long enough for LDL to travel from the vasculature into the interstitium, where higher-affinity binders may be absent.
indeed occurs in vivo is suggested by the hyperactivity of succinate dehydrogenase in COX-negative muscle fibers (10, 17, 62)—which exceeds that ascribable to mitochondrial proliferation (62)—but more stringent tests would be valuable. In vitro it would suffice to establish whether \( \rho^- \) cells ever generate \( \text{CO}_2 \) or if they can grow on pyruvate without glucose. [They have been reported to consume suspiciously little glucose for their growth rate (63), and addition of pyruvate also caused the parental \( \rho^- \) cells to use less glucose when respiring aerobically (63), but \( \text{CO}_2 \) production was not measured.]

The undiminished lifespan of extracellular (EC-) SOD knockout mice (37) is potentially a challenge to RHH, since superoxide is proposed as the intermediary by which COX-negative cells reduce LDL-bound iron. There has been substantial progress in developing histochemical assays for all three variants of SOD (64), so it may now be possible to assess whether EC-SOD colocalizes with COX inactivity; if it does, the role of superoxide is supported but the overall plausibility of RHH is weakened [since an inability to eliminate this superoxide, due to knockout of EC-SOD, produces no phenotype (37)]. EC-SOD levels in skeletal muscle were reported as under 0.5% of total SOD activity by chromatographic separation assays (65), an order of magnitude less than the average for all 11 tissues analyzed, so it remains doubtful whether histochemical analysis will reveal it at significant levels. If EC-SOD is found to be virtually absent from skeletal muscle, perhaps being unable to enter the interstitial space, RHH is not challenged; if it is present but does not colocalize with COX inactivity, a modification of RHH is indicated whereby the PMOR interacts directly with LDL-bound iron, or else via an intermediary other than oxygen.

The oxidation of LDL in the interstitial space is proposed in RHH to lead to import of slightly oxidized LDL by other cells, but it should also lead to the presence of oxidized LDL in the lymphatic system. Indeed, the return of slightly oxidized LDL into the vasculature via the lymphatic system is a requirement of the proposal that this is the source of LDL oxidation that ultimately leads to atherosclerosis. Few studies of lympathic LDL have yet been published, however, and many questions remain concerning its composition, not least its oxidation levels.

Finally, the component of RHH that has often been considered the most tenuous has been greatly reinforced. When a circulating LDL particle binds to the LDL receptor, endocytosis of the particle is followed by acidification of the resulting vacuole, causing the release of cholesterol and its transport to sites of cholesterol utilization elsewhere in the cell. This process seems intuitively unlikely to be sensitive to the presence of peroxidized lipids in the particle: any such contaminants would surely be retained in the vacuolar apparatus and broken down without consequences to the cell. But recent work of Brunk's group (66–68) has demonstrated that the vacuolar apparatus is by no means as robust as is generally imagined; many types of stress can cause lysosomes to rupture, releasing into the cytosol enzymes whose presence there is not in the cell's best interests. This is presumably a rare event in vivo, but one which can considerably increase stress in the cell, with pleiotropic and wide-ranging possible consequences. A lysosome which is already fragile for other reasons may be pushed over the edge by the arrival of a bolus of cholesterol that is undergoing peroxidation.

**CONCLUSION**

The reductive hotspot hypothesis is intricate and in some ways not intuitively plausible. However, to paraphrase Churchill's opinion of democracy, it is arguably the worst elaboration of the mitochondrial free radical theory of aging devised by the wit of man—except for all the others. In contrast to systems of government, RHH has a remarkable paucity of competition, now that the "vicious cycle" theory (19) has been laid to rest by the discovery (10, 16–18, 20) that cells are taken over by expansion of a single species of mutant mtDNA, rather than by a cascade of independent mutations. The only other presently available hypothesis that reconciles the mitochondrial free radical theory of aging (1) [so strongly supported by the viability of non-mitochondrial SOD knockout mice (36, 37)] with the ability of mitochondrial turnover to prevent accumulation of mitochondrial damage other than to the mtDNA (9, 20) is the "mitochondrial–lysosomal axis" theory (69), which proposes that the functional decline of cells is due to the accumulation in lysosomes of largely mitochondrion-derived lipofuscin. This idea now has impressive support from in vitro studies (66–68), but is challenged by reports (70) that vitamin E supplementation, which causes no appreciable extension of lifespan (52), can substantially retard the accumulation of lipofuscin in vivo. Absence of such retardation has also been reported, however (71, 72), so further studies are needed. Provocatively, an age-related decline in lysosomal function might also explain the recent reports of abundant mutant mtDNA (24–26), as well as the startling report that fully 18% of rat liver mtDNA is fragmented (73), if the DNA being analyzed included incompletely degraded mtDNA from lysosomes.

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