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A Mechanism Proposed to Explain the Rise in Oxidative Stress During Aging

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Editor's Note: The question of the underlying cellular mechanism of aging has left two camps: those who feel that changes in nuclear gene expression (telomere shortening, heterochromatin change, senescent gene expression, etc.) are responsible for the alterations in senescent cells, and those who feel that internal changes in the mitochondria themselves are primary. To a large extent, until de Grey's publication in BioEssays in January of this year (see reference 22 in this article), the mitochondria camp lacked any coherent rationale for the preferential accumulation of damaged mitochondrial in aging; the battle appeared to have been won by the senescent gene expression camp, not for reasons of data, but rather by intellectual default. The BioEssays article changed that view for many. In this current article, de Grey (currently at Cambridge University) extends his argument and meets challenges raised by more recent data. Although not proving that accumulation of damaged mitochondria underlies aging, it does offer a consistent hypothesis to support the view. If correct, then anti-aging medicine may need to deal directly with mitochondrial DNA to offer significant clinical benefits. De Grey suggests specific therapeutic avenues which need exploration.

ABSTRACT

Most phenotypes of aging in vertebrates may be caused by a progressive decline in the ability of antioxidant defences to maintain cellular and systemic homeostasis. This is due both to a diminished efficacy of those defences and to an enhanced level of pro-oxidant toxicity; the imbalance between the two has been termed *oxidative stress*. However, the cause of this increasing imbalance remains obscure. This article proposes a mechanism by which spontaneously mutant mitochondrial DNA (mtDNA), despite being present only in very small quantities in the body, may be the main generator of oxidative stress. Mutant mtDNA is distributed very unevenly within a tissue: some cells apparently contain no wild-type mtDNA whatever. Those cells must rely on glycolysis for ATP production; furthermore, they require a system to stabilize their NAD^+/NADH ratio. This can only be achieved by an efflux of electrons from the cell, most probably mediated by the plasma membrane oxidoreductase (PMOR). It is proposed that the required rate of electron efflux from these anaerobic cells exceeds the local electron-accepting capacity of "safe" acceptors in plasma such as dehydroascorbate, with the result that reactive species, such as superoxide, are formed. This leads to increased oxidation of lipids in the plasma, notably of low-density lipoprotein (LDL) particles, which are subsequently imported into mitochondrially healthy cells. This oxidized lipoprotein must be destroyed by the recipient cells' antioxidant defences. That task diverts the cell from the degradation of pro-oxidants that it is itself generating; thus, it imposes oxidative stress on the cell. As the number of anaerobic cells in the body rises, so does oxidative stress in all cells. The consistency of this hypothesis with known facts is discussed, and technically feasible tests are suggested both of the proposed mechanism and of its overall contribution to mammalian aging, including plausible interventions to retard the process.

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INTRODUCTION

THE FUNDAMENTALS OF THE mitochondrial free radical theory of aging were first described by Harman in 1956.[1] Harman proposed that aging results, for the most part, from an ever-increasing level of destructive chemical reactions involving free radicals (molecules with an unpaired electron). He extended this hypothesis in 1972[2] with the idea that mitochondria are the main mediators of this process, in that:

> Of all subcellular components, mitochondria are both the main source of free radicals and the main direct victim of free radical damage;
> Loss of mitochondrial function, and hence bioenergy capacity, is the driving intracellular change underlying aging, causing (rather than caused by) other pro-oxidant changes such as slower protein turnover.

There has since been strong experimental support for these tenets. There have been numerous reports of a decline in ATP synthesis capacity during aging.[3–5] These studies have examined all components of the respiratory chain, and only those that are partly encoded by the mitochondrial DNA (mtDNA) are affected. Many other studies have shown a parallel increase in the levels of mtDNA lesions.[6–11]

Nevertheless, attempts to develop Harman's theoretical framework into a detailed mechanism have met with difficulty. First, it has proven hard to identify a mechanism whereby spontaneously mutant mtDNA could accumulate within a cell. Mechanisms that have been proposed included reduced capacity for mtDNA replication,[12] increased free radical generation accelerating the mtDNA mutation rate,[7] faster replication of grossly deleted mtDNA[13] and preferential replication of mutant mitochondria near the nucleus.[14] All of these have been found to be inconsistent with the observed[15–21] distribution of mutant mtDNA within and between cells: namely, that cells appear to be stochastically and very rapidly taken over by a single, mutant mtDNA genotype—which is not necessarily a deletion—arising from a spontaneous mutation, and that nondividing cells suffer disproportionately. However, a new and detailed mechanism proposed recently[22] (Fig. 1), based on preferential lysosomal degradation of mitochondria driven by self-inflicted membrane damage, appears to be consistent with the evidence and may suffice to bridge this gap in Harman's theory.

Whatever the intracellular kinetics, however, it is clear that any effect of mutant mtDNA molecules on aging at the organismal level must vary with their quantity. Many researchers have consequently attempted to measure the levels of mutant mtDNA in various tissues of young and old individuals.[6–11,23] A popular target for such studies has been a deletion of 4,977 base pairs (nearly a third of the mitochondrial genome) which occurs much more frequently than any other large deletion—indeed, it is usually termed *the common deletion*.[6] It is believed to arise by illegitimate recombination between the sequences at its ends, which are identical over a length of 13 base pairs.[24] The polymerase

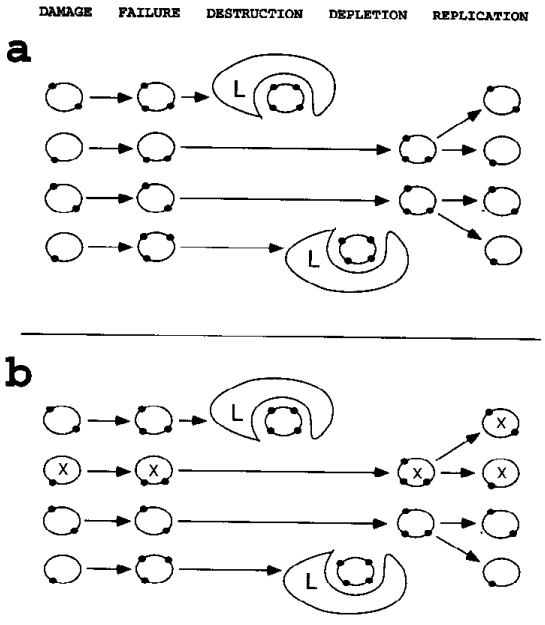

FIG. 1. Possible mechanism driving both normal mitochondrial turnover and the preferential amplification of mutant mtDNA in nondividing cells. (a) Self-inflicted membrane damage drives turnover stably in homoplasmic cells. (b) Mutant mtDNA preferentially escapes destruction. For details see ref. 22. L: lysosome; spots: membrane damage; X: mtDNA damage.

chain reaction (PCR) can be used to establish the ratio of deletion-bearing molecules to full-length ones in a sample of tissue; reported values vary somewhat, but it is generally accepted that no tissue accumulates more than about 0.1% of this particular mtDNA species during normal aging, even by very old age.

The question thus arises of how, if at all, we may extrapolate from this value to an estimate of the "total mutational load" of mtDNA in a given aged tissue.[7] This is difficult even if one restricts oneself to deletions of mtDNA, detectable by Southern analysis. However, there is clear evidence that mtDNA also spontaneously suffers point mutations. Their levels can also be measured by PCR technology. Unfortunately, the technique is much less standardized than for deletions, with the result that hugely divergent estimates have emerged from different groups that employed novel and ingenious refinements designed to improve the accuracy of the technique.[9,25,26] A further factor may also have confounded this issue: most workers have chosen to focus on mutations that are known to cause inherited disease. This is superficially well-motivated because it ensures that the mutation being sought is not phenotypically silent; however, there is good reason to believe that serious loss-of-function mutations in the mtDNA are so deleterious that they cause prenatal mortality and thus can never be passed on. This suggests that the mutations being studied, though not silent, are only very weakly hypomorphic; their segregation behavior may, therefore, not be characteristic of typical somatic mutations that would be more serious. Studies that were able to detect other point mutations[8,9] have provided a somewhat clearer picture which, moreover, is in striking accordance with the predictions of the model just noted,[22] despite originally appearing to be mutually inconsistent.

These points previously led the present author to support[27] the view propounded by others[7] that the true levels of mtDNA point mutations may in fact be much higher than has so far been reported, and that the increase of oxidative stress during aging may result simply from a loss of aerobic function in a significant proportion of cells, whose viability is maintained by their neighbors at the increasing expense of the latter's homeostasis.

However, this view is becoming increasingly difficult to maintain. There has recently been a spate of studies[18–21,28] in which the level of mutant mtDNA has been estimated by methods that are not specific to a single (or a small class of) sequence variant. These studies have taken advantage of the almost complete homoplasmy of all cells,[1] whereby some cells are devoid of wild-type mtDNA and the rest are devoid of mutant mtDNA, with hardly any containing some of each. This homoplasmy results in complete loss of enzymatic function throughout a mitochondrially mutant cell for some or all of those components of the respiratory chain that are partly encoded in the mtDNA. Therefore, either histochemical assays of these enzymes' activity or immunocytochemical assays of their presence can reveal the overall mutational load by direct visualization under the light microscope. Though these studies are somewhat preliminary, with numerous details still remaining to be established, a picture is irresistibly emerging that the overall mutational load is no more than about one order of magnitude higher than has been estimated from studies of mtDNA deletions: that is, that no tissue suffers loss of aerobic respiration in more than about 1% of its cells, and most tissues suffer much less than that.

It is thus clear that, unless the recent studies just noted are found to be methodologically flawed, spontaneous mtDNA mutations in nondividing cells cannot cause the observed levels of oxidative stress simply by soaking up other cells' bioenergetic capacity. Either they are of no great causal significance in aging or else they drive up oxidative stress by some less direct mechanism, which greatly amplifies their toxicity to other cells. This article explores the latter possibility.

The mechanism falls into two parts. The first proposes changes that must occur within cells

[1]An exception is muscle fibers, which are syncytial. Rather than the whole fiber being homoplasmic, there appear to be semipermeable barriers within the fiber, so that large regions of the fiber are homoplasmic but the fiber as a whole is not. Regions neighboring an affected region are preferentially seen to be partly affected, indicating that mitochondria occasionally cross these barriers.[21]

as they become progressively depleted of functional mtDNA, in order that they can survive in the body. The second proposes consequent changes that will occur in the blood stream: in particular, changes to material that is ingested by all cells, be they mitochondrially mutant or healthy. These latter changes are pro-oxidant, so they impose a challenge to the recipient cell's antioxidant system.

HOW DO ANAEROBIC CELLS SURVIVE AT ALL?

Oxidative phosphorylation (OXPHOS) provides over 90% of our cells' ATP synthesis capacity; the remainder arises from earlier steps in glucose (or fatty acid) metabolism. Unlike OXPHOS, glycolysis is performed entirely by enzymes that are encoded in the nuclear DNA. Thus, as has been noted,[14] a cell which has lost mtDNA functionality can satisfy its ATP demand by importing more glucose from the bloodstream.

The most obvious disadvantage of this potential mode of survival is that the pyruvate generated by glycolysis is not further degraded. It must, therefore, be excreted by the cell into the blood either as pyruvate or as its reduced form, lactate. This is not totally implausible because the very small amounts of lactate that so few cells would produce could fairly easily be disposed of. But, in fact, we know that there is another problem.

It is possible to culture human cells lacking functional mtDNA—in fact, lacking any mtDNA whatever.[29] These are usually termed ρ^0 cell lines. However, these cells cannot be kept alive simply by exogenous glucose: they need two other nutrients. One is uridine: this need not concern us because its necessity is not related to energy utilization. The other is ostensibly the last that one would imagine: pyruvate. ρ^0 cells are continually generating pyruvate by breakdown of glucose, so it seems highly curious that they should be auxotrophic for it. But, in fact, there is a simple explanation. Two molecules of pyruvate jointly contain four fewer electrons than one molecule of glucose, so the conversion of glucose to pyruvate is inexorably associated with the conversion of two molecules of NAD^+ to NADH. These can be restored to NAD^+ by the reduction of pyruvate to lactate; thus, if a ρ^0 cell imports glucose and excretes lactate then it can make ATP with a net zero conversion of NAD^+ to NADH. But zero is not sufficient. Many of the vital processes in the cell, such as protein synthesis, convert NAD^+ to NADH; therefore, this must be compensated by a net conversion of NADH to NAD^+ elsewhere. Normally, this is provided by OXPHOS. When OXPHOS is unavailable, exogenous pyruvate can substitute: the cell can import it and reduce it to lactate which it excretes. In this way ρ^0 cells use exogenous glucose to maintain ATP/ADP homeostasis and exogenous pyruvate to maintain NAD^+/NADH homeostasis.

But is this what such cells (either *in vitro* or *in vivo*) actually do? In recent years there has emerged evidence—both *in vitro* and *in vivo*—that, at least in detail, it is not.

The *in vitro* evidence is the discovery of chemicals that can substitute for pyruvate in the culturing of ρ^0 cells.[30] The relevant feature of these chemicals is that they are completely impermeable to the cell: indeed, some—such as ferricyanide—would probably kill any cell that they did penetrate. They perform the same task just described, the conversion of NADH to NAD^+, but they do it "at a distance", through the cell membrane. (NADH is also impermeable to the membrane, so we know that the conversion cannot be taking place in the extracellular medium.) This is mediated by an enzymatic system that has been known for many years,[31,32] called the plasma membrane oxidoreductase (PMOR). The PMOR is present in all cells yet studied. It has been the subject of extensive research with regard to certain other properties, as has another, hormone-responsive PMOR activity which is performed by a different protein.[73] Only one mention, however,[33] has been made of its possible involvement in the survival of spontaneously anaerobic cells *in vivo*.

In vivo evidence also suggests that anaerobic cells may maintain NAD^+/NADH balance by exporting electrons directly rather than by exporting lactate. It arose as a perhaps somewhat fortuitous adjunct to the histochemical studies of the distribution of mtDNA mutations.[17–21]

As a control, these studies have simultaneously assayed for the activity of an enzyme that is wholly encoded in the nuclear DNA but is located, like the partly mt-coded enzymes, in the mitochondrial inner membrane. The one they chose was succinate dehydrogenase (SDH). Indeed, SDH is always found to remain functional in those cells that have lost activity of partly mt-coded enzymes.[17–21] Moreover, some of these studies[17,20,21] have found that such cells usually have substantially *greater* SDH activity than aerobic cells nearby.

The relevance of this to the question of electron export is that SDH is a component of the tricarboxylic acid (TCA) or Krebs cycle. The TCA cycle is an intermediate between glycolysis and OXPHOS: the pyruvate generated from glycolysis is broken down first to acetate and then to carbon dioxide, the latter of those steps being performed by the TCA cycle. The other major product of the TCA cycle is NADH, which (in an aerobic cell) is restored to NAD^+ by the donation of two electrons into the respiratory chain. Additionally, and of great relevance to the present topic, one step of the TCA cycle generates a molecule of ATP (via GTP).

This provides us with a highly plausible explanation for why SDH activity is maintained and even raised in anaerobic cells. Maintenance of the TCA cycle enables the cell to generate twice as much ATP (per imported molecule of glucose) as if it were only performing glycolysis. It also causes the cell to generate several times more NADH; but as we have seen, a mechanism is already in place to respond to that. The TCA cycle also creates one molecule of $FADH_2$, but this can plausibly be interconverted to NADH via mitochondrial ubiquinone and glycerophosphate dehydrogenase requiring no mitochondrially-coded activity. Yet a further advantage of maintaining the TCA cycle is that it allows the utilization of fatty acids as an energy source. Thus, providing only that the PMOR can sustain the necessary rate of oxidation of NADH, the cell is able to make far more efficient use of nutrients (Fig. 2).[2]

CONSEQUENCES IN THE EXTRACELLULAR MEDIUM

This completes a plausible picture of how anaerobic cells survive. (In fact, very recent work indicates that—unsurprisingly—they are not nearly so robust as aerobic cells in that they are prone to atrophy and apoptosis.[21] However, this appears to be strictly a susceptibility, not a programmed response to loss of OXPHOS

[2]The maintenance of the TCA cycle implies an interesting variation on previously suggested mechanisms for the survival of OXPHOS-deficient mitochondria. Mitochondria require both internal ATP and a proton gradient to perform protein import and many other necessary functions; both are normally provided by the respiratory chain. It was previously proposed,[8,27] on the basis of no ATP synthesis within such mitochondria, that the ADP/ATP translocase of mutant mitochondria reverses and imports ATP, whose intramitochondrial hydrolysis (in performing protein import, etc.) causes the phosphate carrier to reverse, thus exporting protons and generating a proton gradient. Because the TCA cycle occurs within mitochondria, its maintenance precludes this means of maintaining a proton gradient, because the phosphate carrier would not reverse. Instead, the rapid production of intramitochondrial NADH causes reversal of the malate/aspartate shuttle, of which one component, the glutamate/aspartate carrier, thereby exports protons as required.

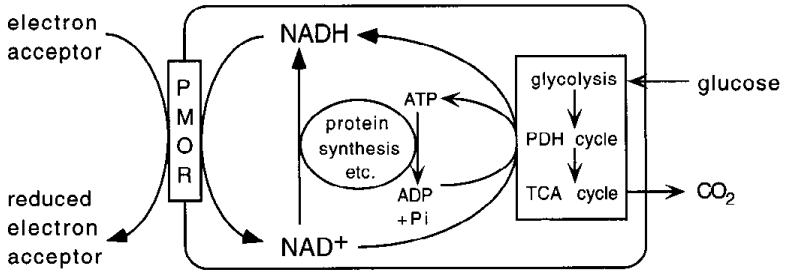

FIG. 2. Electron export allows redox stability without aerobic respiration.

capacity, because the number of anaerobic cells undoubtedly increases with age.[6–11]) It remains, therefore, to explore the caveat which concluded the previous section. How exactly does an anaerobic cell's PMOR manage to process so many electrons? This issue is pivotal to the question that we seek to address. If the PMOR is "hygienic" in disposing of electrons, generating no toxic extracellular products, then it becomes difficult to see how anaerobic cells can be blamed for systemic oxidative stress. In this section, it will be proposed that the PMOR is unhygienic and generates products that are toxic to other cells. Moreover, it will be argued that the scale of this toxicity may be sufficient to constitute the required degree of amplification so that the observed small number of anaerobic cells may indeed suffice to generate the observed levels of age-related oxidative stress.

An important nonenzymatic antioxidant in blood plasma is ascorbic acid (vitamin C). It mainly acts by donating an electron to a lipid radical (generally via tocopherol, vitamin E) so as to terminate a lipid peroxidation chain reaction; in doing so, it becomes ascorbate radical. Pairs of ascorbate radicals rapidly react, undergoing disproportionation, which gives one molecule of ascorbate and one of dehydroascorbate. The latter has no antioxidant capacity and must be turned back into ascorbate by the addition of two electrons. It has been proposed[32] that this last step is a physiological role of the PMOR. No other likely high-affinity acceptor for the electron-translocating activity has yet been identified in plasma.

Plasma carries high levels of one very undesirable potential electron acceptor: oxygen. It has been shown that the PMOR has low affinity for oxygen,[34,32] and indeed this is very necessary, because donation of a single electron to extracellular oxygen would generate superoxide. However, owing to its rapid generation of NADH, an anaerobic cell will be constantly bristling with electrons. If dehydroascorbate is indeed the only high affinity electron acceptor available, it seems inevitable that there will be insufficient flux of it around an anaerobic cell to satisfy its PMOR demand, so that lower-affinity acceptors will also be reduced. There may thus be a substantial rate of superoxide production in the plasma surrounding each anaerobic cell.

This is not directly problematic. One of our three variants of superoxide dismutase is specific to the extracellular medium; it will scavenge most superoxide generated in this way, particularly because it is known to be present at very high levels in the intimal space.[36] The hydrogen peroxide that is thereby produced will, similarly, be converted to water by extracellular glutathione peroxidase and/or catalase.

Some superoxide, however, will inevitably evade this system. Superoxide is a relatively unreactive radical and cannot autonomously initiate lipid peroxidation; but it has a high affinity for ferric iron (Fe^{3+}), which it reduces to ferrous (Fe^{2+}). Ferrous iron, in turn, participates in Fenton reactions: it can react either with hydrogen peroxide, creating the highly reactive hydroxyl radical, or else with lipid hydroperoxides creating a lipid alkoxyl radical.[37] This last reaction is particularly worthy of consideration because it effects the "branching" of lipid peroxidation chain reactions, which is the main reason why they propagate so rapidly.[37]

Because iron is an essential component of many enzymes, it must be provided to all cells after extraction from the diet. This is of course done via the blood stream. But such iron is maintained in the ferric state, almost certainly protected from reduction by superoxide, by its carrier protein transferrin,[38] except possibly during cellular uptake.[39] Another major iron-carrying plasma protein, ferritin, probably also has a low affinity for superoxide because of the protective effect of ceruloplasmin which also binds virtually all plasma copper.[40] A third major source of iron in plasma is haemoglobin, which is released into plasma by red cell lysis, especially at sites of inflammation; but it is both removed by haptoglobin and [according to a recent report[43]] detoxified by haemopexin whenever it assumes the more unstable ferric state, methaemoglobin. A fourth source, however, appears to have less such protection. It is haemin (or hemin).

Haemin is the nonprotein component of haemoglobin, composed of an iron atom in a porphyrin ring. Haemin becomes detached from methaemoglobin at a significant rate and is prone to desorb from its host red blood cell

becoming free in plasma. Once free, it is probably not a significant pro-oxidant, because it is assiduously bound by albumin and haemopexin, the latter of which transports it to the liver for destruction.[42] Recent work,[41] however, has firmly established that haemin which is still suspended in the red cell membrane also binds—albeit transiently—to LDL particles. Crucially, these studies took care to assess the binding affinities in physiologically realistic conditions. The authors concluded that haemin may be heavily involved in LDL oxidation *in vivo*. This is the reason why the Fenton reaction of ferrous iron with lipid hydroperoxides is so likely to be important: most of the lipid hydroperoxides present in plasma are bound to LDL.[44]

The question of exactly how and where LDL is oxidized, and in particular of why its rate of oxidation rises with age, is still open.[45] The implication of haemin just described[41] may be a crucial step forward, but the rate of desorption of haemin from red blood cells is unlikely to rise with age in view of their constant turnover, so another factor—one determining the rate at which haemin-bound iron is reduced to Fe^{2+}—seems to be implied. The PMOR of anaerobic cells seems well placed to be such a factor.

Summary

In this section, we have argued that the accumulation of anaerobic cells is a plausible explanation for the rise in LDL oxidation that occurs with age. Next, we consider what role oxidized LDL may have in aging.

CONSEQUENCES FOR LDL UPTAKE BY DIFFERENT CELL TYPES

Numerous studies of LDL oxidation have been undertaken over many years (see ref. 45 for a concise but comprehensive recent review). Their main motivation was to understand the etiology of atherosclerosis. As a result of these efforts, we now know that atherosclerotic lesions begin with the adherence to the artery wall of a particular class of white blood cell, monocytes, which then undergo transformation into macrophages. These macrophages express a receptor for LDL particles and therefore absorb them. This appears to be a defense mechanism against LDL oxidation, first because the initial adherence of monocytes to the artery wall is due to a change in the endothelial cells that is triggered by oxidised LDL,[46] and second because the LDL receptor expressed by most cells has an affinity dependent on the particle's degree of oxidation, such that highly oxidized particles are not imported, whereas macrophage affinity is just as high for oxidized than unoxidized LDL due to their expression of a different LDL receptor.[47] Macrophages [and also hepatic Kupffer cells[48]] therefore clear oxidized LDL from the blood. However, because macrophages continue to import LDL even when their cholesterol content is very high, their capacity to break down all that they import is eventually exceeded and they transform into "foam cells," forming the fatty streak that is the first visible sign of an emerging atherosclerotic lesion.

This causative role for LDL oxidation in atherogenesis is now accepted,[45] but there is reason to doubt its direct relevance to aging. Atherosclerosis is undoubtedly a major age-related disease involved in the etiology of both heart attack and stroke. However, its rates of onset and progression are highly dependent on diet and moreover are far more variable between individuals than are the rates of many other biomarkers of aging. One may wonder, therefore, whether the mechanism developed above can be held to underlie those other phenotypes of aging. The explanation concerns the *degree* of LDL oxidation.

It was just noted that the standard LDL receptor does not bind oxidized LDL. However, there is a threshold level of oxidation below which LDL is still readily imported by all cells. In a young individual, almost all LDL in plasma is far below this level of oxidation. If the average oxidation of LDL were to double, then the amount that exceeded the threshold for import would rise by a larger factor. But the remainder, which was still below the threshold, would nonetheless have an average oxidation level nearly twice the original (Fig. 3, curves a and b). Only when average oxidation levels reached a far higher—indeed, unphysiological—value (curve c) would the average

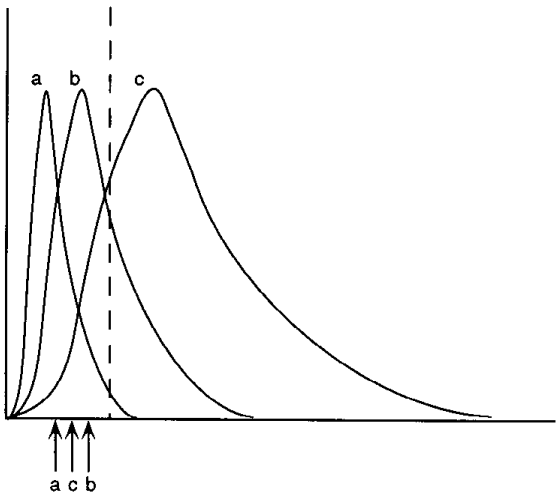

FIG. 3. Rising LDL oxidation only lowers import of oxidised material once average oxidation has become unphysiologically high. The arrows indicate the average degree of oxidation of that part of each distribution which lies to the left of the threshold (dotted line).

oxidation of subthreshold LDL begin to diminish. This means that the blood LDL level (which is set largely by the diet) does not affect the rate of import of oxidized LDL as it affects atherogenesis: the quantity of LDL imported by a given cell is set purely by its cholesterol requirements, so the average oxidation, not the quantity in transit at one time, determines the amount of oxidized material that is imported.

This means that rising LDL oxidation will, despite the efforts of arterial macrophages, translate into rising import of oxidized LDL particles. All major components of LDL particles—phospholipid, cholesteryl ester, protein, and cholesterol—are susceptible to oxidation by Fenton reaction products as well as to further molecular rearrangements and chain reactions.[45] This debris, once inside the cell, is prone to continue initiation of further chain reactions involving intracellular macromolecules.[37,50] Thus, a rise in the quantity of oxidized material imported may cause a disproportionate increase in the levels of intracellular oxidized material. In turn, as has been extensively noted, products of peroxidation are themselves pro-oxidant and amplify the problem still further. Finally, one must take into account the diversion of antioxidant defences from the destruction of pro-oxidants generated as side effects of normal intracellular processes, particularly OXPHOS. This can be—and surely is—compensated by upregulation of these defense systems but never to the extent of restoring pro-oxidant levels to those obtaining earlier, because the assault from these imported toxins continues to escalate (Fig. 4).

This completes the proposed mechanism whereby mtDNA mutations may have a major causative role in aging. Can it be held to provide the degree of amplification of toxicity whose necessity was noted in the Introduction? One can attempt to quantify this by considering the reported factor by which mtDNA deletions increase with age, which is between 1000− and 10000-fold.[6,10,11] Bearing this in mind, it seems reasonable to suggest that age-

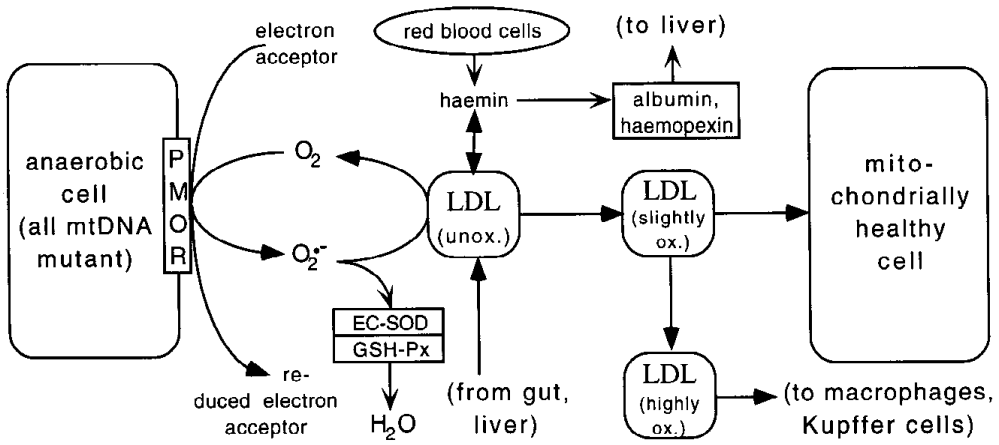

FIG. 4. Anaerobic cells cause import of oxidized lipoproteins by other cells.

related oxidative stress may be predominantly caused by the mechanism proposed here.

Alternative pathways

The pathway just proposed is quite complex and is unlikely to be unique. Some variations are now examined.

It is possible that some electron efflux from anaerobic cells is effected by a lactate/pyruvate couple rather than via the PMOR. However, there is evidence against an age-related increase of either lactate or pyruvate in plasma,[49] so this seems likely to play at most a minor role.

The next step is the transfer of electrons from the PMOR to oxygen forming superoxide. It is possible that electrons might move directly from the PMOR to haemin with no intermediate or, alternatively, via some other intermediate. The involvement of an intermediate seems likely, because it serves as a reservoir that can be filled and tapped asynchronously; it thereby allows haemin-bound iron to receive electrons when at some distance from the anaerobic cell. This permits a greater throughput of electrons than if physical adjacency of the two were necessary. Oxygen is identified above as the likely major intermediate because it is present in much greater amounts in plasma than any other plausible electron acceptor/donor. There have been suggestions that ascorbate itself can act as a pro-oxidant electron donor, but this has been clearly shown not to be the case in physiological conditions.[70]

The role of haemin as the supplier of iron is also probably not unique. Arguments were presented suggesting that transferrin and ferritin are less prone to be involved, but this should not be construed as proof that they are not involved at all. Free iron and copper are hardly present in plasma, due to the activity of ceruloplasmin,[40] but may likewise play a minor part.

Finally, LDL is not the only oxidizable substance imported by cells; they also import free fatty acids from the plasma. This indeed constitutes the major pathway of fatty acid import, far exceeding in volume the amounts of phospholipid or cholesteryl ester imported in LDL. But plasma contains a powerful enzymatic defense against the oxidation of free fatty acids: a selenium-dependent glutathione peroxidase which reverses the incorporation of molecular oxygen into fatty acids that accompanies lipid peroxidation chain reactions. This means that the levels of free [or albumin-bound, as most plasma fatty acid is[51]] lipid hydroperoxides in plasma are extremely low, thus protecting cells from importing them.[52] Glutathione peroxidase can probably also act on phospholipids at the surface of LDL particles,[52] but, crucially, most of the oxidizable material is deep inside the particle and inaccessible to it. Lipid peroxidation must begin at the surface of the particle, but it will then rapidly undergo chain reactions and other molecular rearrangements that spread the damage into the cholesteryl ester core.[37,45] The protein component of LDL is also prone to undergo oxidation,[45] and protein hydroperoxides thus formed can stimulate further free radical production after import into cells, just like lipid hydroperoxides.[50]

For these reasons, it seems quite probable that the particular pathway described earlier, via the PMOR, oxygen, haemin, and LDL, is the primary route transferring oxidative stress from anaerobic cells to aerobic ones.

HOW CAN THIS HYPOTHESIS BE TESTED?

The mechanism proposed here appears to be potentially testable at many different steps. However, the experiments do not appear necessarily straightforward. The various questions that one might seek to ask are now explored in turn.

Is the PMOR upregulated in anaerobic cells in vivo?

Preliminary histochemical assays for the PMOR exist.[53] Studies of muscle tissue may be able, therefore, to establish whether PMOR hyperactivity colocalizes with inactivity of cytochrome c oxidase (the most frequently studied of the partly mt-coded enzymes, abbreviated COX), as has already been shown to be the case for succinate dehydrogenase.[17,20,21]

Is superoxide high near anaerobic cells?

Likewise, there are well-developed assays for superoxide production;[72] but the rate of single-electron reduction of extracellular oxygen by ρ^0 cells has, to my knowledge, been established only under conditions where other electron acceptors are plentiful.[71] Alternatively, the extracellular superoxide dismutase is predominantly bound to endothelial cell surfaces rather than free in plasma,[54] so any preferential colocalization with COX inactivity in tissue may well be directly visualizable.

Is LDL preferentially oxidized near anaerobic cells?

This may likewise be possible to study *in vitro*. The oxidation of LDL by cultured cells is readily inhibited by physiological levels of vitamin E or other antioxidants.[55] Incubation of LDL with ρ^0 cell lines in a physiologically realistic medium should allow measurement of its rate of oxidation.

Is oxidized LDL a major contributor to intracellular oxidative stress?

There are numerous ways to quantify the level of oxidative stress in cells: these include the concentrations of hydrogen peroxide,[56] of lipid peroxidation products,[37] and of oxidatively damaged proteins.[57] The level of oxidation of LDL can also be quite accurately controlled *in vitro*. Thus, an approach to testing the influence of LDL oxidation on intracellular oxidative stress would be to incubate cells for an extended period in conditions where they were induced to import LDL at physiological rates and measure the dependence on LDL oxidation levels of one or more of the aforementioned indicators.

HOW CAN THE EFFECT ON AGING BE TESTED?

If this mechanism is correct, then the rate of advance of most phenotypes of aging would be dramatically retarded by any intervention that could stop electrons from being released into the blood. Two classes of therapy seem plausible enough to justify long-term research and development.

Restoration of OXPHOS

The first is to maintain and/or restore OXPHOS function of mitochondria that have been taken over by mutant mtDNA. This involves the construction and incorporation of the 13 proteins that are encoded in the mtDNA, all of which are essential for OXPHOS. Techniques for achieving this may be divided into two categories: restoring wild-type mtDNA to affected mitochondria [e.g., by skewing mtDNA replication[58] or by importing DNA bound to proteins[59]] or importing wild-type proteins encoded on nuclear transgenes and constructed in the cytosol.[60–62,22,27] Some recent reviews[63–65] have suggested that most mitochondrial pathologies—including age-related spontaneous mutations—can never be addressed by protein import, because it could not rescue defects in mitochondrial RNA genes. This is incorrect, because those genes' sole function is the construction of mitochondrially coded proteins. Conversely, the restoration of wild-type mtDNA faces a hurdle which has not been discussed but which may prove insuperable: the selective advantage of mutant mtDNA which drives its proliferation in the first place. The virtual absence of cells with reduced but non-zero activity for particular enzymes[16–19] reveals that this selection is very strong. It may, therefore, overpower any attempt to import or amplify functional DNA. We might find ways to reverse the selective advantage of mutant mtDNA, but if the mechanism for mutant mtDNA amplification suggested recently[22] is correct then that would be lethal. Protein import may thus hold the most promise for mitochondrial gene replacement. Development of such a protocol would begin by extending the *in vitro* manipulation of mitochondrially coded proteins, followed by evaluation in a transgenic mouse model; thus, it need not await the advances in gene therapy technology that are clearly a prerequisite for application to humans. Indeed, progress has already been made[66,67] in tackling what may be the main hurdle facing this approach, namely, the hydrophobicity of many mito-

chondrially encoded proteins which impedes their import.

Ablation of anaerobic cells

The second plausible approach entails the subversion of a later step in the mechanism. As was noted earlier, under 1% of muscle fibers have become anaerobic at death, even in the most severely affected tissues. Thus, according to the hypothesis outlined here, those anaerobic cells are doing us far more harm than good and we would benefit from simply eliminating them. Because they are so few, it is very unlikely that this elimination would have any deleterious side effects. The benefits would be felt throughout the body as a lower level of adventitious import of peroxidized lipoprotein. According to the hypothesis presented here, that would greatly reduce the oxidative stress in all cells, allowing them to regain the efficiency of macromolecular turnover and other functions that they enjoyed in their (which is to say, our) youth.

The development of such a treatment, therefore, faces only two major hurdles. The first is to construct a vector or vectors capable of inducing apoptosis in all the severely affected cell types: most importantly, muscle fibres and neurons. The second is to identify a targeting mechanism, whereby all (or almost all) anaerobic cells can be removed without simultaneously destroying large numbers of mitochondrially healthy cells.

Our understanding of the mechanism of apoptosis is increasing rapidly. For example, one apoptotic pathway involves *p53*, a gene that has been intensely studied for many years on account of its tumor-suppressing properties. Techniques for manipulating its expression are well advanced, so it holds promise as a tool for targeted apoptosis. The pathway by which it triggers apoptosis has become clearer in the light of recent work.[68,69]

The achievement of acceptable accuracy of targeting may also be relatively simple. Cells that are not respiring aerobically will have, according to this hypothesis, a very high level of activity of the PMOR system. It should be possible to detect this and use it as a signal for the induction of apoptosis in that cell. Furthermore, a reagent is already known—pCMBS, p-chloromercuriphenylsulfonic acid—that selectively kills ρ^0 cells *in vitro* by inhibition of the PMOR.[33] Care is needed in calibrating the detection system to the appropriate level of PMOR activity because it appears that all cells have a residual PMOR, perhaps normally used only to maintain extracellular ascorbate in the reduced, antioxidant state,[32] whose level of expression may vary considerably in response to transient extracellular conditions.

As with mtDNA transfer, this treatment can first be developed *in vitro* and evaluated using mice.

CONCLUSION

I have presented a detailed and testable hypothesis that may reconcile the very low levels of age-related mtDNA decline with the extensive evidence implicating OXPHOS-related damage as a major determinant of the rate of aging. The mechanism proposed here seems potentially amenable to a wide variety of tests and also to intervention that may significantly retard many phenotypes of aging.

The ability of completely OXPHOS-deficient cells to survive in the body is a remarkable phenomenon in itself, which has received only scant attention despite being known for some time. The question that is poses—what deleterious effects, if any, such cells may have on their aerobic neighbors—has, to my knowledge, never been addressed. It is hoped that those with the appropriate expertise and resources will hereafter find this question worthy of exploration.

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