Aging, proteotoxicity, mitochondria, glycation, NAD+ and carnosine: possible inter-relationships and resolution of the oxygen paradox

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It is suggested that NAD+ availability strongly affects cellular aging and organism lifespan: low NAD+ availability increases intracellular levels of glycolytic triose phosphates (glyceraldehyde-3-phosphate and dihydroxyacetone-phosphate) which, if not further metabolized, decompose spontaneously into methylglyoxal (MG), a glycating agent and source of protein and mitochondrial dysfunction and reactive oxygen species (ROS). MG-damaged proteins and other aberrant polypeptides can induce ROS generation, promote mitochondrial dysfunction and inhibit proteasomal activity. Upregulation of mitogenesis and mitochondrial activity by increased aerobic exercise, or dietary manipulation, helps to maintain NAD+ availability and thereby decreases MG-induced proteotoxicity. These proposals can explain the apparent paradox whereby aging is seemingly caused by increased ROS-mediated macromolecular damage but is ameliorated by increased aerobic activity. It is also suggested that increasing mitochondrial activity decreases ROS generation, while excess numbers of inactive mitochondria are deleterious due to increased ROS generation. The muscle- and brain-associated dipeptide, carnosine, is an intracellular buffer which can delay senescence in cultured human fibroblasts and delay aging in senescence-accelerated mice. Carnosine's ability to react with MG and possibly other deleterious carbonyl compounds, and scavenge various ROS, may account for its protective ability towards ischemia and ageing.

Keywords: ageing, methylglyoxal, ROS, exercise, proteolysis, mitogenesis, glycolysis

INCREASED MITOCHONDRIAL DYSFUNCTION AND ALTERED PROTEIN ACCUMULATION – ASSOCIATION WITH AGING

Two strong correlates of aging are mitochondrial dysfunction (Sastre et al., 2003; Sedensky and Morgan, 2006; Figueiredo et al., 2008; Wei et al., 2009) and accumulation of altered proteins (Hipkiss, 2006a; Soskic et al., 2007; Lindner and Demarez, 2009). It is possible that both these apparent effects of aging may also be causal to aging and much age-related pathology. Although many experiments have shown that mitochondrial dysfunction frequently accompanies aging, whether aging causes mitochondrial dysfunction, or whether mitochondrial dysfunction causes aging, is debated. For example, while aging is accompanied by the accumulation of altered proteins, possibly due to a decreased ability of the intracellular proteases (lysosomal and proteasomal) to degrade aberrant polypeptide chains (Carrard et al., 2002; Bergamini et al., 2007; Chondrogianni and Gonos, 2007; Hanson et al., 2008; Kurz et al., 2008a; Yun et al., 2008), it has also been shown that altered proteins can bind to mitochondria and compromise their function (Hansson Petersen et al., 2008; Devi and Anandatheerthavarada, 2009; Rhein et al., 2009; Sun et al., 2009). Furthermore there is strong evidence for the proposal that decreased autophagy and lysosomal activity compromises elimination of dysfunctional mitochondria (Brunk and Terman, 2002; Terman et al., 2003; Kim et al., 2007; Kurz et al., 2008b), thereby promoting their accumulation. Conversely, studies in nematode *Caenorhabditis elegans* (Hanson et al., 2008; Toth et al., 2008), *Drosophila* (Simonsen et al., 2008) and yeast (Alvers et al., 2009) have shown that lifespan in these organisms was extended when autophagy was upregulated, which could improve cellular ability to eliminate dysfunctional mitochondria (i.e. stimulate mitophagy). Alternatively/additionally, mitochondrial dysfunction increases generation of oxygen free-radicals and related reactive oxygen species (collectively termed ROS) which in turn damage proteins to such an extent that cross-linked polypeptides are generated which not only resist proteolytic attack, but also inhibit proteasomal activity, thereby establishing a deleterious cycle which would be likely to eventually result in the cell's demise. However, when lifespan was extended by calorie restriction, proteolysis (autophagic and proteasomal) was found to be stimulated, which again could help to eliminate dysfunctional organelles and altered proteins (Cuervo, 2008). It is also possible that excessive autophagy may be deleterious as increased autophagic activity accompanies aging in progeroid mice (Marino and Lopez-Otin, 2008). Additionally that the β-amyloid peptide (1–42), commonly associated with Alzheimer's disease, can bind to mitochondrial complex-1 NADH dehydrogenase (Munguia et al., 2005), while deficiency in fatty acid beta-oxidation pathway may also be associated with neurodegeneration, possibly due to binding of β-amyloid peptide to hydroxyacyl-CoA dehydrogenase (Oppermann et al., 1999), further illustrate the possible relationships between altered proteins, mitochondrial dysfunction and age-related pathology.

AGING, NAD+ AND METHYLGLYOXAL

Whatever the cause of mitochondrial dysfunction, one consequence of lowered mitochondrial activity will be decreased mitochondrially-mediated regeneration of NAD+ from NADH. Oxidation of the glycolytic intermediate glyceraldehyde-3-phosphate (G3P) by glyceraldehyde-3-phosphate dehydrogenase requires NAD+ (which is converted to NADH). As a continuous supply of NAD+ is required for triose phosphate metabolism to procede, both G3P and dihydroxyacetone-phosphate (DHAP) will accumulate should NAD+ availability be restricted. Accumulation of these trioses is deleterious because both can spontaneously decompose into methylglyoxal (MG) (Turk, 2009), a highly toxic glycating agent which readily damages proteins by reacting with amino and guanidino groups of lysine and arginine residues, respectively (Rabbani and Thornalley, 2008a). Indeed, a number of experiments have demonstrated the damaging effects of MG on mitochondrial activity (Johans et al., 2005; SinhaRoy et al., 2005; Rabbani and Thornalley, 2008b; Wang et al., 2009), including increased ROS formation in mitochondria as well as in the cytoplasm (Desai and Wu, 2008). Increased MG formation is also a likely explanation for many molecular events accompanying cell senescence (Sejersen and Rattan, 2009), ischemia, hyperglycemia and associated pathology (Nicolay et al., 2006; Rabbani and Thornalley, 2008a). Significantly, experiments in *C. elegans* have shown that increased expression of the MG detoxification enzyme, glyoxalase-1, increases organism lifespan, most likely by lowering MG levels and thereby decreasing MG-mediated protein damage and mitochondrial dysfunction (Morcos et al., 2008; Schlotterer et al., 2009). Furthermore, increasing dietary intake of MG-glycated protein can eliminate the beneficial effects on mouse lifespan induced by dietary restriction (Cai et al., 2007). As every-other-day feeding without any overall reduction in calorie intake can also increase organism lifespan (Masternak et al., 2005; Mattson and Wan, 2005; Martin et al., 2006), it is possible that periods of fasting, during which MG formation from glycolytic trioses will be suppressed, may provide an explanation for the observed beneficial effects of dietary restriction on organism lifespan (Hipkiss, 2006b, 2008). Collectively, these observations suggest that factors which regulate either MG formation or its elimination can exert critical roles in aging (Hipkiss, 2009a).

MECHANISMS CONTROLLING MG-MEDIATED MACROMOLECULAR DAMAGE

As noted above, a major influence on intracellular MG formation from the glycolytic trioses G3P and DHAP is the availability of NAD+. Mitochondria provide a major aerobic route for the regeneration of NAD+ from NADH which occurs via the operation of the oxaloacetate-malate shuttle and the glycerol-phosphate cycle. An anaerobic mechanism whereby NAD+ is regenerated from NADH is carried out in yeast by the reduction of acetaldehyde to ethyl alcohol, whereas in animal tissues, due to the absence of the enzyme pyruvate decarboxylase, the conversion of pyruvic acid to lactic acid is the analogous reaction. The latter process, however, imposes the additional metabolic stress of acidosis by increasing the number of hydrogen ions in the cytoplasm. In tissues where this process is particularly important, such as fast-twitch glycolytic muscle, additional buffering capacity is provided by the dipeptide carnosine (β-alanyl-L-histidine) and related imidazole dipeptides

(Abe, 2000). Interestingly, it appears that carnosine, which is also found in brain, especially the olfactory lobe, can react with MG and other metabolic aldehydes and may be generally protective against aldehyde-mediated macromolecular damage (Hipkiss, 2009b,c). Other experiments have demonstrated that carnosine can suppress the deleterious effects of ischemia in brain (Dobrota et al., 2005), liver (Fouad et al., 2007) and kidney (Fujii et al., 2005), possibly mediated by the dipeptide's reaction with MG (Hipkiss and Chana, 1998; Aldini et al., 2005) generated as a consequence of hypoxia-induced failure to regenerate NAD+ from NADH. These anti-ischemic effects of the dipeptide are consistent with the proposal that, by reacting directly with MG, carnosine suppresses dicarbonyl toxicity. That ischemia-related ROS formation and consequent proteotoxicity are prevented by upregulation of glyoxalase-1 in *C. elegans* (Morcos et al., 2008; Schlotterer et al., 2009), reinforces the idea that raised MG levels are significant in ischemia. The observation that defects in triose phosphate isomerase activity, which promotes DHAP accumulation, induces many of the deleterious effects associated with hyperglycemia and aging (Orosz et al., 2009), further supports the proposal that MG plays a causal role in much age-related proteotoxicity (Hipkiss, 2008, 2009a). Significantly, experiments have shown that carnosine delays aging in cultured human fibroblasts (McFarland and Holliday, 1994) and senescence-accelerated mice (Yuneva et al., 1999, 2002). The fact that carnosine has also been reported to (i) possess anti-oxidant activity (Kohen et al., 1988; Bogardus and Boissonneault, 2000; Calabrese et al., 2005), (ii) induce stress-protein expression (when complexed with zinc ions) (Odashima et al., 2006; Ohkawara et al., 2006; Wada et al., 2006) and (iii) decrease telomere shortening (Shao et al., 2004), reinforces the idea that this essentially non-toxic dipeptide is supportive of longevity (Hipkiss, 2009b,c).

NAD+ AND PROTEOTOXICITY

The observations outlined above reinforce the likely importance, to aging and related disorders, of MG, whose generation is affected by NAD+ availability. Additionally, NAD+ is important for stress-protein synthesis (Westerheide et al., 2009), autophagic activity (Lee et al., 2008; Salminen and Kaamiranta, 2009), sirtuin-mediated protein deacetylation (Bordone and Guarente, 2005; Rodgers et al., 2008) and increased mitogenesis (Bonawitz et al., 2007; Cunningham et al., 2007), all of which impact upon the processes influencing proteostasis and aging. Thus the beneficial effects of this virtuous cycle could help suppress the onset of cellular aging by decreasing the potential for MG generation, as well as improving protein quality control. It follows that putative pluripotent protective agents such as carnosine could also help suppress the deleterious effects of age-related disorders such as Alzheimer's disease (Hipkiss, 2007) and type-2 diabetes (Hipkiss, 1998, 2009d), both of which seem to possess a number of overlapping phenotypic characteristics, including increased protein glycoxidation (Maher and Schubert, 2009).

IF ROS ARE CAUSAL TO AGING, WHY IS AGING SUPPRESSED BY INCREASED AEROBIC ACTIVITY? A PARADOX RESOLVED?

Despite the detection of increased levels of oxidatively-damaged macromolecules in aging cells and tissues, it is surely paradoxical that aging and much related pathology can be delayed by

increased mitochondrial activity and aerobic exercise. It is possible to resolve this paradox however. It is suggested that mitochondria, by ensuring NAD+ regeneration from NADH, suppress MG formation (and hence its deleterious effects) and thereby partly explains the beneficial effects of increased aerobic activity towards much age-related dysfunction. Lowered MG generation may also result from the increased synthesis of nucleic acid precursors. This would divert glucose metabolism via the pentose phosphate pathway, which would not only increase glutathione synthesis via increased NADPH generation, but also decrease synthesis of MG precursors, triose phosphates. Furthermore the upregulation in synthesis of mitochondrial proteins may also be accompanied by increased synthesis of the necessary proteases and chaperone proteins to help maintain protein quality (Hipkiss, 2009e). However, it should be pointed out that ROS may also provide a necessary signal for increased mitogenesis in exercised muscle.

It has been shown that limb immobilisation promotes an increase in mitochondrial ROS generation (Pesce et al., 2002; Miller et al., 2007; Bar-Shai et al., 2008), observations consistent with the suggestion that inactive mitochondria generate more ROS than those actively respiring (Van Voorhies, 2004; Barja, 2007; Burhans and Weinberger, 2007; Brooks et al., 2008; Holloszy, 2008; Hood, 2009). Experiments have demonstrated that uncoupling electron transport from ATP synthesis suppresses aging and can extend lifespan (Speakman et al., 2004; Wolkow and Iser, 2006; Hood, 2009); this would increase NAD+ generation from NADH (Liu et al., 2008) and thereby decrease the potential for MG synthesis. Resveratrol and rapamycin, both of which have been shown to exert anti-aging effects, promote mitochondrial activity and mitogenesis, whilst also seeming to suppress glycolysis (Knutson and Leeuwenburgh, 2008; Orallo, 2008; Cox and Mattison, 2009; Harrison et al., 2009) thereby decreasing the potential for MG synthesis. There is also considerable evidence showing that acetyl-L-carnitine, which facilitates entry of fatty acid acyl-units into mitochondria thereby increasing mitochondrial activity, has anti-aging effects and decreases ROS generation (Mollica et al., 2001; Liu et al., 2002; Virmani et al., 2002; Poon et al., 2006). It should also be noted, however, that it is still uncertain whether all age-related increases in ROS formation is intramitochondrial in origin; ROS can be generated outside mitochondria e.g. by MG, glycated proteins and cytoplasmic NAD(P)H oxidase and xanthine oxidase activities.

ARE TOO MANY MITOCHONDRIA DELETERIOUS?

The above proposal suggests that increased mitochondria activity, and in particular mitogenesis, may be considered as anti-aging processes. It is also possible that the presence of excess inactive mitochondria (i.e. those not carrying out electron transport) are actually deleterious. If oxygenated mitochondria are supplied with insufficient electrons, for example when ATP demand is low, this will increase the potential for creation of incompletely reduced oxygen atoms i.e. superoxide O_2^-, an oxygen free-radical. As mentioned above, limb immobilization results in increased ROS generation (Pesce et al., 2002; Miller et al., 2007; Bar-Shai et al., 2008) which may be an example of such a phenomenon. Furthermore increased mitochondrial degradation, which occurs in response to decreased muscle activity in animal and human studies (Hood, 2009), could be a metabolic response to the presence of excess inactive

mitochondria, perhaps stimulated by increased ROS generation in inactive organelles. Indeed ROS-mediated protein modification increases proteolytic susceptibility, both intramitochondrially and in the cytosol, creates a precedent for ROS-induced damage increasing the potential for catabolic attack. These observations can be interpreted as suggesting that excess inactive mitochondria are indeed deleterious.

Regulation of mitochondrial numbers is undoubtably complex, as illustrated in a recent study in *C. elegans* which revealed that the protein prohibitin is important in this process (Artal-Sanz and Tavernarakis, 2009). Significantly, the results showed that the beneficial effect of increased mitogenesis (induced by prohibitin deficiency) upon longevity was strongly influenced by organism metabolism. It was found that raised mitochondrial synthesis promoted an increase in organism lifespan at 25 degrees, and at 35 degrees the organisms were also strongly thermotolerant. While at a lower temperature (20 degrees) decreased longevity was observed in prohibitin-deficient nematodes. These complex and seemingly paradoxical findings can be successfully explained if one assumes that inactive mitochondria produce more ROS than mitochondria actively generating ATP (as proposed above). Hence it can be suggested that many mitochondria are inactive at the lower temperature due to low energy demands and consequently ROS formation is increased and longevity compromised. Whereas, the increased ATP demand at the higher temperature results in increased mitochondrial activity, which, correspondingly, decreases ROS generation and increases lifespan. Additionally, however, temperature-dependent differences in oxygen solubility could influence ROS formation and thereby affect the organism longevity; as oxygen solubility is inversely related to temperature, raising the temperature could decrease mitochondrial oxygen supply and which could decrease the amounts of ROS generated.

CONCLUSION

It is suggested that maintenance of mitochondrial activity helps to suppress aging by ensuring NAD+ availability which decreases MG generation from glycolytic trioses and subsequent formation of toxic MG-modified (glycated) proteins. Furthermore, increased mitogenesis will divert glucose metabolites through the pentose phosphate cycle for ribose and deoxyribose synthesis, which additionally will increase glutathione synthesis, via increased NADPH formation. It is likely that lowering the potential for MG formation will be additionally beneficial by helping to maintain proteostasis and delaying proteostatic collapse, because highly glycated (cross-linked) proteins can compromise protein quality control by inhibiting proteasome activity. Furthermore it is possible that continuous mitogenesis and growth in the young suppress onset of aging phenomena, even when the organism possesses a genetic predisposition for age-related pathology. However, once growth ceases, the potential for MG formation increases, with the inevitable finitude of life pressaged by proteostatic collapse in which altered proteins damage mitochondria, induce ROS formation which then damage more proteins, eventually promoting apoptosis. This would suggest that any process (e.g. increased ROS generation, increased protein glycation, decreased altered protein elimination, increased synthesis of erroneous proteins etc.) that causes intracellular accumulation of altered proteins could provide

the initiating causal event which promotes aging onset. Whilst increased mitogenesis may be beneficial in some circumstances, it is also suggested that excess numbers of inactive mitochondria could be an additional source of ROS and, by overwhelming cytosolic anti-oxidant activity, have adverse effects on stress resistance, cellular viability and organism longevity.

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