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REVIEW

Ageing, oxidative stress, and mitochondrial uncoupling

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Abstract

Mitochondria are a cell's single greatest source of reactive oxygen species. Reactive oxygen species are important for many life sustaining processes of cells and tissues, but they can also induce cell damage and death. If their production and levels within cells is not effectively controlled, then the detrimental effects of oxidative stress can accumulate. Oxidative stress is widely thought to underpin many ageing processes, and the oxidative stress theory of ageing is one of the most widely acknowledged theories of ageing. As well as being the major source of reactive oxygen species, mitochondria are also a major site of oxidative damage. The purpose of this review is a concise and current review of the effects of oxidative stress and ageing on mitochondrial function. Emphasis is placed upon the roles of mitochondrial proton leak, the uncoupling proteins, and the anti-ageing effects of caloric restriction.

Keywords mitochondria, oxidative phosphorylation, proton leak, reactive oxygen species, superoxide, uncoupling protein.

The processes of ageing have intrigued life scientists and citizens alike for centuries, if not millennia. The mechanisms underlying these processes are as yet poorly understood, and it is safe to say that they are multifactorial and complex. One widely subscribed theory that encompasses many of the effects of ageing is referred to as the oxidative stress theory. The theory holds that the progressive declines in physiological function are the result of the accumulation of oxidative damage caused by reactive oxygen species (ROS) (Harman 1956, Sohal & Weindruch 1996).

At the hub of cellular events involving oxidative stress is the mitochondrion. As such, it has been the focus of much research. Indeed, an important correlate of the oxidative stress theory is the mitochondrial theory of ageing, which holds that oxidative stress within mitochondria can lead to a vicious cycle in which damaged mitochondria produce progressively increased amounts of ROS, leading, in turn, to progressive augmentations in damage (Harman 1972, Pak *et al.* 2003). The oxidative stress theory however does not limit the source of the detrimental metabolites to the mitochondrion, or even to oxygen-derived metabolites; moreover its scope extends beyond oxidant damage to include roles for oxidants in signalling and other normal processes (Shigenaga *et al.* 1994, Sohal & Weindruch 1996, Yu 1996, Droge 2002). The overall aim of this paper is to provide a concise and current review of the effects of oxidative stress and ageing on mitochondrial function.

Mitochondria – the major sites of cellular ROS production

It is estimated that approximately 0.2–2% of the oxygen taken up by cells is converted by mitochondria to ROS, mainly through the production of superoxide anion (Chance *et al.* 1979, Hansford *et al.* 1997). Mitochondria consume 85–90% of a cell's oxygen to support oxidative phosphorylation, the major system in cells that harnesses energy from the oxidation of fuels for adenosine triphosphate (ATP) synthesis. The latter

system links the oxidation of fuel substrates in the cytoplasm and in the mitochondrial matrix to the activity of ATP synthase. Fuel oxidation generates reducing equivalents, which funnel into the mitochondrial electron transport chain, which, in turn, pumps protons into the mitochondrial intermembrane space. The resulting protonmotive force is used directly to drive the activity of ATP synthase. Thus the energy from oxidized fuels, such as fatty acids and glucose, is converted to the 'universal energy currency of cells', ATP. Oxidative phosphorylation, however, comes with an additional cost, the production of potentially harmful ROS.

Before outlining sites of mitochondrial ROS production, it should be acknowledged that ROS are also produced to a lesser extent outside of the mitochondrion. Of the oxygen consumed by cells, about 10–15% is used to support non-mitochondrial reactions. Oxygen consumption stems from various oxidases, oxygenases and from direct, non-enzymatic, reactions. The production of ROS concurrent with this activity is thought to be lower than in mitochondria, as cytosolic oxygen-consuming enzymes, in general, have lower oxygen binding efficiencies than mitochondrial cytochrome oxidase (Gutteridge & Halliwell 1994). Examples of extra-mitochondrial ROS-producing reactions include xanthine oxidase, D-amino oxidase, the P450 cytochromes, and the proline and lysine hydroxylases.

Within mitochondria, it is the electron transport chain that is the main source of ROS. The sites of ROS production along the chain have been the subject of many studies. Recent findings show that the two major sites of superoxide production are at complex I and complex III. At complex I (NADH coenzyme Q reductase) the sites are thought to be the iron–sulphur centres (Herrero & Barja 1997, Genova *et al.* 2001) or the 'active site flavin' (Liu *et al.* 2002). Based on inhibitor studies in heart mitochondria, the site of superoxide production at complex III (bc_1 complex) is likely the ubisemiquinone at complex o (Turrens *et al.* 1985). It was later proposed that the site of superoxide production was cytochrome *b* rather than ubisemiquinone (Nohl & Stolze 1992). However, numerous studies have demonstrated that the double inhibition of complex III by inhibitors antimycin A (acts at site Qi, and blocks electron flow from cytochrome b_{560} to ubiquinone or ubisemiquinone) and myxothiazol (acts at site Qo, inhibiting electron flow from ubiquinol to Rieske FeS centre) results in consistent decreases in superoxide production (reviewed in Barja 1999). This strongly supports the idea that unstable ubisemiquinone molecules, and not the b cytochromes, are the major sites of superoxide production at complex III.

The production of superoxide at complex I is widely thought to occur at the matrix side of the inner membrane since the centres are thought to be at this side of the membrane. At complex III, it has been thought that superoxide is also released at the matrix side of the membrane (Turrens 1997). However the X-ray crystal structure of complex III indicates that centre 'o' faces the intermembrane space (Iwata *et al.* 1998, Zhang *et al.* 1998) which would suggest that superoxide is released into the intermembrane space. Moreover, Han *et al.* (2001) demonstrated superoxide production by liver mitoplasts in the presence of antimycin A, supporting the idea that superoxide is released into the intermembrane space. Corroborative results were generated by St-Pierre *et al.* (2002) in a recent study of the topology of mitochondrial ROS production by rat heart and skeletal muscle mitochondria. The significant increase in hydrogen peroxide production with the addition of superoxide dismutase to the antimycin A-treated mitochondria was interpreted as additional evidence that superoxide may be released into the intermembrane space. Cadenas and colleagues have shown that superoxide produced by complex III is released to both the matrix and cytosolic sides of the mitochondria with approximately 80% going to the matrix and 20% going to the intermembrane space (Han *et al.* 2003, Cadenas 2004). The relative contributions of complexes I and III to ROS production appear to be dependent on types of tissues, species and experimental conditions (Barja 1999).

The rate of ROS production is affected substantially by mitochondrial metabolic state. Under state 4 conditions, when oxygen consumption is low and protonmotive force is high, complexes of the electron transport chain are in reduced states. Under these metabolic conditions superoxide production is highest. A common misconception is that the production of ROS is highest when the rate of oxygen consumption is highest, with the notion that superoxide production is a fixed proportion of oxygen consumption. The major factor underlying the protonmotive force-dependent production of superoxide is the occupancy of the outer Q-site of complex III with the semiquinone anion (Nicholls 2002). This is the case when ATP demand is low and protonmotive force is high. Moreover, it has been known for more than 30 years that upon the addition of ADP, and the resulting transition to state 3 respiration, superoxide production diminishes dramatically (Loschen *et al.* 1971, Boveris *et al.* 1972, Boveris & Chance 1973). Thus, because the proportion of oxygen consumed that emerges as ROS is highest under non-phosphorylating, rather than phosphorylating conditions, most determinations of mitochondrial ROS production are made under state 4 conditions.

In an important series of studies in heart and non-synaptic brain mitochondria of five animal species, Barja and colleagues have demonstrated that complex I

continues to produce superoxide during state 3 (reviewed in Barja 1999). While the production of ROS during state 3 is much lower than in state 4 respiration, complex I could be the only producer of ROS when mitochondria are synthesizing ATP at high rates. This, combined with the fact that in intact cells, mitochondria normally respire in metabolic states that are intermediate between states 3 and 4, implies that complex I generation of ROS may be quantitatively important *in vivo*. Clearly more studies of ROS generation in intact cells are needed to elucidate the sites and rates of ROS production.

Many and varied mechanisms of ROS protection

Damage from ROS is normally minimized through a wide array of protective mechanisms. In mitochondria, superoxide anion is converted by manganese superoxide dismutase (MnSOD, SOD2) to hydrogen peroxide, which is subsequently converted to water. The copper- and zinc-containing superoxide dismutase protects against the superoxide that is produced by cytosolic oxidases and cytochrome P450 enzymes. Detrimental effects of hydrogen peroxide in mitochondria and the cytosol are prevented through glutathione peroxidase. The latter removes hydrogen peroxide by using it to oxidize reduced glutathione (GSH) to produce oxidized glutathione (GSSG). Glutathione reductase then converts the GSSG back to GSH. Additional protection is provided by catalase, located largely in peroxisomes, which removes hydrogen peroxide by converting it to water and oxygen.

A possible role for the uncoupling proteins?

While the actual mechanisms of action have yet to be elucidated, an emerging literature strongly supports the idea that mitochondrial uncoupling proteins play a role in the protection from ROS damage (Li *et al.* 2000, Vidal Puig *et al.* 2000, Echtay *et al.* 2002, Brand *et al.* 2004). It is unclear whether or not the novel uncoupling proteins (i.e. UCP2 and UCP3) cause any inward proton translocation, like that described for the original UCP found almost exclusively in brown adipose tissue, UCP1 (Nedergaard & Cannon 2003). Brand and colleagues have proposed that superoxide and lipid peroxides can activate uncoupling protein-mediated proton leak to protect against ROS damage (Echtay *et al.* 2002, Brand *et al.* 2004). However, the absence of UCPs in liver parenchymal cells where ROS production is high, and where proton leak is substantial leads to questions of possible alternative mechanisms. As described below in our recent study (Bevilacqua *et al.* 2004) we have shown that hydrogen peroxide production and leak are decreased in situations where there is increased UCP3 protein.

It has been proposed that UCP3, which is expressed almost exclusively in skeletal muscle and brown adipose tissue, transports fatty acid anions out of the mitochondrial matrix to liberate matrix CoASH, and thereby facilitate high rates of fatty acid oxidation (Himms-Hagen & Harper 2001). Another hypothesis suggests that UCP3 translocates fatty acids from the inner to the outer leaflet of the mitochondrial inner membrane (Schrauwen *et al.* 2001). Results from UCP3 knockout mice and transgenic mice support the idea that UCP3 plays important roles in facilitating fatty acid oxidation, as measured by indirect calorimetry, and muscle enzyme activities (e.g., CPT1 activity; Bezaire *et al.* 2001, V. Bezaire *et al.*, unpublished data). Overall, the increased expression of muscle UCP3 mRNA and protein with caloric restriction (CR) (Lee *et al.* 2002, Bevilacqua *et al.* 2004), is consistent with both an increased reliance on fatty acid metabolism and decreased damage from ROS during CR (see below). Regardless of the molecular mechanism of UCP3 (e.g. proton leak vs. fatty acid anion efflux) it seems clear that the UCPs play some role in protection from ROS.

Finally, there are also protective mechanisms within membranes of cells. There, the protective mechanisms can be grouped into three categories, including free radical scavenging, lipid repair and lipid replacement mechanisms. Despite this extensive array of protective mechanisms, oxidative damage accrues with age, presumably due to slight imbalances between ROS production and protective mechanisms.

Oxidative stress and ageing

As described in the introduction, the term, oxidative stress, refers not only to oxidative damage but also to influences of the oxidative stress on signalling, transcriptional control and other normal processes within cells; the term has also encompassed the effects of oxidants such as reactive nitrogen species (RNS). Oxidative stress impacts upon not only mitochondria, but also upon extra-mitochondrial structures, where effects are observed in lipids, proteins and DNA. Important roles for ROS and RNS in cellular signalling pathways are being increasingly acknowledged (e.g. Landar & Darley-Usmar 2003). Extensive reviews have been written on the subject of oxidative stress and ageing (e.g. Sohal & Weindruch 1996, Beckman & Ames 1998). Here, the emphasis is placed on the effects of oxidative stress on mitochondria, and specifically upon mitochondrial uncoupling.

Mitochondrial membrane lipids are highly susceptible to oxidative damage, especially the long chain polyunsaturated fatty acid components. ROS can lead

directly to the peroxidation of lipids, and the production of highly reactive aldehyde species, such as 4-hydroxy-2,3, trans-nonenal (4HNE), which can then result in secondary detrimental effects (Chen & Yu 1994). Because of the high degree of unsaturation in the fatty acids of cardiolipin, this mitochondrial specific phospholipid is particularly susceptible to oxidative damage (Laganiere & Yu 1993). The content of cardiolipin in the inner membrane is important for mitochondrial energetics, affecting the activities of the adenine nucleotide transporter and cytochrome c oxidase (Hoch 1992, Paradies *et al.* 1998). Thus it is not surprising that oxidative stress and ageing are associated with decreased activities of inner membrane proteins.

Inner membrane proteins are themselves directly susceptible to effects of oxidative stress. Damage to the inner membrane proteins and/or lipids can result in membrane depolarization and impaired mitochondrial function (see: Effects of Ageing on Mitochondrial Energetics section). Mechanisms of damage and/or cell signalling can be direct, for example through the effects of superoxide, or can be secondary through the effects of lipid peroxides, such as 4HNE. *In vitro* studies have also shown that components of the oxidative phosphorylation system, including adenine nucleotide transporter and ATP synthase, are highly sensitive to oxidative stress (Lippe *et al.* 1991, Forsmark-Andree *et al.* 1997). Ageing-induced increases in oxidative damage to mitochondrial proteins have been demonstrated in mitochondria from housefly flight muscles (Sohal & Dubey 1994). The adenine nucleotide transporter is particularly susceptible (Yan & Sohal 1998), as is the matrix enzyme, aconitase (Yan *et al.* 1997). In both of the latter studies, the degree of damage (measured as protein carbonyls) was correlated with the loss of protein function. The collective results demonstrate that mitochondrial protein damage, rather than being a stochastic phenomenon, is a process that selectively affects some proteins over others.

In comparison with nuclear DNA, mitochondrial DNA (mtDNA) is thought to be more sensitive to oxidative stress. This is thought to be because of the fact that mtDNA, unlike nuclear DNA, is not protected by histone proteins. While earlier studies comparing the relative damage between mitochondrial and nuclear DNA have been confounded by artefactual damage to mtDNA that can occur as a result of some mitochondrial isolation methods, more recent studies have confirmed that mtDNA is indeed more susceptible to ageing-related oxidative damage than nuclear DNA (de la Asuncion *et al.* 1996, Barja & Herrero 2000). As discussed by Van Remmen & Richardson (2001), damage to mitochondrial DNA could also have greater implications than damage to nuclear DNA since the whole mitochondrial genome codes for genes that are expressed while nuclear DNA contains a large number of sequences that are not transcribed. Finally, a common misconception regarding mitochondrial DNA repair should be discussed. For quite some time it was thought that mitochondria were unable to repair damage to their DNA. Contrarily, the recent findings of Bohr and colleagues have shown that mitochondria do have effective base-excision repair pathways (Croteau & Bohr 1997, Bohr 2002). They have also demonstrated that mitochondrial mechanisms of repair may even increase with ageing, while nuclear base excision repair (BER) pathways decrease with ageing (reviewed in Bohr 2002). In an interesting related study, these same authors have shown that CR is not associated with an up-regulation of mitochondrial BER, while it is associated with increases in nuclear BER (Stuart *et al.* 2004). The latter findings demonstrate therefore that the mitigating effects of CR on ageing are not due to improved mtDNA repair mechanisms. The effects of CR on ageing processes are discussed further below.

Ageing-induced changes to gene expression profiles: implications for mitochondrial metabolism

The effects of ageing upon gene expression patterns have been examined in recent studies employing complementary DNA (cDNA) microarray technologies to assess the effects of ageing in post-mitotic tissues of mice. These studies also examined the effects of CR, but these aspects will be discussed later in this section.

In a series of studies, skeletal muscle (Lee *et al.* 1999) and heart (Lee *et al.* 2002) gene expression profiles were examined in young and old mice. In skeletal muscle, comparisons were made between 5-month (adult) and 30-month (old) mice. Results overall demonstrated that the ageing process is unlikely to be due to large, widespread alterations in gene expression. Instead, ageing resulted in altered expression of a relatively small number of specific genes. Most of the changes induced by ageing were within groups of genes involved in stress responses including heat shock factors Hsp71 and Hsp27, protease Do and the DNA damage-inducible gene GADD45 (Lee *et al.* 1999). Also induced was the expression of genes associated with muscle injury and re-innervation, including the genes for neurotrophin-3, PEA and HIC-5. With regard to mitochondrial energetics, ageing was associated with decreased expression of subunits in ATP synthase, NADP transhydrogenase, and with decreased expression of the LON protease implicated in mitochondriogenesis. The gene

with expression most greatly affected by ageing (decreased 3.8-fold) was the mitochondrial sarcomeric creatine kinase, a protein with activity already known to be affected by ageing (Stachowiak *et al.* 1998). Finally, ageing was also associated with decreased expression of genes involved in synthesis of fatty acids and cholesterol, and in those involved in protein turnover. The latter findings are consistent overall with the ageing-associated decreases in human muscle mitochondrial density and oxidative capacity identified by Conley *et al.* (2000b).

In the second study, the effects of ageing upon gene expression in the heart were studied again in 5-month and 30-month old mice (Lee *et al.* 2002). Similar to the results from skeletal muscle, only a small proportion (10%) of genes was affected by ageing. Transcriptional alterations were consistent overall with a marked metabolic shift away from fatty acid metabolism, and toward carbohydrate metabolism. Included in the genes that were down-regulated with ageing were those involved in fatty acid uptake and oxidation, and included CD36, hormone sensitive lipase, Cpt1, carnitine acyltransferase, Cpt2, acyl-CoA dehydrogenase, and enoyl-CoA hydratase. Of particular interest is the 2.6-fold down-regulation of UCP3 expression. These striking findings corroborate earlier findings that demonstrated decreased palmitoylcarnitine-driven respiration in heart mitochondria from old rats (McMillin *et al.* 1993, Paradies *et al.* 1995). Consistent with an increased reliance on carbohydrate fuels with ageing were the increases in Glut4, phosphofructokinse, phosphoglycerate kinase and enolase and decreases in fructose 1,6-bisphosphatase 2 and pyruvate dehydrogenase kinase.

The effects of CR on gene expression profiles in rodents and non-human primates have been studied in tissues including skeletal muscle, heart and brain. In skeletal muscle, CR enhances the expression ROS scavenging genes, and decreases the expression of genes associated with stress response (Lee *et al.* 1999; Sreekumar *et al.* 2002). In heart, Lee *et al.* (2002) found that CR initiated in middle-aged rats led to substantial 'reprogramming' of gene expression, consistent with preserved fatty acid metabolism, reduced DNA damage and innate immune activity, the modulation of apoptosis factors, and cytoskeletal reorganization. In the brain, CR was associated with the attenuation of the expression of genes in the neocortex and cerebellum that are specifically involved in inflammatory and stress responses (Lee *et al.* 2000). Gene expression profiles during ageing and CR in non-human primates have recently been reviewed, and results demonstrate overall that the effects of CR on gene expression in non-human primates parallel those observed in rodents (Ingram *et al.* 2004).

Effects of ageing on mitochondrial energetics and their mitigation through CR

It is clear that ageing and oxidative stress have a wide range of effects on mitochondrial components including membrane lipids, proteins and DNA. Progressive declines in mitochondrial function with age have been demonstrated in a variety of tissues, and oxidative stress has been implicated in most cases (reviewed in Sohal & Weindruch 1996, Cadenas & Davies 2000).

Research in our laboratories has examined the effects of ageing and of CR on mitochondrial energetics, and the control of oxidative phosphorylation (e.g. Ramsey *et al.* 2000). Several of our studies have focused on the effects of ageing and CR specifically upon mitochondrial proton leak. Mitochondrial proton leak is a process whose mechanism is still poorly understood (Brand 1990, Brand *et al.* 2003). However, it is clear that it is not an artefact of mitochondrial damage occurring as a result of mitochondrial isolation (Nobes *et al.* 1990). Results from a wide array of studies from isolated mitochondria to cells to tissues support the idea that it accounts for a significant proportion of resting energy expenditure. At the level of the whole body it has been estimated that leak processes would account for approximately 20–25% of resting energy expenditure in the rat (Rolfe & Brand 1997). Moreover, studies in both isolated mitochondria and intact cells have also demonstrated that the proportion of oxygen consumption due to leak is altered in many metabolic situations where basal energy expenditure is modified, such as in altered thyroid states (Nobes *et al.* 1990, Harper & Brand 1993, 1994), in mammals of greatly differing body masses (Porter & Brand 1993), and between ectotherms and endotherms matched for body size and temperature (Brand *et al.* 1994a).

Mechanisms or factors that have been proposed to affect proton leak include the uncoupling proteins and inner membrane lipid composition. The idea that the uncoupling proteins are responsible for basal proton leak has received diminished support given that leak is substantial in mitochondria of liver parenchymal cells where no known UCPs are normally expressed, and given that leak is not increased coincident with fasting-induced increases in UCP3 protein expression in muscle (Cadenas *et al.* 1999, Bezaire *et al.* 2001). Several studies support correlations between inner membrane fatty acid composition and leak, to the extent that leak is positively correlated to docosahexanoic acid and negatively correlated to linoleic acid content (Brand *et al.* 1994b, Porter *et al.* 1996, Brookes *et al.* 1998). Proton leak is also positively correlated with inner membrane surface area (Porter *et al.* 1996).

Ageing is associated with changes in fatty acid composition of the inner membrane. Indeed, some

theories correlating membrane lipid composition with ageing have centred on the inverse correlation between long-chain polyunsaturated fatty acids and maximum lifespan. These theories suggest that the peroxidation of the long-chain fatty acids underlies cellular damage and senescence (Pamplona *et al.* 2002, Yu *et al.* 2002, Hulbert 2003). The effects of ageing on the composition of the inner membrane include increased proportions of 22 : 4 and 22 : 5 and decreases in 18 : 2, 18 : 1 and 16 : 1. As described above, it is thought that linoleic acid (18 : 2) is needed to optimize the interactions of cardiolipin with transporters and other components of the inner membrane (Hoch 1988, 1992). Shigenaga *et al.* (1994) proposed that the decreases in 18 : 2 and altered interactions of cardiolipin and inner membrane proteins were responsible for their observed decreases in mitochondrial respiratory control ratios (RCR; state 3 rate/state 4 rate) with ageing. Furthermore it has been specifically demonstrated that membrane lipid peroxidation results in increased proton leak (Brookes *et al.* 1998).

Our findings have established a direct correlation between proton leak and ageing. For example, we demonstrated an age-dependent increase in proton leak rate and a decrease in ATP turnover reactions in isolated hepatocytes from 30-month old mice compared with 3-month old mice (Harper *et al.* 1998). Our findings were similar in nature to those of Sastre *et al.* (1996) and Hagen *et al.* (1997) who demonstrated in intact hepatocytes that mitochondrial protonmotive force is decreased with age coincident with increased production of hydrogen peroxide. Because each of these three studies was conducted in intact hepatocytes, results cannot be due to increased damage to mitochondria during isolation.

Also examined were the effects of ageing on the control and regulation of the three blocks of reactions in the oxidative phosphorylation pathway: substrate oxidation, proton leak, and ATP synthesis and turnover reactions. Cellular oxygen consumption was 15% lower ($P < 0.05$) in hepatocytes of older compared than in those from younger mice. This was attributed to a decrease in the oxygen used to support ATP synthesis and turnover. The proportion of oxygen used to support mitochondrial proton leak reactions increased. At all values of mitochondrial membrane potential examined, proton leak was indeed higher in cells of older mice. Metabolic control analysis demonstrated a general shift with ageing in the control over respiration and phosphorylation away from substrate oxidation reactions toward increased control by leak and by ATP turnover. The latter is consistent with the idea that oxygen consumption and the efficiency of ATP synthesis become increasingly sensitive to changes in proton leak with ageing.

In a subsequent study, we examined the effects of ageing in Wistar rats on mitochondria from a post-mitotic tissue, skeletal muscle (Lal *et al.* 2001). Ageing was associated with increased state 4 respiration in mitochondria from 33-month old rats compared with mitochondria from 33-month old CR rats (33% CR, beginning at 10 months of age). Results from young controls were between values from old control and old CR rats. Results also demonstrated that the overall kinetics of proton leak reactions in skeletal muscle mitochondria of old rats were far more variable than the leak kinetics of either young controls or old CR rats.

A more detailed study ensued into the effects of short- (2 weeks; 2 months) and medium- (6 months) term CR (40%) on rat muscle mitochondrial proton leak, ROS production, and whole animal oxygen consumption in FBNF1 rats (Bevilacqua *et al.* 2004). We hypothesized that CR would result in decreased mitochondrial H_2O_2 production, decreased proton leak, and concomitant decreases in resting oxygen consumption. Further, we hypothesized that decreased oxidative damage to the mitochondrial inner membrane may mitigate the previously documented ageing-induced increases in proton leak (Harper *et al.* 1998, Lal *et al.* 2001). Results showed that mitochondrial H_2O_2 production was indeed lower in all three CR groups, compared with controls. While several previous studies have examined effects of CR on H_2O_2 production in heart mitochondria, ours was the first to describe such short-term effects in muscle mitochondria. Drew *et al.* (2003) found that H_2O_2 production in isolated gastrocnemius muscle mitochondria tended to decrease ($P < 0.066$) following lifelong CR in 26-month old Fisher 344 rats compared with *ad libitum* fed controls (no changes were observed in heart mitochondria). Gredilla *et al.* (2002) reported a decrease in rat heart mitochondria H_2O_2 production after 40% CR for 1 year, but not following 6 weeks or 4 months of CR. In rat liver mitochondria from long-term (1 year) CR rats, Lopez-Torres *et al.* (2002) documented a 47% decrease in H_2O_2 production and a 46% decrease in oxidative damage to mtDNA. In liver mitochondria from rats subjected to 1 or 6 months of 40% CR, Ramsey *et al.* (2004) demonstrated no differences in H_2O_2 production. Our results from skeletal muscle mitochondria demonstrate the rapidity with which CR reduces H_2O_2 production in this tissue. Given this rapidity, CR regimens of shorter duration merit further study. The differences between liver, heart and skeletal muscle H_2O_2 production may reflect differences in duration and degree of CR, and differences between mitotic (liver) and post-mitotic (skeletal muscle, heart) tissues.

At each time point in muscle, maximal leak-dependent O_2 consumption was lower in CR rats compared with age-matched controls (Bevilacqua *et al.* 2004).

However, proton leak kinetics indicated however that mechanisms of adaptation to CR were different between short- and medium-term treatments, with the former leading to decreases in protonmotive force (Δp) and state 4 O_2 consumption, and the latter to increases in Δp and decreases in state 4 O_2 consumption. Maximum leak-dependent oxygen consumption was reduced by 26, 42 and 53% in the 2 week, 2 and 6 month CR rats, respectively, compared with age-matched controls. The rapid decreases in protonmotive force, concurrent with decreases in state 4 O_2 consumption are novel and the mechanisms are under further investigation in our laboratories.

To determine whether there were any coincident changes in the levels of UCP3 protein, we conducted immunoblots with appropriate positive and negative controls (recombinant UCP3, and mitochondria from UCP3 knockout mice). Even following 6 month CR, UCP3 protein expression was increased (doubled) compared with control fed rats (Bevilacqua *et al.* 2004). These increases in UCP3 in muscle are consistent with the increased UCP3 mRNA expression in heart following 16 months of 41% CR in 30-month old mice observed by Lee *et al.* (2002). Increased UCP3 protein expression concurrent with the clearly decreased proton leak at 6 months of CR is inconsistent with the hypothesis that UCP3 causes basal proton leak. Thus, we have observed clear reductions in proton leak concomitant with a doubling of UCP3 content in muscle mitochondria. Two previous studies into the effect of fasting on leak and UCP3 expression have demonstrated that proton leak in muscle mitochondrial is not simply a function of UCP3 level (Cadenas *et al.* 1999, Bezaire *et al.* 2001, Harper *et al.* 2001). Stuart *et al.* (2001) found that the uncoupling induced in many *in vitro* systems following transfection with UCPs is due to artefactual uncoupling (i.e. disrupted integrity of the mitochondrial inner membrane).

Results from metabolic control analyses of oxidative phosphorylation following short- and medium-term CR demonstrate that the control by proton leak over substrate oxidation, phosphorylation, and proton leak reactions tends to be lower at all time points in mitochondria of CR rats. At all three time points, control by phosphorylation reactions under state 3 conditions was increased in CR animals. Our findings on the overall control by phosphorylation reactions over muscle mitochondrial energy expenditure (i.e. substrate oxidation flux) indicate that ATP turnover becomes a stronger controlling factor following CR and leak a weaker controlling factor than in mitochondria from control animals. While our studies did not include measurements of ATP production, other groups have assessed ATP production following CR. Drew *et al.* (2003) recently studied the effects of ageing and lifelong CR on ATP content and the rate of ATP production in rat skeletal muscle and heart. They studied 12-month old *ad libitum* fed, 26-month old *ad libitum* fed, and 26-month old CR (lifelong 40% restriction) rats, and demonstrated decreased ATP content and ATP production with age in skeletal muscle (50% decrease in gastrocnemius), but not in heart. Age-associated decreases in ATP content are consistent with the results of an NMR study of *in vivo* ATP production in quadriceps muscle of elderly subjects where ATP production was 50% that of younger subjects (Conley *et al.* 2000a). CR had no effect on ATP content or production in either tissue. The absence of any CR-induced alterations in ATP production is consistent with the results of Sreekumar *et al.* (2002), who found no changes in muscle following 36 weeks of 40% CR in rats. Our metabolic control analysis approaches have not addressed ATP content, only the distribution of control for its production. Overall, our findings detail the rapid effects of short- and medium-term CR on proton leak, ROS production and on metabolic control of oxidative phosphorylation in muscle mitochondria.

Kerner *et al.* (2001) demonstrated that skeletal muscle mitochondria from 24-month old Fisher 344 rats have lower amounts of UCP3 protein than mitochondria of 6-month old controls. These findings are in general consistent with the results from gene expression profiling of mouse heart in which UCP3 mRNA levels fall with age in normally fed mice (but were much higher in old CR mice than in young controls; Lee *et al.* 2002). Such changes in UCP3 expression with age and CR are interesting in light of the idea that UCP3 plays some role in facilitating fatty acid oxidation (Dulloo *et al.* 2001, Himms-Hagen & Harper 2001). They are also in line with the results of *in vivo* studies in humans in which decreased fat oxidation during exercise was documented in elderly subjects compared with young adults undergoing the same absolute or relative degree of exercise intensity (Sial *et al.* 1996). Kerner *et al.* (2001) however found that state 4 respiration was decreased in the aged rats compared with 6-month old controls; unfortunately there were no assessments of mitochondrial protonmotive force values with these measures. There were no age-associated decreases in carnitine-palmitoyl transferase I and carnitine palmitolyl transferase II, but there was a small but significant drop in carnitine content with ageing. The authors proposed that increased production of free radicals with ageing results in decreased content of UCP3 in mitochondrial inner membrane and decreased state 4 respiration. This possibility is, however, inconsistent with the findings of Cadenas *et al.* (1999) and Bezaire *et al.* (2001) who demonstrated clear increases in muscle UCP3 protein in the absence of any significant change in state 4 respiration and proton leak.

We have recently investigated the effects of long-term CR, initiated in adulthood, on muscle mitochondrial energetics and H_2O_2 production and on characteristics of whole body energy metabolism (L. Bevilacqua *et al.*, unpublished data). CR (40%) began in $FBNF_1$ rats at 6 months of age and continued for 12 or 18 months. Indirect calorimetry demonstrated decreased whole body oxygen consumption (V_{O_2}) and respiratory exchange ratios (RER; V_{CO_2}/V_{O_2}) in CR rats compared with age-matched control-fed rats. Moreover, RER was lower in 18-month old vs. 24-month old control-fed rats, indicating that ageing induced shifts in metabolism away from fatty acid oxidation toward carbohydrate oxidation. Production of H_2O_2 by isolated muscle mitochondria was lower ($P < 0.01$) in CR than in control-fed mitochondria at both time points and was increased with ageing. The 12-month CR rats had 40% lower ($P < 0.001$) mitochondrial state 4 respiration compared with age-matched control-fed rats. Following 18 month CR there were no differences in state 4 rates between CR and control-fed rats. Proton leak kinetics were affected by 12 months of CR such that leak-dependent respiration was lower in CR mitochondria at protonmotive force values exceeding 170 mV. Following 18 months of CR, leak-dependent oxygen consumption was only slightly, but consistently, lower than control-fed mitochondria over the entire range of protonmotive force values. These findings corroborate and significantly extend earlier results on the effects of short- and medium-term CR. Results from whole body energetics are consistent with the idea of decreased reliance on fatty acids with age, and mitigation of such decreases by CR.

Studies of liver mitochondria isolated from FBNF1 rats subjected to the same 40% CR initiated at 6 months of age reveal the importance of tissue type and duration of CR in affecting changes in proton leak. Ramsey and colleagues demonstrated that proton leak was not altered significantly following short-term (2 months) or medium-term (6 months) CR, but was significantly decreased following 12 and 18 months of CR (V. Ramsey *et al.*, unpublished data). These findings corroborate and extend earlier findings of decreased proton leak in hepatocytes of long-term CR C57Bl6 mice (Harper *et al.* 1998).

Conclusion

Results overall demonstrate that with ageing proton leak increases in mitochondria of mitotic (e.g. liver) and post-mitotic (skeletal muscle) tissues. Effects of ageing and the mitigating effects of CR occur more rapidly in post-mitotic tissues than in mitotic tissues. Findings from gene expression studies in muscle and heart demonstrate that UCP3 expression decreases with ageing (Kerner *et al.* 2001, Lee *et al.* 2002). These findings, plus the fact that UCPs are not normally expressed in parenchymal liver cells, are inconsistent with the idea that the novel UCPs cause proton leak. However, results are generally consistent with a mechanism related to increased oxidative stress-induced damage to lipids and specific proteins of the mitochondrial inner membrane. CR results in phasic alterations in proton leak in muscle mitochondria. Following short-term CR, maximum leak-dependent respiration is decreased, but there are also clear decreases in mitochondrial protonmotive force suggesting that more than one process affecting leak kinetics is involved. Following medium term CR, there are clear decreases in proton leak kinetics (i.e. consistent decreases in respiration and increases in protonmotive force). Finally, following long-term CR, the effects in muscle of CR on the kinetics of proton leak become less pronounced, as if the decreases in proton leak in muscle by CR are temporary. In the liver, however the effects of CR in mitigating the ageing induced increases in leak do not occur with short- or medium-term CR, but require long-term CR. Clearly further studies will be needed to investigate the latter possibilities.

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