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Membrane phospholipid composition may contribute to exceptional longevity of the naked mole-rat (*Heterocephalus glaber*): A comparative study using shotgun lipidomics

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Abstract

Phospholipids containing highly polyunsaturated fatty acids are particularly prone to peroxidation and membrane composition may therefore influence longevity. Phospholipid molecules, in particular those containing docosahexaenoic acid (DHA), from the skeletal muscle, heart, liver and liver mitochondria were identified and quantified using mass-spectrometry shotgun lipidomics in two similar-sized rodents that show a ~9-fold difference in maximum lifespan. The naked mole rat is the longest-living rodent known with a maximum lifespan of >28 years. Total phospholipid distribution is similar in tissues of both species; DHA is only found in phosphatidylcholines (PC), phosphatidylethanolamines (PE) and phosphatidylserines (PS), and DHA is relatively more concentrated in PE than PC. Naked mole-rats have fewer molecular species of both PC and PE than do mice. DHA-containing phospholipids represent 27–57% of all phospholipids in mice but only 2–6% in naked mole-rats. Furthermore, while mice have small amounts of di-polyunsaturated PC and PE, these are lacking in naked mole-rats. Vinyl ether-linked phospholipids (plasmalogens) are higher in naked mole-rat tissues than in mice. The lower level of DHA-containing phospholipids suggests a lower susceptibility to peroxidative damage in membranes of naked mole-rats compared to mice. Whereas the high level of plasmalogens might enhance membrane antioxidant protection in naked mole-rats compared to mice. Both characteristics possibly contribute to the exceptional longevity of naked mole-rats and may indicate a special role for peroxisomes in this extended longevity.

Keywords: Longevity; Membrane lipids; Docosahexaenoic acid; Polyunsaturated fatty acids; Maximum lifespan; Lipid peroxidation; Naked mole-rat

1. Introduction

The laboratory mouse (*Mus musculus*) is the most common mammalian model for studying aging and has a maximum lifespan of 3–4 years, depending upon strain (e.g. Miller et al., 2002). Such a relatively short lifespan is experimentally advantageous when examining treatments that alter lifespan, such as caloric restriction or genetic manipulation. However, this short-lived rodent may not be the most appropriate model for human aging: *Homo sapiens* is an exceptionally long-living mammal with a maximum life span record of 122 years resulting in a longevity quotient (LQ = ratio of actual maximum life span to that predicted from body mass) of ~5 while that for the mouse is ~0.7. Short-living mammals such as the mouse may not be able to give full insight into the mechanisms responsible for such exceptional longevity. A more suitable rodent

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model for human aging may be the longest-living rodent known, the naked mole-rat (*Heterocephalus glaber*). This mouse-sized rodent has a maximum lifespan of ~28 years resulting in a similar LQ to that of humans (Buffenstein, 2005) and may employ similar mechanisms involved in slow and successful aging.

Longer living mole-rats maintain physiological and reproductive function well into their third decade of life (O'Connor et al., 2002; Buffenstein, 2005) and in addition maintain body composition and vascular youthfulness in keeping with attenuated rates of aging (Csiszar et al., 2007). Basal metabolic rate is 30% lower than predicted by body mass, but this reduction in oxygen consumption with its inevitable by-product, oxidative damage, is not sufficient to fully account for the 5-fold difference in extended longevity, and not surprisingly, naked mole-rats have the highest mass specific lifetime energy expenditure of any known mammal (O'Connor et al., 2002). Antioxidant defences of naked mole-rats are not superior to those of mice; naked mole-rats show similar levels of catalase activity, moderately higher levels of superoxide dismutase activity, but surprisingly show a 70-fold lower level of glutathione peroxidase activity (Andziak et al., 2005) and significantly lower levels of glutathione (Andziak and Buffenstein, 2006). Furthermore, reactive oxygen species production as indicated by hydrogen peroxide production in isolated heart mitochondria (Lambert et al., 2007) or superoxide production in vascular endothelial cells (Labinskyy et al., 2006) is similar to that of mice and surprisingly the longer living species exhibits greater levels of oxidative damage to macromolecules than do mice, even at a young age (Andziak et al., 2006). Nevertheless, they do show attenuated age related changes in ROS production (Csiszar et al., 2007) and accrued oxidative damage (Andziak and Buffenstein, 2006). One area where naked mole-rats do agree with theoretical predictions for such a relatively long-living species is in respect of the fatty acid composition of their cellular membranes (Hulbert et al., 2006a).

It has been known for many years that fatty acids differ dramatically in their susceptibility to peroxidation. Only polyunsaturated fatty acids (PUFA) can be peroxidised, and the greater the degree of polyunsaturation of a PUFA molecule the greater it's susceptibility to peroxidation, while both saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) are essentially incapable of being peroxidised (Holman, 1954). It was not until the 1990s however, that it became known that mammals differ in the fatty acid composition of their membranes in a systematic manner (Couture and Hulbert, 1995) and that the fatty acid composition of mammalian mitochondrial membrane lipids was shown to be correlated with their maximum lifespan (Pamplona et al., 1998, 1999). These observations have been used to modify the 'oxidative stress' theory resulting in the 'membrane pacemaker' theory of aging (Hulbert, 2005) which emphasises the importance of lipid peroxidation in the processes of aging. The connections between the metabolic rate, membrane composition and maximum lifespan of animals has been recently reviewed (Hulbert et al., 2007).

Measurement of the fatty acid composition of phospholipids from tissues of naked mole-rats reveal that their cellular membranes are much more resistant to peroxidative damage than those of mice (Hulbert et al., 2006a). Indeed it has been suggested that this difference in membrane composition may underly their exceptional longevity. The most polyunsaturated fatty acid commonly found in mammalian membranes is docosahexaenoic acid (DHA or 22:6 n−3), which has six double bonds per molecule and is 320-times more susceptible to peroxidation than the monounsaturated oleic acid (see Hulbert, 2005; Hulbert et al., 2007). The phospholipids from naked mole-rat tissues have similar total amounts of unsaturated fatty acids as do mice tissue phospholipids, but the amount of DHA is approximately one-nineth that measured for the mice. This difference results in membranes with a much lower peroxidation index and may account for the approximately 9-fold greater longevity of naked mole-rats compared to mice (Hulbert et al., 2006a).

That the fatty acid composition of membranes is specified by the individual's genome is manifest from the observation that membrane fatty acid composition of wild-derived strains of mice with extended longevity differs from that of a genetically diverse shorter-living laboratory strain even when they are fed the same food and housed under identical conditions (Hulbert et al., 2006b). Relatively little is known of the precise mechanisms regulating membrane fatty acid composition. Studies in rat hepatocytes show, despite the presence of a very large number of phospholipid molecular species found in membrane bilayers, that only four types of both phosphatidylcholine and phosphatidylethanolamine are synthesised *de novo*. The remaining phospholipid molecular species are made by deacylation–reacylation remodelling of the *de novo* synthesised phospholipid molecules (Schmid et al., 1995a).

In one group of phospholipids, the acyl chain at the C1 (sn-1) of glycerol is attached by a vinyl-ether linkage in place of an ester linkage. These phospholipids are known as plasmalogens and were originally thought of as biological peculiarities, present in myelin sheaths of nerve fibers, cell membranes of muscle, and platelets (Brites et al., 2004). However recently they have been implicated in health and many age-related degenerative diseases such as Alzheimers (Gorgas et al., 2006; Farooqui et al., 2006; Brosche and Platt, 1998). These ether phospholipids have been proposed to be important membrane antioxidants and are also implicated in some cell signalling pathways.

The first steps of plasmalogen synthesis take place in peroxisomes which are essential for their synthesis. These ubiquitous organelles also play important roles in other aspects of lipid metabolism as well as being involved in both the generation and neutralization of reactive oxygen species (ROS). Peroxisomes are also critically involved in the formation of DHA where the final step of DHA synthesis involves a single cycle of β-oxidation in the peroxisome.

Indeed, peroxisomes are considered indispensable for the proper functioning of cells and their dysfunction has been implicated in several aspects of aging (Perichon et al., 1998; Terlecky et al., 2006).

This study sets out to elucidate how long-living naked mole-rats maintain membrane composition more resistant to lipid peroxidation than those of mice, by using shotgun lipidomics to analyse in depth the molecular composition of DHA-containing phospholipids in skeletal muscle, heart, liver and liver mitochondria from naked mole-rats and mice. Shotgun lipidomics is a technique whereby the individual phospholipid molecules that make up complex phospholipid mixtures (such as those found in biological membranes) can be identified and quantified by electrospray ionisation mass spectrometry (ESI-MS) and tandem mass-spectrometry (ESI-MS/MS) (Ekroos et al., 2002; Pulfer and Murphy, 2003; Han and Gross, 2005). We hypothesise that the longest-living rodent will have low levels of DHA-containing molecules of both phosphatidylcholines (PC), phosphatidylethanolamines (PE) and higher levels of plasmalogens than shorter-living rodents.

2. Materials and methods

Young adult naked mole-rats (2 years old) were obtained from a captive colony maintained in the Department of Biology, City College of City University of New York. Animals were housed under simulated burrow conditions (as previously described O'Connor et al., 2002; Andziak et al., 2005, 2006). Briefly, animals were kept under ambient conditions of 30 °C, 75% relative humidity with dim incandescent lighting, and were fed a diet of fresh fruits and vegetables supplemented with a protein-rich moist cereal (Pronutro; Bokomo Foods, Cape Town, South Africa). No free water was supplied. Young adult mice (CB57BL6, 0.25 years old) were purchased from Charles River Laboratories (Wilmington, MA, USA) and were housed under standard rodent conditions for approximately one month prior to use in this investigation.

Animals were killed by cardiac exsanguination following anesthesia (sodium pentobarbital 60 mg/kg). Tissues were rapidly removed and flash frozen in liquid nitrogen prior to storage at −80 °C. Mitochondria were prepared from a portion of fresh liver by methods described previously (Brand et al., 1991), immediately following the killing of the animal. Tissue and mitochondrial samples were kept frozen at −80 °C until extraction of lipids (<2 weeks). Total lipids were extracted by hand using glass/glass homogenizers and ultrapure grade chloroform/methanol (2/1, vol/vol) containing butylated hydroxytoluene (0.01% wt/vol) as an antioxidant. Total lipid extracts were dried and sealed in small glass vials under a nitrogen atmosphere. These were transported from City College of New York to University of Wollongong for the lipidomic analysis by mass-spectrometry.

The lipid extracts were reconstituted in ultrapure grade chloroform/methanol (2/1, vol/vol) containing butylated hydroxytoluene (0.01% wt/vol) and aliquots taken for GC analysis (Hulbert et al., 2006a). The remainder of each extract was dried under nitrogen in a 37 °C water bath and reconstituted in ultrapure grade methanol/chloroform (2/1, vol/vol) and total phospholipid concentration determined by the method of Mills et al. (1984). Extracts where diluted to 40 pmol/μL using methanol/chloroform (2/1, vol/vol) containing 625 fmol/μL each of internal standards (dinonadecanoyl phosphatidylcholine, 19:0/19:0 PC; diheptadecanoyl phosphatidylserine, 17:0/17:0 PS; diheptadecanoyl phosphatidylethanolamine, 17:0/17:0 PE; diheptadecanoyl phosphatidylglycerol, 17:0/17:0 PG; diheptadecanoyl phosphatidic acid, 17:0/17:0 PA; tetramyristoyl cardiolipin, $14:0_4$ CL) and 1.4% ammonia, pH 10. The diluted extracts where analysed by mass spectrometry immediately.

Electrospray ionization mass spectrometry was performed on a Waters Quattro Micro (Waters, Manchester, U.K.) equipped with an electrospray ion source. Capillary voltage was set to 3000 V, source temperature 80 °C and desolvation temperature 120 °C. Cone voltage was set at −50 V in negative ions and 35 V in positive ions. Nitrogen was used as the drying gas at a flow rate of 320 mL/min. Samples were infused (10 mL/min) using the instrument's syringe pump and phospholipid classes analysed using head group-specific precursor ion and neutral loss scans using laboratory frame collision energies ranging from 22 to 50 eV as described previously (Brugger et al., 1997). Argon was used as the collision gas at a pressure of 3 mTorr. Precursor ion scans for the fatty acid carboxylate anions were performed under the same conditions using a laboratory frame collision energy of 50 eV for saturated fatty acids and 35 eV for unsaturated fatty acids. To identify the molecular composition of phosphatidylcholines in positive ions, neutral loss scans of the fatty acyl chains were performed after the addition of 200 μM lithium acetate (LiOAc) to the lipid extracts. Typically, 120 spectra were averaged for each head group scan. Each mass spectrum was normalised to the internal standard after correction for isotope contributions and each molecular phospholipid is presented as a percentage of total phospholipids. Fatty acid precursor ion scans were performed for identification purposes only; accordingly, the minimum number of scans were averaged in order to obtain clean spectra. Tandem mass spectrometry was performed on isobaric phospholipids and the relative contribution of each isobaric species was determined from the ratio of fatty acid ion abundances. Since no internal standard for sphingomyelin (SM) or phosphatidylinositol (PI) were available they where normalised to other internal standards. SM was normalized by comparing to 17:0/17:0 PC and multiplying by a correction factor (1/0.317) determined by comparison of signal response of 16:0 and 18:1 SM and 16:0/18:1 and 18:1/18:1 PC species over a concentration range of 0.8–1.6 μM (each PL species). PI was normalized using the mean abundance of 17:0/17:0 PA and 17:0/17:0 PG as the ionisation efficiency of PI is known to sit between PA and PG. There was also no appropriate internal standard

for plasmenyl phospholipids available, i.e., one not present in the native samples. Accordingly, the loss of plasmenyl phospholipids during extraction was tested using 16:0p/18:1 PE. We found an approximate loss of 50% in this phospholipid during extraction, likely a consequence of acid hydrolysis. As the neutral loss of 141 Da from PE is known to be less efficient from plasmenyl than diacyl PE (Zemeski-Berry and Murphy, 2004) this was also tested using 16:0p/18:1 PE in comparison to 17:0/17:0 PE and 18:1/18:1 PE. The fragmentation efficiency for the plasmenyl PE was found to be approximately 29% of the diacyl PEs. As a consequence the percentages of plasmenyl phospholipids described in this study are underestimated, particularly for PE, however the differences between animals are not affected.

3. Results

The distribution of phospholipid classes was essentially the same in tissues of mice and naked mole-rats (see Fig. 1). In skeletal muscle, heart, liver and liver mitochondria of both species, phosphatidylcholines (PC) were the most abundant type of phospholipid (constituting 57–66% of total phospholipids) while phosphatidylethanolamines (PE) were the second most abundant phospholipid type (constituting 18–33% of all phospholipids). The differences between the tissues were generally greater than the difference between the two species. In both species, all other phospholipids classes combined constituted 17–20% of the total phospholipids in skeletal muscle, 15–17% in heart, 8–15% in liver and 4–10% in liver mitochondria.

The relative distribution of DHA-containing phospholipids is shown in Fig. 2 as percent of all DHA-containing phospholipids, and in Table 1 as percent of total tissue phospholipids. As can be seen in Fig. 2, the relative distribution of DHA-containing phospholipids did not mirror the distribution of all phospholipid classes (shown in Fig. 1). DHA was only found in three phospholipid classes; phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS). There were no DHA-containing molecules of any other phospholipid class in any tissue from either species [with the exception of phosphatidic (PA) acid in which 18:0/22:6 PA was 0.2% of all phospholipids in skeletal muscle of mice and 18:1/22:6 PA was 0.1% of all phospholipids in liver and liver mitochondria of mice]. DHA was relatively more concentrated in PE and relatively less concentrated in PC than would be expected from the general abundances of PE and PC (Fig. 2 compared to Fig. 1). This was true of all tissues but was most pronounced in skeletal muscle and heart. Within both types of muscle this was more pronounced in the naked mole-rat than the mice. For instance, although PE constituted, respectively, only 18% and 21% of all phospholipids in skeletal muscle and heart of the naked mole rat, DHA-containing PE constituted 54% and 62%, respectively, of all DHA-containing phospholipids. While in the same tissues from the same species, PC constituted, respectively, 66% and 59% of all tissue phospholipids and DHA-containing PC made up 37% and 36% of all DHA-containing phospholipids. In liver and liver mitochondria, the shift to PE from PC was not as pronounced for either species. For example, PC were 59% and 64% of all phospholipids in

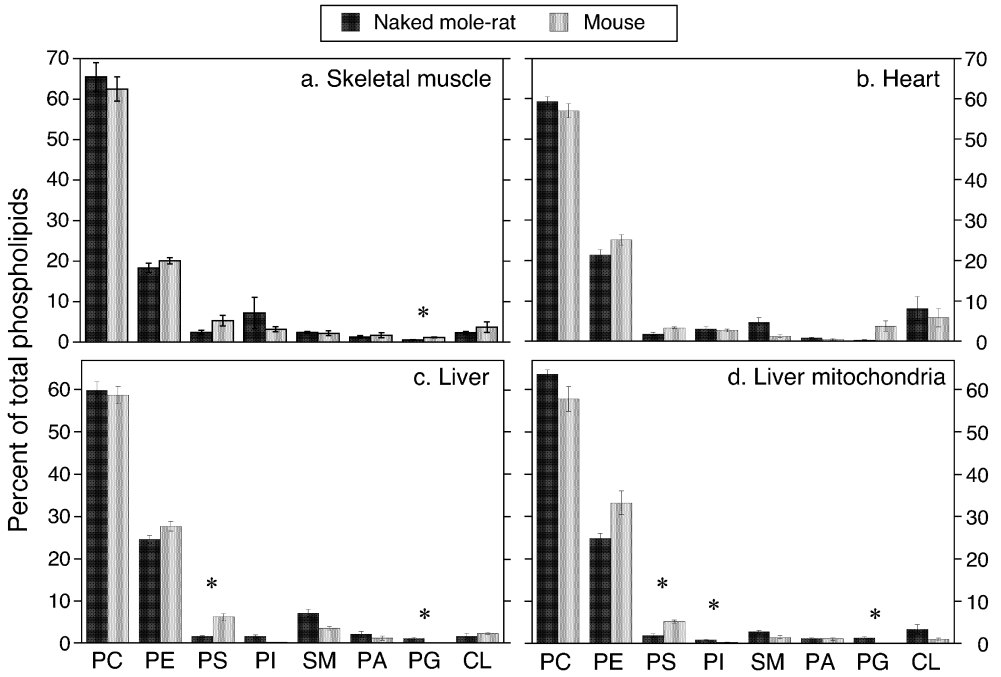

Fig. 1. The relative distribution of phospholipid classes in the (a) skeletal muscle, (b) heart, (c) liver and (d) liver mitochondria of naked mole-rats (*H. glaber*, $N = 4$) and laboratory mice (*M. musculus*, $N = 4$). PC, phosphatidylcholines; PE, phosphatidylethanolamines; PS, phosphatidylserines; PI, phosphatidylinositols; SM, sphingomyelins; PA, phosphatidic acid; PG, phosphatidylglycerols; CL, cardiolipins. Error bars represent ±SEM. *Marks those phospholipids that are significantly different ($P < 0.01$) between naked mole-rats and mice.

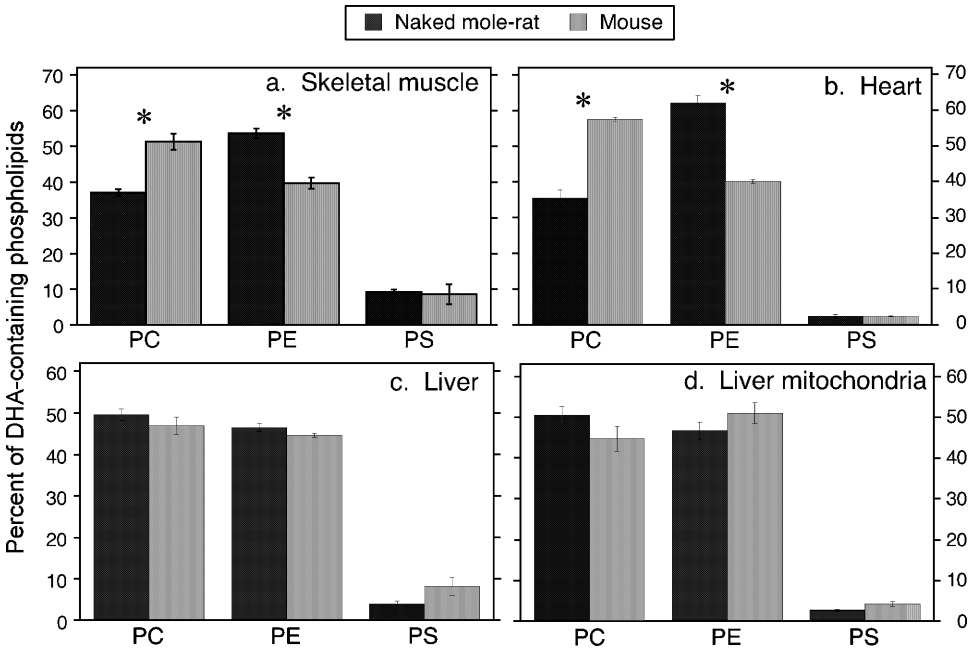

Fig. 2. The relative distribution of DHA-containing phospholipids in the (a) skeletal muscle, (b) heart, (c) liver and (d) liver mitochondria of naked mole-rats (*H. glaber*, $N = 4$) and laboratory mice (*M. musculus*, $N = 4$). PC, phosphatidylcholines; PE, phosphatidylethanolamines; PS, phosphatidylserines. Error bars represent ±SEM. *Marks those DHA-containing phospholipids that are signficantly different ($P < 0.01$) between naked mole-rats and mice.

liver and liver mitochondria, respectively, from the naked mole-rat while DHA-containing PC were, respectively, 50% and 51% of all DHA-containing phospholipids. In the same tissues from the same species, PE were 24% and 25% of all phospholipids while DHA-containing PE was, respectively, 46% and 47% of all DHA-containing phospholipids. DHA-containing PS were of approximately the same relative abundance as the relative abundance of PS

Table 1
Molecular species composition of docosahexaenoic acid-containing phospholipids from skeletal muscle, heart, liver and liver mitochondria of naked mole-rats (NMR) and mice

	Skeletal muscle		Heart		Liver		Liver mitochondria	
	NMR	Mice	NMR	Mice	NMR	Mice	NMR	Mice
DHA-containing PC								
16:0/22:6	0.7 ± 0.1	16.5 ± 0.4*	0.5 ± 0.1	16.5 ± 0.6*	1.1 ± 0.1	8.5 ± 0.3*	1.3 ± 0.1	8.7 ± 0.4*
18:0/22:6	0.5 ± 0.1	2.2 ± 0.2*	0.2 ± 0.0	13.9 ± 0.5*	1.5 ± 0.2	3.1 ± 0.4*	1.7 ± 0.1	3.2 ± 0.4*
16:1/22:6	–	0.6 ± 0.1*	–	0.1 ± 0.0*	–	0.2 ± 0.0*	–	0.1 ± 0.0*
18:1/22:6	0.1 ± 0.0	0.9 ± 0.1*	–	1.3 ± 0.1*	0.2 ± 0.0	1.0 ± 0.1*	0.2 ± 0.0	1.1 ± 0.1*
18:2/22:6	–	0.6 ± 0.1*	–	1.7 ± 0.1*	–	0.1 ± 0.0*	–	0.1 ± 0.0*
Total PC	1.3 ± 0.1	20.8 ± 0.7*	0.7 ± 0.1	33.4 ± 1.1*	2.8 ± 0.2	12.9 ± 0.7*	3.1 ± 0.2	13.2 ± 0.7*
DHA-containing PE								
16:0/22:6	0.3 ± 0.0	4.6 ± 0.3*	0.3 ± 0.1	4.9 ± 0.3*	1.1 ± 0.1	7.2 ± 0.3*	1.2 ± 0.2	9.0 ± 0.5*
18:0/22:6	1.1 ± 0.1	8.3 ± 0.5*	0.2 ± 0.0	12.0 ± 0.6*	0.8 ± 0.1	2.9 ± 0.1*	1.0 ± 0.1	3.3 ± 0.1*
16:1/22:6	–	–	–	0.1 ± 0.0*	–	0.3 ± 0.0*	–	0.4 ± 0.1*
18:1/22:6	0.4 ± 0.1	2.1 ± 0.1*	0.7 ± 0.1	3.6 ± 0.2*	0.8 ± 0.1	1.9 ± 0.1*	0.7 ± 0.1	2.4 ± 0.2*
18:2/22:6	–	0.8 ± 0.1*	–	0.7 ± 0.0*	–	–	–	–
Total PE	1.8 ± 0.1	15.9 ± 0.7*	1.2 ± 0.1	21.6 ± 1.1*	2.6 ± 0.3	12.2 ± 0.5*	2.9 ± 0.3	15.1 ± 0.8*
DHA-containing PS								
16:0/22:6	–	–	–	–	–	0.3 ± 0.1*	–	0.3 ± 0.1*
18:0/22:6	0.3 ± 0.0	3.6 ± 1.1*	0.1 ± 0.0	1.4 ± 0.1*	0.2 ± 0.1	1.9 ± 0.4*	0.2 ± 0.0	1.0 ± 0.1*
total PS	0.3 ± 0.0	3.6 ± 0.7*	0.1 ± 0.1	1.4 ± 0.1*	0.2 ± 0.1	2.2 ± 0.5*	0.2 ± 0.0	1.3 ± 0.2*
All DHA-containing PL								
Total PL	3.4 + 0.3	40.5 + 1.3*	2.0 + 0.2	56.5 + 2.3*	5.7 + 0.5	27.4 + 1.1*	6.2 + 0.5	29.5 + 0.6*

Values are expressed as percent of total phospholipids. Values are presented as means ± SEM ($N = 4$). DHA, docosahexaenoic acid; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PS, phosphatidylserines; PL, phospholipids. The values for DHA-containing phosphatidic acid and DHA-containing plasmenyl PE are not shown as they were only found in mice and were negligible % of total phospholipids.

* indicates NMR and mouse values are significantly different ($p < 0.01$).

in all phospholipids (2–6% vs 2–8%) except for skeletal muscle in both species where DHA-containing PS were 9% of all DHA-containing phospholipids in both species while PS constituted only 2% and 5% of all phospholipids in naked mole-rats and mice, respectively.

The specific DHA-containing PC, PE and PS molecules quantified in this study are listed in Table 1. As can be seen from this Table, phospholipid molecules containing DHA were many times more abundant in mice than in naked mole-rats. Whereas DHA-containing phospholipids, respectively, constituted 40.5% and 56.5% of all phospholipids in skeletal muscle and heart of the mice, they constituted only 3.4% and 2.0%, respectively, in the naked mole-rat. In liver and liver mitochondria, DHA-containing phospholipids were 27.4% and 29.5% of all phospholipids in the mice but only 5.7% and 6.2% in the naked mole-rats. The large difference between the two species in the relative abundance of DHA-containing phospholipids was manifest in all tissues and all three phospholipid classes (i.e. in PC, PE and PS). The large species-difference in relative abundance of DHA-containing phospholipids was also manifest in the number of DHA-containing molecules identified in each species. While mice had four-to-five DHA-containing PC and DHA-containing PE, only two-to-three were found in phospholipids from the naked mole-rat tissues. While mice had one-to-two types of DHA-containing PS, only a single DHA-containing PS molecule was measured in naked mole-rat tissues. While both 16:0/22:6 PC and 18:0/22:6 PC were measured in all tissues from both species, the di-polyunsaturated 18:2/22:6 PC was only observed in tissues from the mice and was absent in those from the naked mole-rats. Similarly, while both 16:0/22:6 PE and 18:0/22:6 PE were found in all tissues from both species, the di-polyunsaturated 18:2/22:6 PE was only measured in skeletal and heart from mice and was absent in naked mole-rat tissues.

For some membrane lipids, the sn-1 acyl chain is linked to the glycerol backbone by a vinyl ether linkage (rather than an ester linkage) with a double bond between C-1 and C-2 of the acyl chain. Such membrane lipids are known as plasmenyl phospholipids (also called plasmalogens) and are generally restricted to the choline and ethanolamine phospholipid classes. In Fig. 3 the relative abundance of both types of plasmenyl phospholipids are shown for the four sources of phospholipids from both species, as well as the relative abundance of DHA-containing plasmenyl phospholipids. As can be seen from this figure, the relative abundance of plasmenylcholines was significantly greater (4 to 8-fold greater) in all the tissues from the naked mole-rat compared to the same tissue from mice. The plasmenylethanolamines were in greater relative abundance in skeletal muscle and liver from naked mole-rats

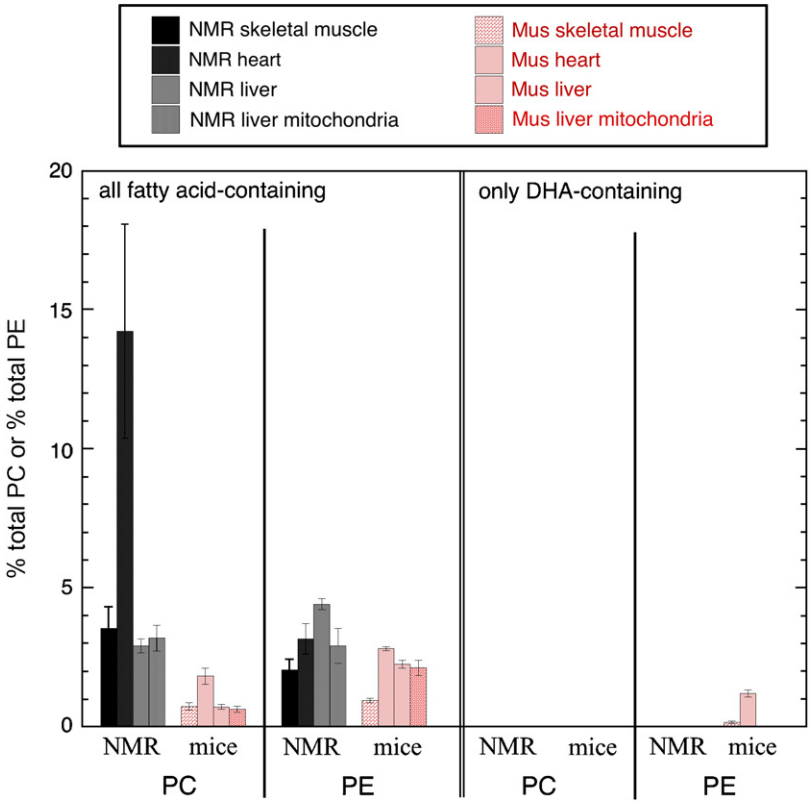

Fig. 3. The relative distribution of vinyl ether-linked plasmenyl phospholipids in the skeletal muscle, heart, liver and liver mitochondria of naked mole-rats (NMR – *H. glaber*) and laboratory mice (*M. musculus*). Left hand graph shows values for all plasmenyl phospholipids while the right hand graph shows only DHA-containing plasmenyl phospholipids. PC, phosphatidylcholines; PE, phosphatidylethanolamines. Values of PC plasmenyls are presented as % of total PC, while PE plasmenyls are presented as % of total PE. Error bars represent ±SEM ($N = 4$).

compared to the same tissues in mice. Although plasmenyl phospholipids were relatively more abundant in naked mole-rats, none contained DHA while 17% and 43% of the plasmenylethanolamines from the skeletal muscle and heart of mice contained DHA. There were no DHA-containing plasmenylethanolamines in liver and liver mitochondria from mice and no DHA-containing plasmenylcholines in any of the mice or naked mole-rat tissues.

4. Discussion

Previous studies have shown that the fatty acid composition of mitochondrial phospholipids is related to the maximum lifespan of a variety of mammalian species (Pamplona et al., 1998, 1999) and furthermore the patterns relating fatty acid composition of liver mitochondrial phospholipid to lifespan of these mammal species were manifest in both phosphatidylcholines and phosphatidylethanolamines (Portero-Otin et al., 2001). More recently, the exceptional longevity of the naked mole-rat relative to the similarly-sized mouse has also been related to differences in the fatty acid composition of phospholipids and the calculated susceptibility to peroxidation of the membranes from these two species (Hulbert et al., 2006a)

This study investigated the phospholipid molecular species primarily involved in the peroxidation resistance of naked mole-rat membranes. Our hypothesis that the longest living rodent will have low levels of *de novo* DHA-derived molecular species phosphatidylcholines (PC), phosphatidylethanolamines than shorter-living rodents held true in this first report of phospholipid molecular species for naked mole-rats. We know of only one study investigating phospholipid molecular species from mice tissues and that is for murine myocardium (Han et al., 2004). The values reported for the fed control mice in that study are similar to those reported for mice in our present investigation. Han et al. (2004) report that the following molecular species; 16:0/22:6 PC, 18:0/22:6 PC, 16:0/22:6 PE and 18:0/22:6 PE, respectively, constitute 17%, 12%, 7% and 16% of all heart phospholipids in mice. Our values for the same molecular species in mouse heart are 17%, 14%, 5% and 12%, respectively (see Table 1). These mice data are markedly different from those of naked mole-rats.

The current results confirm the previous report that phospholipids from naked mole-rats contain very low levels of DHA, the most polyunsaturated and most peroxidation-prone fatty acid naturally occurring in membranes (Hulbert et al., 2006a). DHA-containing phospholipids of mice tissues are 5 to 28-fold higher than in naked mole-rats; the greatest difference (28-fold) is evident in heart tissues; whereas a 12-fold difference is observed in skeletal muscle and there is a 5-fold difference in both liver and liver mitochondria (see Table 1). The very large difference in this highly peroxidisable fatty acyl chain is primarily responsible for the low peroxidation index calculated for naked mole-rat membrane lipids compared to mice. Indeed, naked mole-rats membrane lipids have the same level of unsaturated fatty acids as predicted for a mammal of their body size and essentially the same as mice. The low $n-3$ PUFA in naked mole-rat tissues is compensated by elevated MUFA in heart, brain and liver, and by elevated $n-6$ PUFA levels in skeletal muscle and heart phospholipids (see Fig. 2, Hulbert et al., 2006a). The very high $n-6$ PUFA levels in skeletal muscle and heart, coupled with the body size-predicted levels of $n-6$ PUFA in other tissues of naked mole-rats likely explain the high levels of lipid peroxidation products previously reported for the naked mole-rat (Andziak and Buffenstein, 2006). The markers used in this previous report were urinary isoprostanes and liver malondialdehyde both of which are products specific to the peroxidation of the $n-6$ PUFA arachidonic acid (Halliwell and Gutteridge, 1999) while levels of peroxidation products of $n-3$ PUFAs are lower in naked mole rats compared to mice (Buffenstein, unpublished data).

Other differences in the maximum longevity both among and within species have also been related to differences in membrane fatty acid composition. For example, birds are generally longer-living than similar-sized mammals and this is associated with a shift from $n-3$ PUFA to the less peroxidation-prone $n-6$ PUFA and consequently a lower peroxidation index of membranes compared to mammals (Hulbert, 2003, 2005). Wild-derived strains of *M. musculus* that have an extended longevity compared to a laboratory *Mus* strain, have peroxidation-resistant membrane fatty acid composition (Hulbert et al., 2006b), as do more longevous queen honeybees compared to worker bees (Haddad et al., 2007). The egg-laying mammal, the monotreme *Tachyglossus aculeatus*, is similar to the naked mole-rat in that it lives much longer than predicted from its body size, having a maximum lifespan of ~50 years (Carey and Judge, 2000). This corresponds to a LQ of ~5, and this species too has very low levels of DHA in its phospholipids and consequently a low membrane peroxidation index commensurate with its exceptional longevity (Hulbert and Beard, unpublished results).

To gain insight into how naked mole-rats maintain low DHA levels in their membrane lipids was one of the reasons for the current study. There is very little knowledge of how different species regulate their different membrane lipid compositions. That membrane lipid composition is regulated is manifest from studies showing that variation in dietary fatty acid composition has limited influence on membrane fatty acid composition (see Hulbert et al., 2005). That different strains of mice have different membrane fatty acid compositions when fed the same diet and maintained under identical conditions (e.g. Hulbert et al., 2006b) suggests that membrane fatty acid composition is likely controlled by enzymatic mechanisms with characteristics determined by the genome specific to the individual animal. The turnover of membrane lipids is more rapid than generally appreciated. For example, in the endoplasmic reticulum of liver cells, the half-lives of phospholipids

is shorter than that of many of the proteins in the membrane (Finean et al., 1974). The rapid remodelling of membrane phospholipids by deacylation/reacylation is suggested to be a rapid non-selective repair mechanism that when rat liver cells were subjected to oxidative stress removes damaged fatty acids from peroxidised phospholipids, replacing them with undamaged PUFA from the triacylglycerol pool (GironCalle et al., 1997).

Studies of rat hepatocytes show that only single DHA-containing molecular species of both phosphatidylcholine (16:0/22:6 PC) and phosphatidylethanolamine (16:0/22:6 PE) are synthesised *de novo* and that other DHA-containing molecular species are formed from remodelling of these *de novo* species by deacylation–reacylation processes (Schmid et al., 1995a). The incorporation of exogenous fatty acids into molecular species of PC in rat hepatocytes is also via the *de novo* pathway and extensive remodelling of such newly synthesized molecular species of PC occurs within 1 h (Schmid et al., 1995b). To our knowledge, these studies on rat liver cells provide the only quantitative information differentiating between those phospholipid molecular species that are derived from *de novo* synthesis and those derived from remodelling. Although it is speculative, we have assumed these findings also apply to skeletal muscle and heart cells. Thus assuming that, in naked mole-rats and mice, only the 16:0/22:6 PC and 16:0/22:6 PE are *de novo* synthesised and that other DHA-containing PC and PE are formed by deacylation/reacylation remodelling of these *de novo* produced molecules, we have plotted the relative distribution of these two classifications (*de novo* vs remodelled) of DHA-containing PC and PE for both species in Fig. 4. It can be seen from this figure that the *de novo*/remodelling division is identical in liver and liver mitochondria and similar for both PC and PE within each species although there is a small difference between species. In naked mole-rats the split is 40% *de novo*:60% remodelled, while in mice it is 60–65% *de novo*:35–40% remodelled. For both skeletal muscle and heart, there is a difference in the split between PC and PE. For skeletal muscle PC, the *de novo*:remodelled ratio was 54:46 for naked mole-rats and 79:21 for mice while for skeletal muscle PE it was 17:83 for naked mole-rats and 29:71 for mice. For heart PC, the ratio was 71:29 for naked mole-rats and 49:51 for mice. For heart PE, it was 25:75 for naked mole-rats and 23:77 for mice. Thus the balance between '*de novo*' and 'remodelled' DHA-containing phospholipids is shifted away from the '*de novo*' molecular species in naked mole-rats, compared to mice, in all tissues but the heart. In heart PE the balance is equal in both naked mole-rats and mice while for heart PC the balance is shifted to the 'remodelled' PC in mice compared to naked mole-rats. Taken together these suggest that it is likely that the low level of DHA in naked mole-rat membranes is due to a low rate of *de novo* synthesis of DHA-containing phospholipids. This may, in turn, be due to low levels of DHA synthesis and consequently implicate difference in the activities of desaturase and elongase enzymes in the tissues of naked mole rats compared to mice. Interestingly, the ratio of 20:4/18:2 which is sometimes used as a very approximate indicator of the relative activity of the desaturase/elongase systems is not significantly different between naked mole rats and mice for heart, liver or liver mitochondrial phospholipids but is significantly lower in naked mole rat skeletal muscle phospholipids but significantly higher in naked mole rat kidney phospholipids compared to the respective mice tissues (see supplementary Tables in Hulbert et al., 2006a).

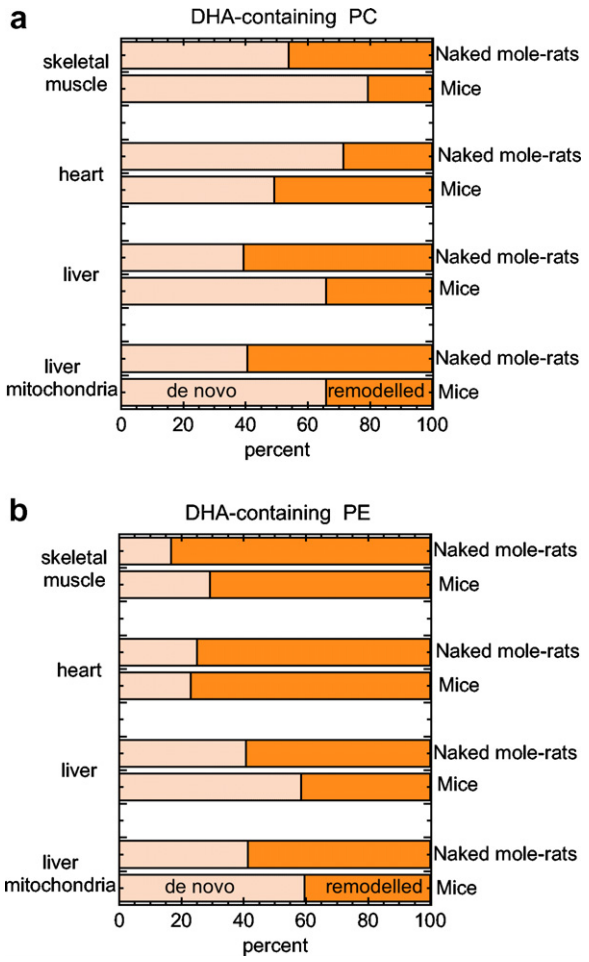

Fig. 4. The relative distribution between assumed '*de novo* synthesised' (light stippled bar) and 'remodelled' (dark solid bar) molecular species of DHA-containing phospholipids in the skeletal muscle, heart, liver and liver mitochondria of naked mole-rats (*H. glaber*) and laboratory mice (*M. musculus*). PC, phosphatidylcholines; PE, phosphatidylethanolamines. (see text for assumptions).

DHA is an unusual membrane fatty acid in that it differs from other acyl chains in its synthesis. In mammals, $n-3$ PUFA are essential components of the diet and generally the most common $n-3$ PUFA in the diet is linolenic acid (18:3 $n-3$). The synthesis of DHA (22:6 $n-3$) from the 18-carbon linolenic acid first involves the synthesis of a 24-carbon PUFA (24:6 $n-3$) by the same enzymes systems used to make other PUFA (namely elongases and desaturases). The unusual feature of DHA synthesis is that this 24-carbon PUFA is then transferred to the peroxisomes

where a single cycle of β-oxidation converts it to DHA (Sprecher, 2000).

Another interesting aspect of the phospholipid molecular species distribution was the presence of di-polyunsaturated phospholipids (18:2/22:6 PC and 18:2/22:6 PE) in small quantities in mouse tissues but their complete absence in naked mole-rats. As essentially only PUFA are capable of being peroxidised and the products of PUFA peroxidation are themselves powerful reactive oxygen species, which in turn, can initiate peroxidation of other PUFA, then it may be particularly deleterious for the membrane bilayer to have two PUFA fixed adjacent to one another in the same phospholipid molecule. This may be the reason such phospholipid molecular species are absent in naked mole-rats.

An unexpected finding was that, although naked mole rat tissues had no DHA-containing plasmenyl phospholipids, they had much higher levels of total plasmenyl phospholipids (especially plasmenylcholines) than mice. This is of interest because the functional significance of these phospholipids in membranes is not precisely known but one suggested function for which there is some evidence is that they are membrane antioxidants (Brosche and Platt, 1998). Plasmenyl phospholipids have been shown to be highly sensitive to oxidative attack at the vinyl ether linkage and this property has resulted in their suggested role as scavenger antioxidants. In a seminal study, Zoeller et al. (1988) showed that mutant chinese hamster ovary cells deficient in plasmalogens were killed when labelled with pyrene and subjected to ultraviolet light exposure (which produced reactive oxygen species). Restoration of the plasmalogen levels in these cells restored their resistance to UV-light-induced oxidative stress and these authors suggested that the vinyl ether linkage of plasmalogens may protect animal cell membranes against some oxidative stresses (Zoeller et al., 1988). While most of the evidence for such an antioxidant function of plasmenyl phospholipids (plasmalogens) comes from *in vitro* studies (e.g. Zommara et al., 1995; Sindelar et al., 1999), the recent production of the "ether lipid-deficient" mouse will likely be helpful in ascertaining the *in vivo* importance of the suggested antioxidant function of such lipids (Gorgas et al., 2006). Whether they have such a function and whether their high levels in naked mole-rats is functionally related to the exceptional longevity of these rodents compared to mice is of course as yet unknown.

It is of interest that, in mammals, the synthesis of the ether bond in these lipids requires two peroxisomal enzymes and that the first steps in the synthesis of plasmenyl phospholipids takes place in the peroxisomes (Gorgas et al., 2006). When this is coupled with the fact that the last steps of DHA synthesis also occur in peroxisomes (Sprecher, 2000), although catalysed by completely different enzymes to those involved in ether phospholipid synthesis, is it intriguing to speculate that there may be something special about peroxisomes in naked mole-rats that is crucial to their exceptional longevity.

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