Oxidative damage and phospholipid fatty acyl composition in skeletal muscle mitochondria from mice underexpressing or overexpressing uncoupling protein 3

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Five markers of different kinds of oxidative damage to proteins [glutamic semialdehyde, aminoadipic semialdehyde, N^{ϵ}-(carboxymethyl)lysine, N^{ϵ}-(carboxyethyl)lysine and N^{ϵ}-(malondialdehyde)lysine] and phospholipid fatty acyl composition were identified and measured in skeletal muscle mitochondria isolated from mice genetically engineered to underexpress or overexpress uncoupling protein 3 (UCP3). Mitochondria from UCP3-underexpressing mice had significantly higher levels of oxidative damage than wild-type controls, suggesting that UCP3 functions *in vivo* as part of the antioxidant defences of the cell, but mitochondria from UCP3-overexpressing mice had unaltered oxidative damage, suggesting that mild uncoupling *in vivo* beyond the normal basal uncoupling provides little protection against oxidative stress. Mitochondria from UCP3-underexpressing mice showed little change, but mitochondria from UCP3-overexpressing mice showed marked changes in mitochondrial phospholipid fatty acyl composition. These changes were very similar to those previously found to correlate with basal proton conductance in mitochondria from a range of species and treatments, suggesting that high protein expression, or some secondary result of uncoupling, may cause the observed correlation between basal proton conductance and phospholipid fatty acyl composition.

Key words: peroxidation, protein oxidation, proton leak, reactive oxygen species, superoxide.

INTRODUCTION

Mitochondria pump protons from the matrix to the cytoplasm, and couple their return to ATP synthesis. However, protons also leak back through other pathways. Mitochondrial proton leak is significant in hepatocytes and skeletal muscle and it may be responsible for up to 20 % of basal metabolic rate in rats [1,2]. It is useful to recognize two types. Inducible proton conductance is exemplified by the fatty acid-activated uncoupling catalysed by uncoupling protein (UCP) 1 in mitochondria from brown adipose tissue [3] and the superoxide-activated uncoupling catalysed by UCP3 in mitochondria from skeletal muscle [4]. Basal proton conductance is the constitutive pathway present in all mitochondria [2].

It is unclear whether basal proton leak has a function. Although the futile cycle of proton pumping and proton leak is thermogenic, its primary function is not thermogenesis (except in brown adipose tissue), since it occurs in ectotherms as well as endotherms [5]. An attractive hypothesis is that basal proton leak lowers mitochondrial membrane potential and decreases local oxygen concentration, thereby lowering mitochondrial production of superoxide and other reactive oxygen species (ROS) and protecting against oxidative damage and aging [6].

The mechanism of basal proton conductance remains unresolved. Three hypotheses cover most of the current ideas [2,7]: conductance is a basal, activator-independent activity of specific proteins, it is a more general property of mitochondrial proteins or protein–lipid interactions, or it occurs through the phospholipid bilayer. Skeletal muscle mitochondria from *UCP3*-knockout mice can be used to test whether UCP3 contributes to basal proton conductance. Two groups [8–10] have reported that the proton conductance of skeletal muscle mitochondria from *UCP3*$^{(-/-)}$ mice was decreased compared with controls. However, we found that basal proton conductance was not altered [11]. In addition, the basal proton conductance did not change when UCP3 concentration was changed physiologically [10,12], indicating that UCP3 does not contribute to this reaction. Here we use mitochondria from *UCP3*$^{(-/-)}$ and *UCP3*$^{(+/-)}$ mice to examine whether endogenous UCP3 protects mitochondria from oxidative damage through decreases in ROS production caused by basal uncoupling catalysed by UCP3 [8–10], or through an antioxidant activity related to activation of UCP3 by superoxide [4].

Skeletal muscle mitochondria from mice overexpressing human UCP3 are more uncoupled than controls *in vivo* [13] and *in vitro* [11]. However, they cannot be used to test for the physiological effects of the interaction of UCP3 with superoxide, since the

Abbreviations used: AASA, aminoadipic semialdehyde; CEL, N^{ϵ}-(carboxyethyl)lysine; CML, N^{ϵ}-(carboxymethyl)lysine; d4-AASA, [$^2H_4$]AASA; d4-CEL, [$^2H_4$]CEL; d4-CML, [$^2H_4$]CML; d5-GSA, [$^2H_5$]GSA; d8-lysine, [$^2H_8$]lysine; d8-MDA-lys, [$^2H_8$]MDA-lys; GSA, glutamic semialdehyde; MDA-lys, N^{ϵ}-(malondialdehyde)lysine; MUFA, mono-unsaturated fatty acid; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; SFA, saturated fatty acid; UCP, uncoupling protein; UFA, unsaturated fatty acid.

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uncoupling is an artifactual result of UCP3 overexpression and native superoxide sensitivity is lost [11]. They can, however, be used to test the effects of mild uncoupling *in vivo* on the accumulation of oxidative damage. Here we use mitochondria from UCP3-overexpressing mice to examine whether mild uncoupling, superimposed on the endogenous basal proton leak, can protect mitochondria *in vivo* from oxidative damage.

We also test whether UCP3 underexpression has effects on the fatty acyl composition of the mitochondrial phospholipids. If UCP3 underexpression *in vivo* raises ROS production and causes increased oxidative damage to the most highly unsaturated peroxidizable fatty acyl chains, we might expect to see evidence of this in decreased steady-state abundance of the most sensitive fatty acyl groups.

Phospholipid fatty acyl composition correlates significantly with basal proton conductance in mitochondria from different animal species and thyroid hormone states [5,14–16]. Greater basal proton conductance is associated with lower proportions of mono-unsaturated fatty acids (MUFAs) (notably $18:1n-9$) or $18:2n-6$, and higher proportions of $n-3$ polyunsaturated fatty acids (PUFAs), notably $22:6n-3$ [5,16–20]. This correlation led to the hypothesis that differences in fatty acyl composition cause differences in proton conductance [21]. There is some support for this hypothesis [22,23], but proton conductance is the same in liposomes formed from phospholipids isolated from a range of mitochondria with very different proton conductances [24], suggesting that fatty acyl composition cannot affect basal proton conductance through purely phospholipid-based mechanisms. Here we investigate the correlations between fatty acyl composition and basal proton leak by asking whether uncoupling *in vivo* caused by overexpression of UCP3 affects the fatty acyl composition of the mitochondrial phospholipids.

The results show that underexpression of UCP3 *in vivo* leads to increased oxidative damage to mitochondrial proteins, supporting a role for UCP3 in protection against oxidative stress, and that overexpression of UCP3 *in vivo* leads to changes in phospholipid fatty acyl composition, suggesting that overexpression of UCP3, or some secondary result of the consequent artifactual uncoupling, leads to the observed compositional patterns that correlate with basal proton conductance.

EXPERIMENTAL

Animals

Mice underexpressing UCP3 (10–15 weeks old) were homozygous $UCP3^{(-/-)}$ [4] or heterozygous $UCP3^{(+/-)}$ littermates [11]. Mice overexpressing human UCP3 (10–15 weeks old) were homozygotes from the *UCP3*-tg strain [13]. Controls were wild-type littermates of the underexpressers or overexpressers.

Mitochondria

Skeletal muscle mitochondria were prepared as described in [11]. Protein content was determined by the biuret method. All samples were from the mitochondrial preparations used for the proton conductance measurements reported previously [4,11,13]. They were kept on ice for several hours and then stored at -20 °C for 0.5–2 years before compositional analysis. There was no trend for markers of oxidative damage to be higher or phospholipid fatty acyl composition to be different in older samples, indicating that storage of samples did not affect the results reported here. Because of limited sample volume and availability, some samples were pooled before assay. For the wild-type, seven individual mitochondrial preparations, one pooled sample of two preparations and one pooled sample of three preparations were used, making $n = 9$. For UCP3 overexpression, six individual *UCP3*-tg mitochondrial preparations were used ($n = 6$). For UCP3 underexpression, $UCP3^{(-/-)}$ and $UCP3^{(+/-)}$ preparations showed the same compositional trends, so the values were averaged to give greater statistical power. Two individual $UCP3^{(+/-)}$ preparations, one $UCP3^{(-/-)}$ preparation, one pooled sample of two $UCP3^{(-/-)}$ preparations and one pooled sample of three $UCP3^{(-/-)}$ preparations were used, making $n = 5$. We have previously shown using Western blots that skeletal muscle mitochondria from $UCP3^{(-/-)}$ mice lack detectable UCP3 protein [25], and skeletal muscle mitochondria from $UCP3^{(+/-)}$ mice have less than 10 % of the UCP3 protein content of mitochondria from wild-type mice [11].

GC/MS measurements

Glutamic semialdehyde (GSA), aminoadipic semialdehyde (AASA), N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(carboxyethyl)-lysine (CEL) and N^{ε}-(malondialdehyde)lysine (MDA-lys) concentrations in mitochondrial proteins were measured by GC/MS as described previously [26–28]. Samples containing 0.5–1 mg of protein were delipidated using chloroform/methanol (2:1, v/v), and protein was precipitated by adding trichloroacetic acid to a 10 % (v/v) final concentration, followed by centrifugation. Protein samples were reduced by overnight reduction with 500 mM $NaBH_4$ in 0.2 M borate buffer, pH 9.2, containing 1 drop of hexanol as an anti-foam reagent. Protein was reprecipitated by adding 1 ml of 20 % (v/v) trichloroacetic acid, followed by centrifugation. The following isotopically labelled internal standards were then added. [$^2H_8$]Lysine (d8-lysine) was from CDN Isotopes, [$^2H_4$]CML (d4-CML), [$^2H_4$]CEL (d4-CEL) and [$^2H_8$]MDA-lys (d8-MDA-lys) were prepared as described in [29–31], and [$^2H_5$]GSA (d5-GSA) and [$^2H_4$]AASA (d4-AASA) were prepared as described in [28]. The samples were hydrolysed at 155 °C for 30 min in 1 ml of 6 M HCl, then dried *in vacuo*. The *N*,*O*-trifluoroacetyl methyl ester derivatives of the protein hydrolysate were prepared as described previously [29]. GC/MS analyses were carried out on a Hewlett-Packard model 6890 gas chromatograph equipped with a HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) coupled to a Hewlett-Packard model 5973A mass selective detector. The injection port was maintained at 275 °C; the temperature program was 5 min at 110 °C, then 2 °C/min to 150 °C, 5 °C/min to 240 °C, 25 °C/min to 300 °C, and finally it was held at 300 °C for 5 min. Quantification was performed by external standardization using standard curves constructed from mixtures of deuterated and non-deuterated standards. For each sample, the amounts of internal standard finally injected for quantification were 3 nmol of d8-lysine, 12 pmol of d4-CML, 10 pmol of d4-CEL, 10 pmol of d8-MDA-lys, 16 pmol of d5-GSA and 3.5 pmol of d4-AASA. Standard curves of non-deuterated standards ranged as follows: lysine, 2.5–25 nmol; CML, 5–80 pmol; CEL, 5–75 pmol; MDA-lys, 5–75 pmol; d5-GSA, 2–20 pmol and d4-AASA, 2–20 pmol.

Analyses were carried out by selected ion-monitoring GC/MS. The specificity of this procedure was ensured by (i) identity in retention time of analysed peaks with reference to isotopically labelled internal standards and (ii) previous analyses of mass spectra supporting the use of characteristic prominent peaks for each compound [28–31]. The characteristic ions used were: lysine and d8-lysine, m/z 180 and 187, respectively; GSA and d5-GSA, m/z 280 and 285, respectively; AASA and d4-AASA, m/z 294 and 298, respectively; CML and d4-CML, m/z 392 and 396, respectively; CEL and d4-CEL, m/z 379 and 383, respectively; and MDA-lysine and d8-MDA-lys, m/z 474 and 482, respect-

ively. The amounts of product were expressed as the ratio μmol of GSA, AASA, CML, CEL or MDA-lys/mol of lysine.

Fatty acid analysis

Phospholipid fatty acyl groups were analysed as described in [32]. Mitochondrial phospholipids were extracted with chloroform/methanol (2:1, v/v) in the presence of 0.01 % (w/v) butylated hydroxytoluene. The chloroform phase was evaporated under nitrogen and the fatty acyl groups were transesterified by incubation in 2.5 ml of 5 % (v/v) methanolic HCl for 90 min at 75 °C. The resulting fatty acid methyl esters were extracted by adding 2.5 ml of *n*-pentane and 1 ml of saturated NaCl solution. The *n*-pentane phase was separated and evaporated under nitrogen. The residue was dissolved in 75 μl of carbon disulphide and 1 μl was used for GC/MS analysis. Separation was performed in a SP2330 capillary column (30 m × 0.25 mm × 0.20 μm) in a Hewlett Packard 6890 Series II gas chromatograph. A Hewlett Packard 5973A mass spectrometer was used as detector in the electron-impact mode. The injection port was maintained at 220 °C, and the detector at 250 °C; the temperature program was 2 min at 100 °C, then 10 °C/min to 200 °C, 5 °C/min to 240 °C, and finally it was held at 240 °C for 10 min. Identification of fatty acyl methyl esters was made by comparison with authentic standards and on the basis of mass spectra. Results are expressed as mol %. The following fatty acyl indices were calculated: saturated fatty acids (SFAs); unsaturated fatty acids (UFAs); $n-3$ PUFAs and $n-6$ PUFAs; double-bond index = [(Σmol % monoenoic) + (2 × Σmol % dienoic) + (3 × Σmol % trienoic) + (4 × Σmol % tetraenoic) + (5 × Σmol % pentaenoic) + (6 × Σmol % hexaenoic)]; and peroxidizability index = [(0.025 × Σmol % monoenoic) + (Σmol % dienoic) + (2 × Σmol % trienoic) + (4 × Σmol % tetraenoic) + (6 × Σmol % pentaenoic) + (8 × Σmol % hexaenoic)] [33].

Statistics

Values were compared using Student's *t* test. P values < 0.05 were considered to be significant.

RESULTS AND DISCUSSION

Oxidative damage

Five markers of different kinds of oxidative damage were measured in skeletal muscle mitochondria from wild-type mice and mice overexpressing or underexpressing UCP3. We used markers of direct oxidation of proteins (GSA, AASA) and of carbonyl-amine reactions, such as lipoxidation and glycoxidation (CML, MDA-Lys, CEL). GSA and AASA are the main carbonyl products of metal-catalysed protein oxidation [28], and are derived from proline and arginine, and lysine respectively [34]. CML [29] and MDA-lys [31] are derived from protein adducts of lipid peroxidation products. CEL is derived from the reaction of methylglyoxal with proteins [30]. In cells, methylglyoxal is generated from metabolic processes such as base-catalysed phosphate elimination from glyceraldehyde 3-phosphate and dihydroxyacetone phosphate.

Mild uncoupling has been proposed to protect mitochondria and their host cells from oxidative damage by limiting formation of ROS due to decreased mitochondrial membrane potential and decreased local oxygen availability [6]. In support of this hypothesis, isolated mitochondria produce less ROS when uncoupled [35–37] or when membrane potential is lowered [6,38–40]. However, there were no significant differences in any of the markers of oxidative damage between mitochondria from wild-type and UCP3-overexpressing mice (Figure 1), indicating that the observed increased *in vivo* uncoupling caused by overexpression of UCP3 [11,13] did not protect the mitochondria against oxidative damage. There are several possible explanations. Perhaps uncoupling does not protect *in vivo* against free-radical-induced oxidative damage, or such protection is already optimal and increased uncoupling has no further effect, or our methods are insufficient to detect such protection. Perhaps the *UCP3*-tg model, which causes artifactual uncoupling without increasing native UCP3 function, does not allow possible protective effects of native uncoupling against protein oxidation to be observed, because it has secondary effects (such as effects due to the observed higher rates of fatty acid oxidation [13]) that increase oxidative damage and mask the *in vivo* protection.

In contrast, two of the markers, AASA and CML, were significantly increased in mitochondria with lowered UCP3 content (Figure 1). The other three markers also increased, but without quite reaching statistical significance. These results indicate that the endogenous UCP3 in skeletal muscle mitochondria protects against protein oxidative damage *in vivo*. They are consistent with observations that skeletal muscle mitochondria from *UCP3*-knockout mice produce more ROS and have more damaged aconitase [8]. They also fit the pattern seen with UCP2: macrophages from *UCP2*$^{(-/-)}$ mice produce more ROS than controls [41], and clonal pancreatic β-cells transiently transfected with *UCP2* are less sensitive than controls to H_2O_2 [42].

Protection against oxidative damage by endogenous UCP3 could be mediated by an uncoupling activity of UCP3 leading to a lower steady-state mitochondrial membrane potential, lower ROS production and lower oxidative damage. In support of this, the basal proton conductance of skeletal muscle mitochondria from *UCP3*$^{(-/-)}$ mice has been reported to be decreased compared with controls [8–10]. However, we have observed no difference in the basal proton conductance of such mitochondria [11], and the basal proton conductance does not change when UCP3 protein concentration is altered by physiological manipulations [10,12], so we do not favour this explanation.

A more attractive hypothesis is that the increased oxidative damage in mitochondria from *UCP3*$^{(-/-)}$ mice is related to the superoxide-sensitive inducible proton conductance catalysed by UCP3. Exogenous superoxide stimulates proton conductance in skeletal muscle mitochondria from wild-type but not *UCP3*$^{(-/-)}$ mice [4]. This may reflect a physiological feedback mechanism to limit oxidative damage by endogenous superoxide: under transient conditions of activated substrate supply but low ATP demand, which will cause mitochondrial membrane potential to be high (such as adrenergic stimulation), mitochondrial superoxide production may rise. In these conditions, superoxide would activate inducible uncoupling by UCP3 to lower membrane potential and local oxygen concentration, leading to a negative feedback control over superoxide production [4]. The lowered UCP3 concentration in mitochondria from UCP3-underexpressing mice would diminish this feedback control, causing greater oxidative damage to mitochondria *in vivo*. Alternatively, the exogenous superoxide activation of uncoupling by UCP3 may be an *in vitro* manifestation of a more subtle endogenous superoxide–UCP3 interaction that is part of the mitochondrial ROS defences. For example, UCP3 might function physiologically to protect against ROS by transporting superoxide anion from the mitochondrial matrix to the intermembrane space for disposal; the uncoupling by exogenous superoxide would be caused by an unphysiological cycle of massive uptake of protonated exogenous superoxide followed by UCP3-dependent export of superoxide anion [4]. Again, lack of UCP3 would result in the observed oxidative damage *in vivo*. The hypothesis that UCP3 ablation

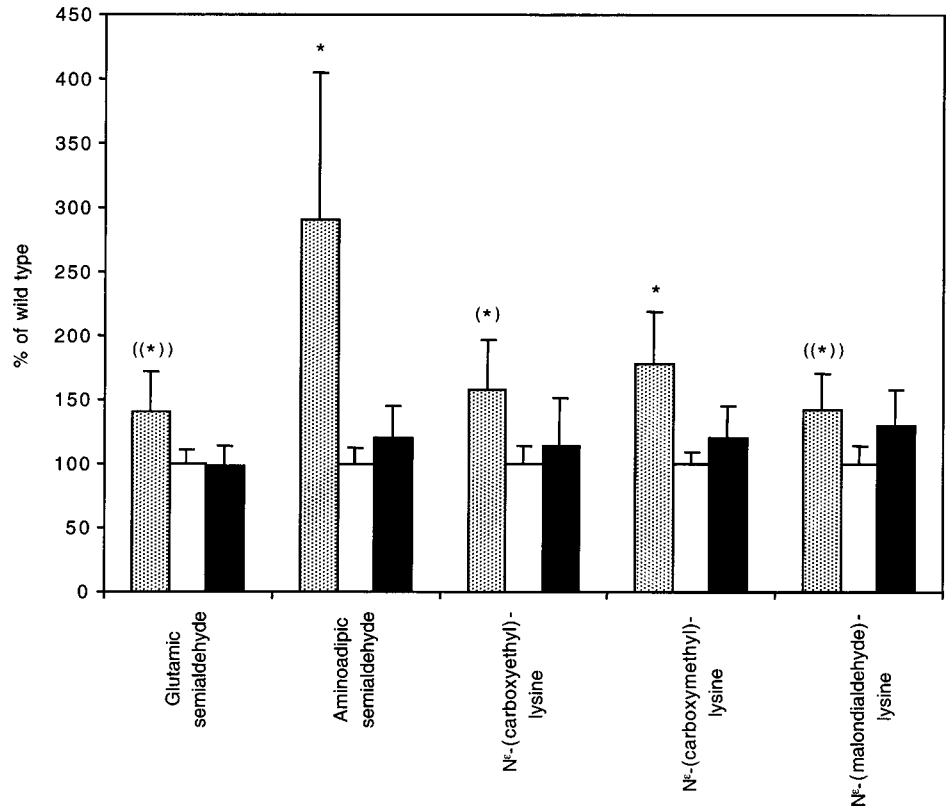

Figure 1 Markers of oxidative, glycoxidative or lipoxidative stress in skeletal muscle mitochondrial proteins from wild-type, *UCP3*$^{(-/-)}$/*UCP3*$^{(+/-)}$ and *UCP3*-tg mice

For details see the Experimental section. Values, expressed as percentages of the wild-type value, are means ± S.E.M. from $n = 9$, 6 and 5 samples for wild-type, *UCP3*$^{(-/-)}$/*UCP3*$^{(+/-)}$ and *UCP3*-tg mice respectively. Stippled bars, UCP3-underexpressing mice; white bars, wild-type mice; black bars, UCP3-overexpressing mice. Raw 100% mean values (in μmol/mol of lysine) were: GSA, 14030; AASA, 526; CEL, 1459; CML, 3408; MDA-lys, 2357. *$P < 0.05$, (*)$P < 0.1$, ((*))$P < 0.15$ versus wild-type mice.

leads to oxidative damage not through simple basal uncoupling but by the interaction of superoxide with UCP3 could explain the lack of protection against oxidative damage seen in mitochondria from UCP3-overexpressing mice. In these mitochondria UCP3 uncouples because of an overexpression artifact, and the native superoxide-dependent activity is absent [11].

Phospholipid fatty acyl composition

Remarkably, the effects of altered expression of UCP3 *in vivo* on phospholipid fatty acyl composition were the opposite of the effects on oxidative damage: UCP3 underexpression had no effect on fatty acid profiles, while UCP3 overexpression caused significant changes. Since UCP3 underexpression had significant effects on the markers of oxidative damage (above), but not on fatty acyl composition, it seems that fatty acyl compositional changes are a less sensitive index of ROS-induced damage to mitochondria than the markers of oxidative damage studied here.

Figure 2 and Table 1 show that there were no significant differences in the abundance of individual phospholipid fatty acyl groups (except for the minor component 22:4$n-6$), and Figure 3 shows that there were no significant differences in any of the global fatty acid indices in skeletal muscle mitochondria from UCP3-underexpressing mice compared with controls. These observations fail to support the suggestion [10], based on single fatty acid analyses of pooled samples, that $n-3$ PUFAs and $n-6$ PUFAs are higher, and that SFAs and 16:0 are lower, in mitochondria from *UCP3*$^{(-/-)}$ mice. Our UCP3 underexpressers did have lower average SFAs and 16:0 (and higher UFAs) than wild-type mice, but the differences were not significant (Table 1). However, comparison of our three samples from *UCP3*$^{(-/-)}$ mice with controls did reveal significant decreases in SFAs and 18:0, and significant increases in UFAs and 16:1$n-7$ ($P < 0.05$; results not shown).

In contrast, when we compared phospholipid fatty acyl composition between skeletal muscle mitochondria from wild-type and UCP3-overexpressing mice, there were numerous significant differences (Table 1). Figure 2 shows that there were changes in relative abundance of all five of the fatty acyl groups that contributed more than 10 mol% to mitochondrial phospholipid fatty acyl composition. Mitochondria from UCP3 overexpressers had significantly lower amounts of 16:0, 18:1$n-9$ and 18:2$n-6$, and significantly higher amounts of 18:0 and 22:6$n-3$. These changes led to significantly lower proportions of total MUFAs, significantly higher proportions of total $n-3$ PUFAs, and significantly greater average chain length, double-bond index and peroxidizability index in the UCP3-overexpressing mice (Figure 3).

In the present experiments we overexpressed UCP3 in mouse skeletal muscle, and this primary change led secondarily to artifactual *in vivo* and *in vitro* increases in basal mitochondrial proton conductance [11,13] and to significant changes in mito-

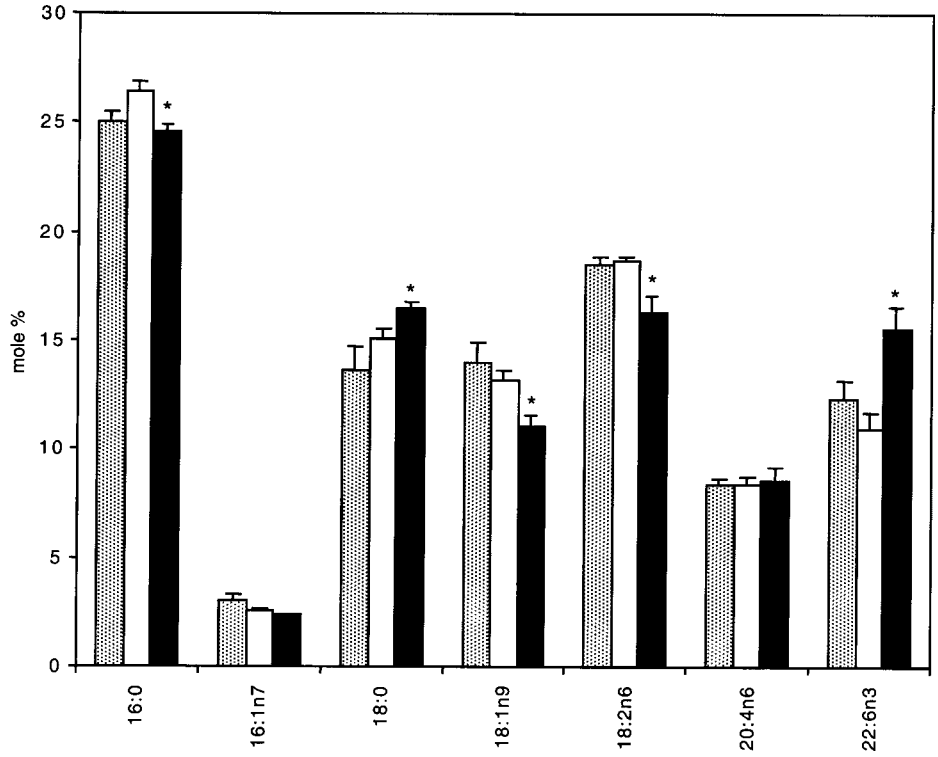

Figure 2 Major fatty acyl groups in phospholipids of skeletal muscle mitochondria from wild-type, *UCP3*$^{(-/-)}$/*UCP3*$^{(+/-)}$ and *UCP3*-tg mice

Only fatty acyl groups comprising more than 2 mol% are shown. For details see Table 1. Values are means ± S.E.M. from $n = 9$, 6 and 5 samples for wild-type, *UCP3*$^{(-/-)}$/*UCP3*$^{(+/-)}$ and *UCP3*-tg mice respectively. Stippled bars, UCP3-underexpressing mice; white bars, wild-type mice; black bars, UCP3-overexpressing mice. $^*P < 0.05$ versus wild-type.

Table 1 Fatty acyl composition of phospholipids of skeletal muscle mitochondria from wild-type, *UCP3*$^{(-/-)}$/*UCP3*$^{(+/-)}$ and *UCP3*-tg mice

For details see the Experimental section. Values are means ± S.E.M. from $n = 9$, 6 and 5 samples for wild-type, *UCP3*$^{(-/-)}$/*UCP3*$^{(+/-)}$ and *UCP3*-tg mice, respectively. *P* values are shown for comparison with the respective wild-type samples; $^*P < 0.05$.

Fatty acyl group	Wild-type mice mol%	UCP3-underexpressing mice		UCP3-overexpressing mice	
		mol%	*P* value	mol%	*P* value
14:0	0.2 ± 0.0	0.1 ± 0.0	0.141	0.2 ± 0.0	0.857
14:1	0.4 ± 0.0	0.3 ± 0.1	0.089	0.3 ± 0.0	0.055
16:0	26.4 ± 0.5	25.0 ± 0.5	0.117	24.5 ± 0.3	0.020*
16:1*n*−7	2.6 ± 0.1	3.0 ± 0.3	0.111	2.4 ± 0.1	0.155
18:0	15.1 ± 0.4	13.6 ± 1.1	0.170	16.5 ± 0.3	0.045*
18:1*n*−9	13.1 ± 0.5	14.0 ± 1.0	0.411	11.0 ± 0.5	0.015*
18:2*n*−6	18.7 ± 0.2	18.5 ± 0.4	0.677	16.3 ± 0.7	0.002*
18:3*n*−3	0.3 ± 0.0	0.3 ± 0.0	0.079	0.3 ± 0.0	0.851
20:3*n*−6	0.4 ± 0.0	0.5 ± 0.0	0.801	0.4 ± 0.0	0.746
20:4*n*−6	8.4 ± 0.4	8.4 ± 0.2	1.000	8.5 ± 0.6	0.835
22:4*n*−6	0.4 ± 0.0	0.6 ± 0.1	0.013*	0.3 ± 0.0	0.191
22:5*n*−3	0.8 ± 0.1	1.0 ± 0.1	0.216	1.1 ± 0.0	0.020*
22:5*n*−6	0.6 ± 0.1	0.6 ± 0.1	0.966	0.5 ± 0.1	0.336
22:6*n*−3	10.9 ± 0.8	12.3 ± 0.8	0.277	15.6 ± 0.9	0.002*
24:0	1.5 ± 0.1	1.6 ± 0.1	0.636	1.9 ± 0.1	0.032*
24:1*n*−9	0.3 ± 0.0	0.3 ± 0.1	0.841	0.3 ± 0.0	0.668
SFAs	43.2 ± 0.8	40.4 ± 1.2	0.078	43.0 ± 0.3	0.906
UFAs	56.8 ± 0.8	59.6 ± 1.2	0.078	57.0 ± 0.3	0.904
MUFAs	16.4 ± 0.6	17.6 ± 1.1	0.306	14.0 ± 0.5	0.013*
PUFAs	40.5 ± 1.0	42.0 ± 0.6	0.285	43.0 ± 0.6	0.081
n−3 PUFAs	12.0 ± 0.8	13.6 ± 0.8	0.215	16.9 ± 0.9	0.002*
n−6 PUFAs	28.4 ± 0.5	28.4 ± 0.4	0.943	26.1 ± 1.4	0.078
Average chain length	18.2 ± 0.0	18.3 ± 0.0	0.211	18.4 ± 0.0	0.001*
Double-bond index	163.4 ± 5.0	174.1 ± 4.2	0.181	185.1 ± 2.8	0.006*
Peroxidizability index	151.3 ± 6.7	163.8 ± 6.1	0.247	187.1 ± 4.9	0.002*

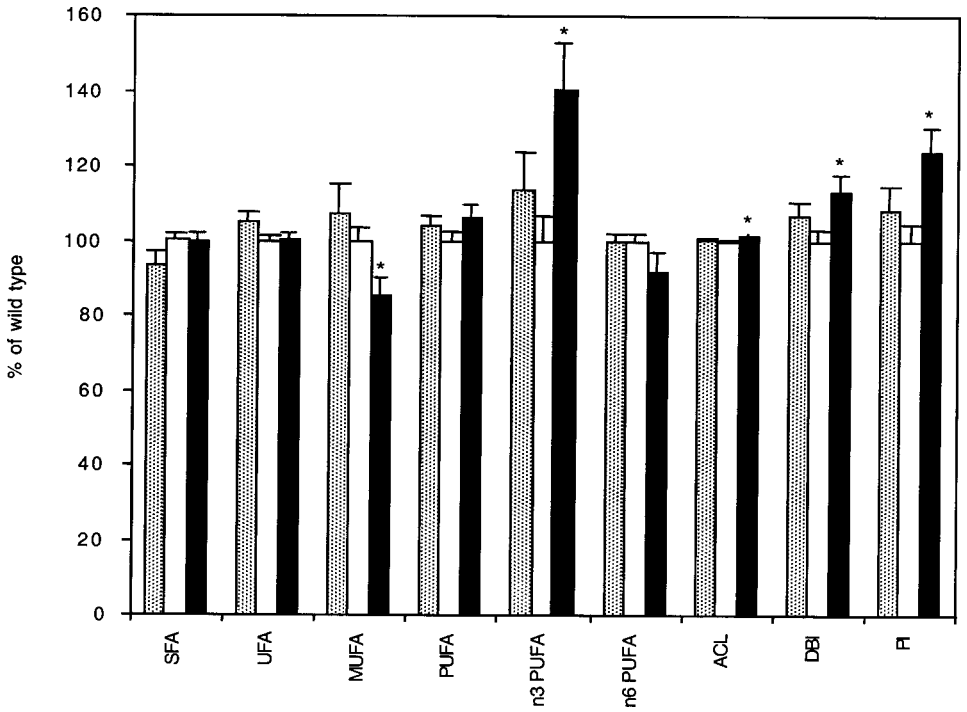

Figure 3 Global fatty acyl group parameters in phospholipids of skeletal muscle mitochondria from wild-type, *UCP3*$^{(-/-)}$/*UCP3*$^{(+/-)}$ and *UCP3*-tg mice

For details see Table 1. Values, expressed as percentages of the wild-type value, are means ± S.E.M. from $n = 9$, 6 and 5 samples for wild-type, *UCP3*$^{(-/-)}$/*UCP3*$^{(+/-)}$ and *UCP3*-tg mice respectively. Stippled bars, UCP3-underexpressing mice; white bars, wild-type mice; black bars, UCP3-overexpressing mice. *$P < 0.05$ versus wild-type. DBI, double-bond index; PI, peroxidizability index; ACL, average chain length.

chondrial fatty acyl composition (Table 1). The relationship between proton conductance and fatty acyl composition in Table 1 is remarkably similar to the relationship seen when mitochondria are compared across a wide range of animal species, tissues and thyroid hormone states [5,14–16,21]. In general, greater basal proton conductance in cross-species comparisons is associated with lower proportions of MUFAs, notably 18:1$n-9$, and higher proportions of $n-3$ PUFAs, notably 22:6$n-3$ [5], while the hypothyroid to euthyroid transition leads to increased liver mitochondrial proton conductance and is particularly associated with lower 18:2$n-6$ [16–20,23]. These are exactly the changes caused by artifactually increasing basal proton conductance by overexpression of UCP3 (Table 1).

We originally suggested that the hormonal and cross-species correlation reflected a mechanistic relationship in which fatty acyl composition caused the differences in proton conductance [21]. We tested this hypothesis by isolating mitochondrial phospholipids with different fatty acyl profiles from mitochondria with different proton conductances from a range of animals, and measuring the proton conductance of liposomes formed from these phospholipids. The test showed that fatty acyl composition had no influence on liposome proton conductance, and failed to support the hypothesis [24]. While this result can be explained in other ways, the present results suggest the opposite hypothesis, that differences in phospholipid fatty acyl composition are a secondary result of primary differences in proton conductance. In this hypothesis, UCP3 overexpression causes artifactual uncoupling, leading secondarily to the compositional changes seen in Table 1; thyroid hormones physiologically increase mitochondrial proton conductance [17], leading secondarily to the observed changes in mitochondrial phospholipid fatty acyl composition [20] and different species have different mitochondrial proton conductance because they need to match proton conductance to metabolic rate [14,15,21], leading to the observed differences in fatty acyl composition and the observed correlations between proton conductance and membrane fatty acyl composition [5]. Alternatively, fatty acyl composition and mitochondrial proton conductance might be affected independently by some other factor that is modified by UCP3 overexpression, by thyroid hormone action or by evolutionary pressure to raise metabolic rates. This factor might be the density of proteins (or thyroid hormones) in the mitochondrial inner membrane, leading to alterations in phospholipid–protein interactions that change both proton conductance and phospholipid processing by desaturases and other enzymes.

In conclusion, our results suggest that the native function of UCP3 is related to protection against damage by ROS, since such damage increased in skeletal muscle mitochondria from *UCP3*$^{(-/-)}$ mice. Our results fail to support the hypothesis that increased basal proton conductance protects against ROS in skeletal muscle, since overexpression of UCP3, which causes uncoupling *in vivo* and *in vitro*, did not decrease oxidative damage to skeletal muscle mitochondria. Our finding that UCP3 overexpression led to changes in the fatty acyl composition of mitochondrial phospholipids supports the hypotheses that changes in fatty acyl composition can be caused by changes in proton conductance, or that composition and conductance are correlated because they both change in response to some other property (such as the protein composition) of the mitochondrial membrane.

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