Improved glucose homeostasis in mice overexpressing human UCP3: a role for AMP-kinase?

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SHORT COMMUNICATION

Improved glucose homeostasis in mice overexpressing human UCP3: a role for AMP-kinase?

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OBJECTIVE: An unexplained phenotype of mice overexpressing human UCP3 is their improved glucose homeostasis. Since overexpression of UCP3 might affect the energy charge of the cell, we investigated whether these mice have an increased AMP-activated protein kinase (AMPK) activity.
METHODS: Mitochondrial localisation of UCP3 was determined by immunoelectronmicroscopy and AMPK activity was measured in medial gastrocnemius of control mice and mice overexpressing human UCP3.
RESULTS: Mice overexpressing human UCP3 had 5.8 fold higher levels of UCP3 protein, for which mitochondrial localisation was confirmed by immunoelectronmicroscopy. The ATP/AMP ratio was significantly lower in mice over-expressing UCP3 compared to the wild-type (10.9 ± 1.6 *vs* 20.4 ± 1.9 AU, $P = 0.03$). Over-expression of UCP3 resulted in increased AMPK $\alpha 1$ activity (1.23 ± 0.05 *vs* 1.00 ± 0.06 normalized values, $P = 0.004$) and a tendency towards increased AMPK $\alpha 2$ activity (1.18 ± 0.08 *vs* 1.00 ± 0.10 normalized values, $P = 0.08$).
CONCLUSION: Increased AMPK activity provides a plausible explanation for the improved glucose tolerance characteristic for these mice.
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Introduction

The human uncoupling protein-3 (UCP3) has been associated with the regulation of glucose metabolism and type II diabetes mellitus. Concerted upregulation of UCP3 and GLUT4 mRNA upon cold exposure[1] and acute exercise[2] has been interpreted as evidence for a role of UCP3 in glucose metabolism. Furthermore, we have recently shown that type II diabetic subjects have 2-fold lower UCP3 protein levels compared to healthy controls, suggesting that here also UCP3 might be involved in the disturbances in glucose homeostasis characteristic for type II diabetes mellitus.[3] In addition to these associations between UCP3 and glucose metabolism, it has also been shown that overexpression of UCP1 in skeletal muscle (to a level of only 1% of the UCP expression naturally occurring in brown adipose tissue) increases skeletal muscle glucose transport and protects against insulin resistance and hyperglycemia induced by high-fat feeding.[4] Also, mice overexpressing human UCP3 in skeletal muscle are characterized by reduced fasting glucose concentration, improved glucose tolerance following an oral glucose load and lower insulin levels.[5] However, the mechanism by which UCP3 can affect glucose homeostasis is presently unknown. Nowadays, the prevailing opinion is that the primary physiological function of UCP3 is most likely in the prevention of ROS production[6] or in fatty acid metabolism.[7–9] It is, therefore, possible that through these putative functions in ROS production and/or fatty acid metabolism, UCP3 could indirectly interact with glucose metabolism, explaining the observed associations between UCP3 and glucose metabolism. However, it is also important to note that as a side effect of its primary role, UCP3 might still be able to uncouple mitochondria, especially when the

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(over)expression of UPC3 is very high.[10] Indeed, supraphysiological overexpressing UCP3 in yeast[11] and mice,[5] reduces the proton gradient across the inner mitochondrial membrane, which is used to generate the energy for the phosphorylation of ADP to form ATP**.** In such conditions the adenylate kinase reaction ($2\,ADP \rightleftharpoons ATP + AMP$) may help to maintain cellular ATP levels with a concomitant increase in AMP. The latter is a well-known activator of the enzyme AMP-activated protein kinase (AMPK), an enzyme responsible for the phosphorylation of key enzymes that control metabolic flux, among which is glucose uptake (via GLUT4 translocation).[12] Therefore, the beneficial effect of (surpraphysiological) UCP3 overexpression on glucose homeostasis could reflect an effect of mitochondrial uncoupling on AMPK activity. In this context, chemical compounds that uncouple mitochondrial respiration have also been shown to increase glucose uptake in muscle cells.[13] Therefore, we used mice overexpressing human UCP3 (UCP3-Tg), which were shown to have improved glucose tolerance[5] and their wild-type littermates (wild-type) to test the hypothesis that improved glucose homeostasis in these mice can be accounted for by increased activity of the $\alpha 1$ and/or $\alpha 2$ isoform of AMPK. In addition, we examined the localisation of UCP3 in these UCP3-Tg mice, to confirm that UCP3 indeed was located in the inner mitochondrial membrane.

Materials and methods

Animals

A total of 18 UCP3-Tg mice (aged: 21.4 ± 1.2 weeks) and 16 wild-type littermates (aged: 22.2 ± 1.4 weeks) were used. Creation of UCP3 overexpressing mice has been described previously.[5] Animals were housed individually on a 12:12 h light–dark cycle (light from 0700 to 1900 hours), at 21–22°C, and allowed unlimited access to standard chow diet. The studies were approved by the Institutional Animal Care and Use Committee of the Maastricht University and complied with the principles of laboratory animal care. Under general anaesthesia (1.5–2.0% halothane in O_2 and N_2O (3:1, 4.0 l/min), medial gastrocnemius muscles were rapidly excised unilaterally and freeze-clamped within 7 s after cutting the nerve and blood vessels.

UCP3 protein

Endogenous and human UCP3 protein content was determined by Western blotting, using rabbit polyclonal antibodies against mouse (code 1338) and human UCP3 (code, 1331, kindly provided by LJ Slieker, Eli Lilly.)[10] Immunogold electron microscopy was performed on an ultrathin cryosections of medial gastrocnemius essentially according to Martinez–Menarguez.[14] By combining our antibody specifically detecting human UCP3 with the antibody detecting mice UCP3, we were able to differentiate between endogenous UCP3 expression and expression of hUCP3 in the transgene.

AMPK activity and nucleotides

Immunoprecipitate kinase assays were performed as described previously, using AMPK anti-$\alpha 1$ and anti-$\alpha 2$ antibodies.[15] Samples of frozen muscle of eight UCP3-Tg and wild-type mice were ground to powder under liquid nitrogen with a pestle and mortar. The powdered muscle was homogenized with an equal volume of 5% perchloric acid, and then centrifuged at 14 000 rpm and 4°C, for 3 min to remove acid-insoluble material. The supernatant was then extracted with two washes of a 10% excess (volume) of 1:1 tri-*n*-octylamine and 1,1,2,-trichlorotrifluoroethane. Nucleotides were separated by capillary electrophoresis with on-column isotachophoretic concentration, using run buffers consisting of 100 mM sodium phosphate, and 50 mM NaCl (pH 5.7; leading buffer); and 100 mM MES/Tris pH 5.7 (tailing buffer). To each buffer 0.2% hydroxyethyl-cellulose was added to decrease the electro-osmotic flow. Before running each sample, a 10% volume of leading buffer was added containing 8-Br-cAMP as a reference compound. Nucleotide peaks were detected by UV absorbance at 260 nM (ref. 350 nM), and integrated using System Gold software (Beckman). Nucleotide ratios were calculated from peak areas after correction for retention times. Peaks due to added ATP, ADP and AMP coincided with the main sample peaks supporting their assignment as such.

Statistical analysis

Results are presented as mean ± s.e. Differences between groups were evaluated by unpaired *t*-tests. Pearsons' correlation coeffficients were calculated to determine the relationship between selected variables. Outcomes were regarded statistically significant if $P<0.05$.

Results

UCP3-Tg mice had 5.8-fold higher levels of total UCP3 protein (human + endogenous) compared to wild types ($P<0.0001$). Moreover, we confirmed the mitochondrial localisation of human UCP3 by immunolectronmicroscopy (Figure 1). UCP3-Tg mice had significantly lower body weight compared to the wild-type (22.4 ± 0.9 *vs* 25.9 ± 0.8 g in UCP3-Tg and wild-type mice respectively, $P<0.01$) and total UCP3 protein content correlated significantly with body weight ($r=-0.53$, $P<0.005$, Figure 3a). ATP concentration was not affected by overexpression of UCP3 (relative fraction of the total adenine nucleotide HPLC signal: 0.78 ± 0.01 *vs* 0.79 ± 0.02, in UCP3-Tg and wild-type mice respectively, NS), indicating that UCP3-Tg mice, despite the proton leak present are able to maintain levels of ATP, perhaps via the adenylate kinase reaction. In line with this, we observed a significant decrease in ADP levels (relative fraction: 0.14 ± 0.01 *vs* 0.20 ± 0.02, in UCP3-Tg and wild-type mice respectively, $P=0.03$), suggesting that ADP was converted to AMP to maintain ATP levels. Indeed, AMP levels

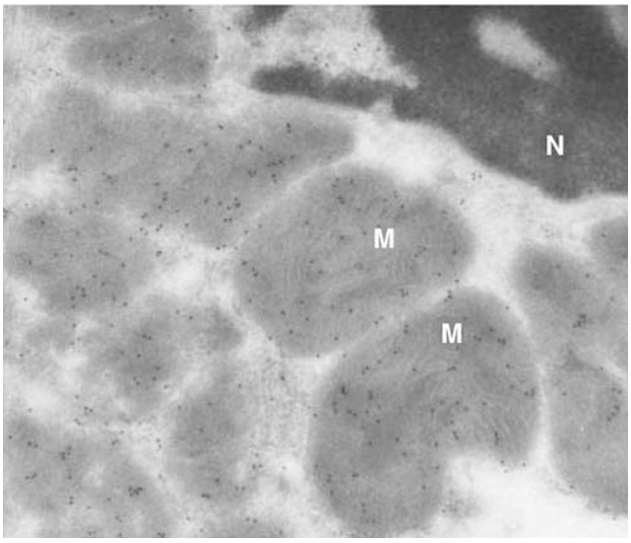

Figure 1 Immunogold electron microscopy confirming mitochondrial localisation of human UCP3 in the UCP3-Tg mice (n = nucleus, m = mitochondrion).

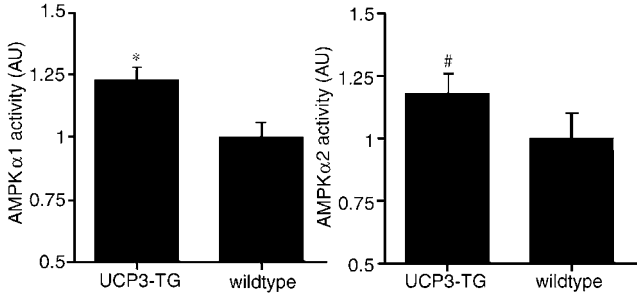

Figure 2 AMPK α1 and α2 activity in m. gastrocnemius of wild-type ($n=16$) and UCP3-Tg mice ($n=18$). AMPK activity was measured as described in Materials and methods. Data are means $\pm$ s.e.m. *$P<0.005$, # $P=0.08$ compared to wild-type.

doubled in UCP3-Tg compared to wild-type mice (0.08 ± 0.01 *vs* 0.04 ± 0.00 AU, $P=0.08$). The ATP/AMP ratio, an important regulator of AMPK activity, was significantly lower in UCP3-Tg compared to wild-type mice (10.9 ± 1.6 *vs* 20.4 ± 1.9, $P=0.03$), creating the subcellular conditions to activate AMPK.

Overexpression of UCP3 indeed resulted in increased AMPK α1 activity (1.23 ± 0.05 *vs* 1.00 ± 0.06 normalized values, $P=0.004$, Figure 2) and a tendency towards increased AMPK α2 activity (1.18 ± 0.08 *vs* 1.00 ± 0.10 normalized values, $P=0.08$, Figure 2). The UCP3 protein content correlated with AMPK α1 activity ($r=0.43$, $P<0.05$) and tended to correlate with AMPK α2 activity ($r=0.3$, $P=0.09$). Both AMPK α1 ($r=-0.52$, $P<0.005$, Figure 3b) and α2 ($r=-0.69$, $P<0.001$, Figure 3c) activity correlated with body weight. No differences in protein content of AMPK (α1 or α2) were detected between mice overexpressing human UCP3 and wild-type mice (data not shown).

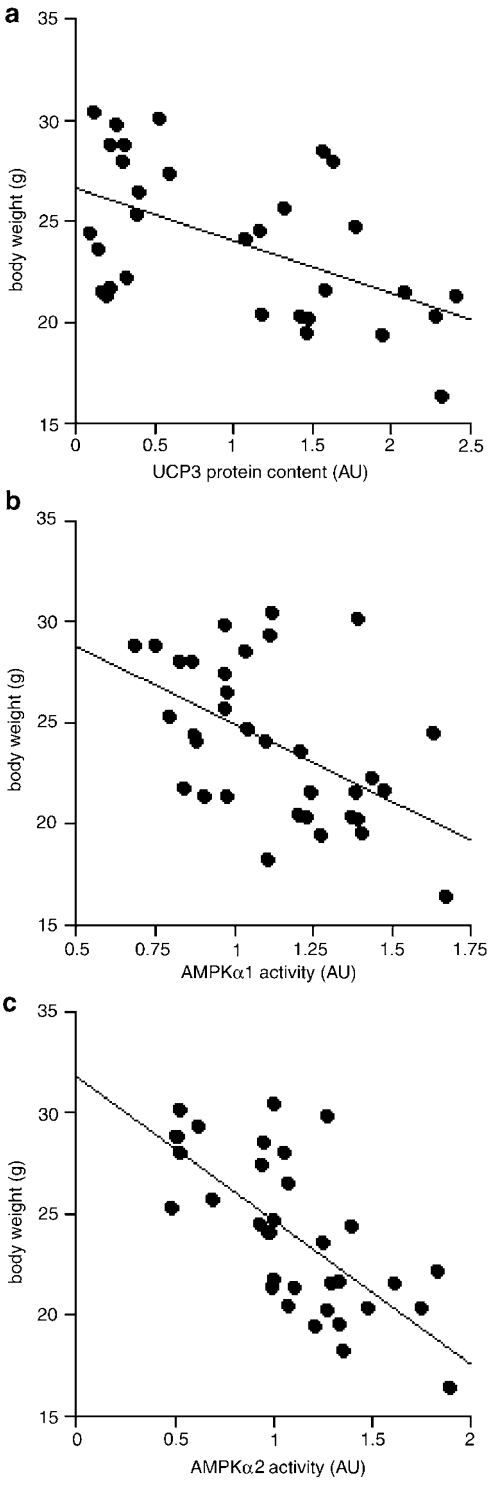

Figure 3 Relationship between m. gastrocnemius (**a**) UCP3 protein content ($r=-0.53$, $P<0.005$, (**a**), (**b**) AMPK α1 ($r=-0.52$, $P<0.005$) and (**c**) AMPK α2 activity and body weight ($r=-0.69$, $P<0.001$) in wild-type ($n=16$) and UCP3-Tg mice ($n=18$). AMPK activity was measured as described in Materials and methods. Data are means $\pm$ s.e.

Discussion

In recent years, AMPK has been recognized as a novel target for the pharmacological treatment of type II diabetes mellitus.[12] Chemical activation of AMPK with the compound AICAR increases glucose uptake[16,17] and insulin sensitivity.[18,19] Here we show that overexpressing human UCP3 in skeletal muscle of mice affects the cellular energy charge and induces activation of AMPK α1 and tends to induce the activation of AMPK α2. Why AMPK α1 was more affected compared to AMPK α2 cannot be deduced from the present study, but is remarkable considering that especially AMPK α2 is involved in glucose homeostasis in muscle.[20] In addition to the increased AMPK activity, UCP3 overexpression also affected body weight, which previously was shown to be due to an increase in metabolic rate.[5] Interestingly, AMPK activity was tightly correlated with the body weight. This suggests that UCP3-induced AMPK activation stimulates substrate metabolism necessary to provide fuels for the increased metabolic rate, and diverts glucose towards oxidation rather than towards storage as glycogen and fat. This suggestion is also consistent with the decreased adipose tissue mass reported in UCP3-Tg mice.[5] Most important, the increased AMPK activity observed in the present study, after supraphysiological overexpression of human UCP3 in skeletal muscle, provides a plausible explanation for improved glucose tolerance in these UCP3-Tg mice,[5] and suggests that mitochondrial uncoupling improves glucose metabolism by activating AMPK. The latter could also explain the improved glucose handling that has been shown in muscle cells treated with chemical uncouplers[13] and in mice overexpressing UCP1 in skeletal muscle.[4] Therefore, our data indicate that agents able to (lifetime) activation of AMPK, for example by uncoupling mitochondria, are powerful tools in the prevention or treatment of type II diabetes mellitus. The results of the present study do not, however, indicate that the primary physiological function of UCP3 is to regulate glucose metabolism by AMPK activation. We have recently shown that upregulation of UCP3 in humans in the physiological range does not affect mitochondrial uncoupling,[10] and it is now generally accepted that the primary physiological function of UCP3 is not to uncouple mitochondria.[7,6,9] It has therefore been suggested that overexpressing UCP3 in the supraphysiological range does not represent native uncoupling, possibly due to improper incorporation of UCP3 in the mitochondrial membrane.[6] In contrast to this suggestion, we show in the present study, using immunogold electron microscopy, that the vast majority of UCP3 was associated with the innermitochondrial membrane and the cristae and that extramitochondrial labelling was almost absent (see Figure 1). However, regardless of the nature of uncoupling (either non-native or due to the supraphysiological level), as it is the change in energy charge, which is responsible for AMPK activation, our data also provide a plausible explanation why ablation of UCP3 (that does not affect cellular energy charge) does not result in apparent disturbances in glucose homeostasis.[21] Therefore, the previously observed beneficial effects of UCP3 on glucose metabolism seems to be due to an effect of (supraphysiological concentrations of) UCP3, via mitochondrial uncoupling, on AMPK activity. However, whether the reduction of UCP3 protein levels in type II diabetic subjects[3] also affects AMPK activity seems doubtful given that UCP3 content in the physiological range in humans does not affect mitochondrial uncoupling,[10] but this should be further investigated. In this context, the reduction in UCP3 protein levels in type II diabetic patients could well be related to alteration in fatty acid metabolism in this disease, as we previously suggested.[9]

In conclusion, we have shown that mice overexpressing human UCP3 have increased AMPK activity, which provides a plausible explanation for the improved glucose tolerance characteristic for these mice. This suggests that affecting mitochondrial uncoupling, whether or not via UCPs, might be a target in the prevention or treatment of type II diabetes mellitus.

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