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PAPER

# Up-regulation of muscle uncoupling protein 3 gene expression in mice following high fat diet, dietary vitamin A supplementation and acute retinoic acid-treatment

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**OBJECTIVE:** To analyse the impact of vitamin A supplementation of both a normal fat (NF) diet and a high fat (HF) diet and of acute retinoic acid (RA)-treatment on the expression of uncoupling protein 3 (UCP3) in mice.
**DESIGN:** C57BL/6J mice were fed for 18 weeks a NF or a HF diet (10 and 45 energy% as fat, respectively), both with the normal vitamin A content or an excess vitamin A (8 mg and 320 mg retinyl palmitate/kg diet, respectively). Body weight and energy intake were recorded periodically. UCP3 mRNA and UCP3 protein levels in skeletal muscle (*soleus/gastrocnemius*) were analysed, as well as UCP1, UCP2 and UCP3 mRNA levels in interscapular brown adipose tissue (BAT), and UCP2 mRNA, UCP2 protein and leptin mRNA levels in white adipose tissue (WAT) depots. The effect of acute RA-treatment (100 mg/kg/day, 4 days) on UCP3 mRNA levels in skeletal muscle and BAT of NMRI mice was also assessed.
**RESULTS:** Vitamin A supplementation of a NF diet led to increased levels of UCP3 mRNA and UCP3 protein in muscle, UCP1 mRNA in BAT, and UCP2 mRNA in inguinal WAT, but had no impact on body weight or adiposity of B6 mice. HF diet promoted obesity and increased levels of UCP3 mRNA and UCP3 protein in skeletal muscle, and of the mRNAs for all three UCPs in BAT. Supplementing the HF diet with vitamin A had little effect on the final obesity reached and did not lead to further increases of muscle UCP3 mRNA nor BAT UCP1 mRNA over the levels achieved with the non-supplemented HF diet. Adipose leptin mRNA levels were down regulated after vitamin A supplementation, independently of the fat content of the diet. Up-regulation of muscle, but not BAT, UCP3 mRNA levels was also found after acute RA-treatment in NMRI mice.
**CONCLUSION:** The results provide evidence of a stimulatory effect of retinoids on muscle UCP3 expression *in vivo*, and a differential retinoid-regulation of the UCP3 gene in muscle and BAT.
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**Keywords:** vitamin A; retinoic acid; high fat diet; UCP3; UCP1; UCP2

## Introduction

Uncoupling protein 3 (UCP3) and 2 (UCP2) genes (reviewed in ref. 1) were cloned in 1997 as genes encoding proteins with a high sequence homology to UCP1, the inner mitochondrial membrane protein of brown adipocytes that constitutes the molecular basis of adaptive thermogenesis in brown adipose tissue (BAT). When active, UCP1 can dissipate energy as heat by uncoupling oxidative phosphorylation.[1,2] Different reports (see ref. 1) showed that UCP2 and UCP3 lower mitochondrial membrane potential when ectopically expressed in yeast, and, more recently, analysis of UCP3 knockout mice provided evidence of uncoupling activity by UCP3 in skeletal muscle *in vivo*,[3,4] although results are conflicting.[5]

Considering that BAT plays an important role in energy homeostasis in rodents, but is scarce in adult humans, the discovery of novel putative uncoupling proteins that are expressed in different mammalian tissues apart from BAT led to a renewed interest in UCPs as potential players in

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energy metabolism. UCP2 is expressed in a variety of tissues, and UCP3 selectively in BAT and skeletal muscle, the latter tissue a major contributor to human metabolic rate.[6] A role of the novel UCPs in regulated energy expenditure is, however, controversial. Against such a role is the fact that the expression of UCP3 and UCP2 is up-regulated in fasting (reviewed in ref. 1), a condition where whole body energy is conserved, and the fact that, like UCP1 deficient mice,[7] UCP3[3,8] and UCP2[9] deficient mice are not obese nor especially sensitive to developing diet-induced obesity. Up-regulation of muscle UCP3 and UCP2 during fasting, when lipid oxidation in muscle is increased, and down-regulation below control levels upon refeeding, when body fat deposition is increased, have suggested a role for the novel UCPs in the handling of lipids as fuel substrates.[10] The UCPs may also be involved in the protection against excessive reactive oxygen species (ROS) production. There is evidence that high coupling of respiration enhances mitochondrial superoxide generation (reviewed in ref. 11) and, indeed, higher levels of ROS were found in tissues from both UCP3[3] and UCP2[9] deficient mice.

The regulation of the UCPs by dietary factors is a topic of interest both in lipid metabolism research and obesity research that, up to recently, received little attention.[12] Two dietary factors of particular relevance are fat and vitamin A. There are many reports indicating that high fat diets induce the expression of the UCPs in rodent tissues,[13–17] an effect that appears to be selectively potentiated by particular types of fat, such as polyunsaturated fatty acids,[18] olive oil[19] or coconut oil.[20] On the other hand, retinoic acid (RA), the carboxylic acid form of vitamin A and a well-known modulator of gene transcription, has been shown to up-regulate the expression of the UCPs in cell model systems,[21–25] an effect that, in the case of UCP1, has also been demonstrated for certain pro-vitamin A carotenoids.[26] Up-regulation of BAT UCP1[22,27,28] and UCP2[28] expression was also reported after acute RA-treatment in intact rodents, where it correlated with a significant reduction of body weight and body adiposity.[22,28,29] Moreover, mice fed a vitamin A-deficient diet had reduced levels of expression of UCP1 and UCP2 in BAT, increased levels of expression of adipogenic transcription factors in white fat depots, and a higher adiposity and body weight than control mice,[28,29] suggesting that the vitamin A status can affect both the thermogenic and the adipogenic capacity of rodents.

In this work, we have studied the effects of chronic dietary vitamin A supplementation on the expression of the UCPs and on the development of high fat diet-induced obesity in obesity-prone mice. We have focused on UCP3 because its possible regulation by retinoids had not been previously addressed in experiments *in vivo*. Chronic vitamin A supplementation had little effect on the development of diet-induced obesity in obesity-prone mice, but our results show that muscle UCP3 expression responds to dietary factors and in particular to the fat and the vitamin A load of the diet, and evidence a differential regulation of UCP3 by retinoids in muscle and BAT.

## Methods

### Vitamin A supplementation experiment

Four-week-old C57BL/6J (B6) male mice (CRIFFA, Barcelona, Spain) were used (four animals/group). The animals were fed during 18 weeks one of the following diets (supplied by Research Diets Inc, New Brunswick, USA): a normal fat normal vitamin A diet (diet D12450, NF group), a normal fat high vitamin A diet (NF + A group), a high fat normal vitamin A diet (diet D12451, HF group) or a high fat high vitamin A diet (HF + A group). In an independent experiment, 4-week-old B6 male mice were fed the diets for 4 weeks before being killed. The normal fat diet contained 10% of total energy as fat and the high fat diet, 45% of total energy as fat (mainly lard). The normal vitamin A content was 4000 IU/kg diet (8 mg retinyl palmitate/kg diet); the supplemented diets contained 40 times as much (160 000 IU/kg diet, 320 mg retinyl palmitate/kg diet). The supplementation dose was chosen on the basis of a previous report in rats.[30] The vitamin A-supplemented groups did not show any of the known signs of vitamin A toxicity, which include occasional bleeding from the nose or partial paralysis of the legs. The animals were caged 4 per cage at 22°C, with a 12 h light/12 h dark cycle. Body weight was measured periodically over the experimental period. Energy intake was estimated on a per-cage basis for 24 h, once per week throughout the experimental period, from the actual amount of food consumed by the animals and its energy equivalence.

### Acute RA-treatment experiment

Twelve-week-old NMRI male mice (CRIFFA, Barcelona, Spain) that had been fed with regular laboratory chow (Panlab, Barcelona, Spain) and kept at 22°C with a 12:12 h light – dark cycle were used. The animals were acclimated to near thermoneutrality (28°C) during one week before being randomly allocated to two experimental groups: RA-treated animals, which received a daily subcutaneous injection of 100 mg/kg of all-trans-RA (Sigma, Madrid, Spain) during the 4 days immediately before they were killed, and control animals, that were injected with vehicle (olive oil). Two independent experiments were conducted (four and five animals per group, respectively).

### Tissue collection

The animals were killed with $CO_2$ followed by cervical dislocation and decapitation, at the start of the light cycle. Blood was collected from the neck and serum prepared. Interscapular BAT (BAT), inguinal WAT (iWAT), epididymal WAT (eWAT) and retroperitoneal WAT (rWAT) were excised in their integrity, weighted, rapidly frozen in liquid nitrogen

and stored at −70°C; leg muscle (*gastrocnemius*/*soleus*) was also sampled and frozen in liquid nitrogen.

### RNA extraction and northern blotting analysis

Reagents and probes used were from Boehringer Mannheim (Barcelona, Spain). Total tissue RNA was isolated using Tripure™ reagent, and 10–20 μg was fractionated by agarose gel electrophoresis, transferred onto a nylon membrane by capillary blotting in 20 × SSC and fixed with UV light, all according to Roca *et al*.[14]

The RNAs of interest were analysed by a chemiluminiscence-based procedure, using antisense oligonucleotide probes end-labeled with digoxigenin.[31] The following probes were used: for UCP1 mRNA, 5′-GTTGGTTTTATTCGTGGTCTCCCAGCATAG-3′;[14] for UCP2 mRNA, 5′-GGCAGAGTTCATGTATCTCGTCTTGACCAC-3′;[14] for UCP3 mRNA, 5′-GACTCCTTCTTCCCTGGCGATGGTTCTGTAGG-3′; for leptin mRNA, 5′-GGTCTGAGGCAGGGAGCAGCTCTTGGAGAAGGC-3′;[32] and for 18S rRNA, 5′-CGCCTGCTGCCTTCCTTGGATGTGGTAGCCG-3′.[32]

Fixed membranes were pre-hybridized at 42°C for 15 min in DIG-Easy Hyb, and then hybridized with the corresponding oligonucleotide probe (34 ng/ml, except the 18S rRNA probe, which was used at 70 pg/ml) in DIG-Easy Hyb at 42°C overnight and submitted to 2 × 15 min washes in a solution of 2 × SSC/0.1% (w/v) SDS at room temperature, followed by 2 × 15 min washes in 0.1 × SSC/0.1% (w/v) SDS at 48°C. After blocking, the membranes were incubated with an anti-digoxigenin–alkaline phosphatase conjugate, then with the chemiluminescent substrate CDP-Star™ and, finally, exposed to Hyperfilm™ ECL (Amersham, Buckinghamshire, UK). Bands in films were analysed by scanner photodensitometry, quantified using the BioImage program (Millipore, Bedford, MA, USA), and normalized using the corresponding 18S rRNA values. The RNAs of interest were analysed sequentially on the same membrane, after stripping with boiling 0.1% (w/v) SDS.

### Western blotting analysis

Samples of frozen eWAT, iWAT and leg muscle were homogenized in phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 6.5 mM $Na_2HPO_4$ and 3.5 mM $KH_2PO_4$) in a tefflon/glass homogenizer. The homogenates were centrifuged at 500 g for 10 min at 4°C and total protein content in the supernatants was determined by the method of Bradford.[33] Protein (50 μg) was fractionated by SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels according to Laemmli[34] and then electroblotted to a nitrocellulose membrane. Ponceau S staining provided visual evidence for correct loading and electrophoretic transfer of proteins to the nitrocellulose membrane. Blocking and development of the immunoblots were performed using an enhanced chemiluminiscence western blotting analysis system (Amersham Pharmacia Biotech, Buckinghamshire, UK). Primary rabbit antibodies against UCP2 and UCP3 were purchased from Alpha Diagnostics (San Antonio, TX, USA); the specificity of these antibodies was previously confirmed by Sivitz *et al*.[35] Bands in films were analysed by scanner photodensitometry and quantified using the BioImage program (Millipore, Bedford, MA, USA).

### Other parameters determined

Muscle lipids were determined by the method of Folch.[36] Serum nonesterified fatty acid (NEFA) levels were quantified using a colorimetric acyl-CoA synthetase and acyl-CoA oxidase-based method (Wako Chemicals GmbH, Neuss, Germany).

### Statistical analysis

Values are expressed as means ± s.e.m. In the vitamin A supplementation experiments, differences between groups were assessed by two-way analysis of variance (ANOVA); Student's *t*-test was applied for mean comparisons when two-way ANOVA revealed interaction between the variables. In the acute RA-treatment experiments, differences between control and tRA-treated animals were assessed by Student's *t*-test. Results were considered statistically significant at $P < 0.05$. The analyses were performed with SPSS® software for Windows (SPSS Inc, Chicago, IL/USA).

## Results

### Impact of long-term vitamin A supplementation on high fat diet-induced obesity in B6 mice

We chose the C57BL/6J (B6) strain to test the possible impact of chronic vitamin A supplementation on the development of obesity. These mice are particularly sensitive to developing obesity upon high fat diet feeding due to multiple genetic factors, but do not develop obesity when fed a regular chow.[37,38] As expected, body weight and the size of all fat pads analysed, especially the visceral fat pads, were significantly increased in B6 mice after 18 weeks on a high fat diet, as compared with B6 mice fed a normal fat diet (Figure 1A and Table 1). Gross total energy intake was similar in the high fat diet-fed groups and the normal fat diet-fed groups (Figure 1B); this may be in accordance with previous reports indicating that high fat diet-induced obesity in B6 mice is not dependent on excess energy intake, but on an increment of feed efficiency (body weight gain/energy consumed) with fat.[37,38] Supplementation of the diet with vitamin A did not significantly affect energy intake nor the final body weight or adiposity of B6 mice, whether fed the normal fat diet or the high fat diet (Table 1). However, from the growth curves in Figure 1A it is apparent that the development of overweight upon high fat diet feeding was retarded when the diet was supplemented with vitamin A, and in Table 1 it can be seen that inguinal (but not visceral) fat mass tended to be reduced in the vitamin A-supplemented groups. Muscle lipid content was slightly but significantly higher in the groups fed the

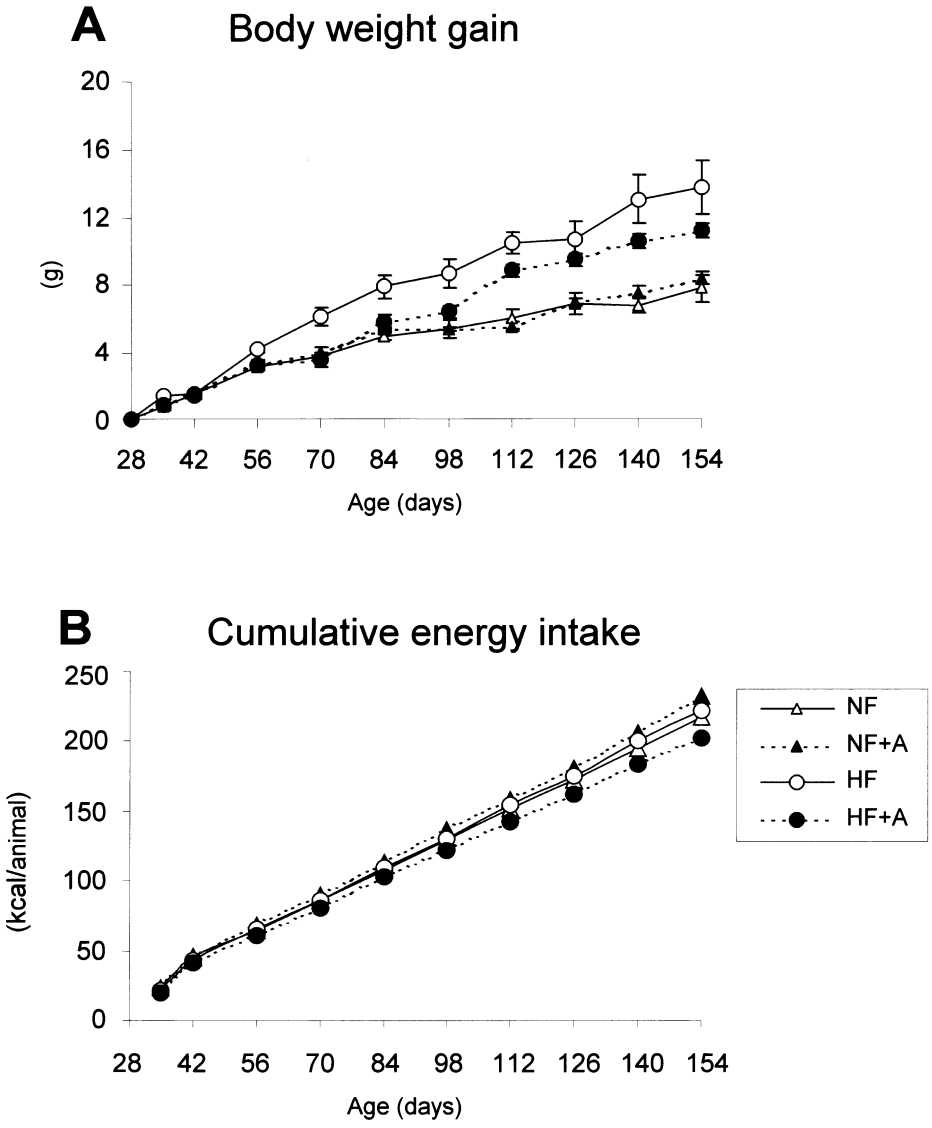


**Figure 1** Effects of high fat diet and vitamin A supplementation on body weight gain (A) and cumulative energy intake (B) of B6 mice. The animals were fed a normal fat normal vitamin A diet (NF), a normal fat high vitamin A diet (NF + A), a high fat normal vitamin A diet (HF) or a high fat high vitamin A diet (HF + A). Details of the diets are given in the materials and methods section. Data in A are the means ± s.e.m. of four animals per group. Energy intake was estimated on a per-cage basis (four animals/cage) for 24 h, from the actual amount of food consumed by the animals and its caloric equivalence.

high fat diet than in those fed the normal fat diet and was unaffected by dietary vitamin A load (Table 1). Serum NEFA levels were not affected by long-term high fat diet or vitamin A supplementation (Table 1).

### Effects of long-term high fat diet feeding and vitamin A supplementation on leptin and UCP2 expression in WAT depots of B6 mice

Leptin mRNA levels were increased in the hypertrophied iWAT and eWAT depots of high fat diet-fed B6 mice, in accordance with the well known general positive correlation between leptin expression and fat content, and tended to be reduced after dietary vitamin A supplementation independently of the fat content of the diet, a tendency that reached statistical significance in eWAT (Table 2).

UCP2 mRNA levels in WAT depots were unaffected in both iWAT and eWAT by high fat diet feeding, and were increased in iWAT after dietary vitamin A supplementation, independently of the fat content of the diet (Table 2). Of note, UCP2 mRNA levels in WAT depots, especially in iWAT, appeared to be very low, semi-quantification by northern

**Table 1** Effects of 18 weeks of high fat diet and dietary vitamin A supplementation on general parameters of B6 mice

| | *NF* | *NF + A* | *HF* | *HF + A* | *ANOVA* |
|---|---|---|---|---|---|
| Pre diet body weight (g) | 19.5 ± 0.6 | 19.8 ± 0.5 | 20 ± 0.3 | 20.5 ± 0.5 | |
| Post diet body weight (g) | 28.4 ± 1.5 | 29.4 ± 0.7 | 35.7 ± 2.1 | 33.3 ± 0.9 | F |
| Body weight gain (g) | 8.9 ± 0.9 | 9.6 ± 0.4 | 15.7 ± 1.8 | 12.8 ± 0.5 | F |
| BAT weight (mg) | 115 ± 14 | 113 ± 12 | 159 ± 40 | 147 ± 8 | |
| eWAT weight (mg) | 596 ± 123 | 663 ± 206 | 1777 ± 199 | 1776 ± 101 | F |
| rWAT weight (mg) | 158 ± 46 | 171 ± 71 | 531 ± 44 | 569 ± 36 | F |
| iWAT weight (mg) | 446 ± 54 | 317 ± 79 | 943 ± 174 | 797 ± 52 | F |
| Muscle lipids (mg/g of tissue) | 51 ± 8 | 53 ± 6 | 71 ± 7 | 69 ± 1 | F |
| Serum NEFA (mEq/l) | 1.12 ± 0.11 | 1.01 ± 0.15 | 1.15 ± 0.08 | 1.33 ± 0.09 | |

Four-week-old B6 mice were fed for 18 weeks a normal fat normal vitamin A diet (NF group), a normal fat high vitamin A diet (NF + A group), a high fat normal vitamin A diet (HF group) or a high fat high vitamin A diet (HF + A group). Details of the diets are given in the materials and methods section. Data are the means ± s.e.m. of four animals per group. ANOVA significances, $P < 0.05$: F, effect of high fat diet. BAT, interscapular brown adipose tissue; NEFA, nonesterified fatty acids; eWAT, rWAT and iWAT: epididymal, retroperitoneal and inguinal white adipose tissue.

blotting requiring long-time exposition of the films to the blots. UCP2 protein levels as measured by western blotting also appeared to be very low and, both in iWAT and eWAT, were found to be unaffected by high fat diet feeding or dietary vitamin A supplementation (results not shown). Thus, increased UCP2 mRNA levels in the iWAT of vitamin A-supplemented animals were not accompanied by parallel increases of the tissue UCP2 protein levels.

### Vitamin A supplementation up-regulated muscle UCP3 expression in B6 mice

Skeletal muscle UCP3 mRNA levels were found to be increased in B6 mice after 18 weeks of dietary vitamin A supplementation of a normal fat diet (by +116%, significant by Student's *t*-test) and, more markedly (by +174%), after 18 weeks of high fat diet feeding; there was a clear trend to interaction between the two diet variables, with the up-regulating effect of vitamin A supplementation being apparent under the normal fat diet only (Figure 2A). Changes of skeletal muscle UCP3 protein levels showed a similar profile, with increments in response to both vitamin A supplementation and high fat diet that were not cumulative in the HF + A group (Figure 2B). In a separate experiment, we found muscle UCP3 mRNA levels already increased in B6 mice after 4 weeks of dietary vitamin A supplementation of a normal fat diet (by +124%) and after 4 weeks of high fat diet (by +46%); as in the long-term experiment, in this short-term experiment the effect of vitamin A supplementation was apparent only under the normal fat diet (relative levels of expression: NF group, 100 ± 7; NF + A group, 224 ± 11; HF group, 146 ± 3; HF + A group, 155 ± 20, $n = 4$ for each group. ANOVA significances, $P < 0.05$: effect of vitamin A, interaction between vitamin A and fat. Student's *t* test significances, $P < 0.05$: NF + A *vs* NF, HF *vs* NF, HF + A *vs* NF + A).

**Table 2** Effects of 18 weeks of high fat diet and dietary vitamin A supplementation on the relative leptin and UCP2 mRNA levels in white adipose tissue depots of B6 mice

| | *NF* | *NF + A* | *HF* | *HF + A* | *ANOVA* |
|---|---|---|---|---|---|
| Leptin mRNA levels | | | | | |
| iWAT | 100 ± 17 | 53 ± 26 | 478 ± 229 | 219 ± 32 | F |
| eWAT | 100 ± 22 | 49 ± 18 | 170 ± 21 | 69 ± 24 | F, A |
| UCP2 mRNA levels | | | | | |
| iWAT | 100 ± 25 | 306 ± 26 | 96 ± 40 | 204 ± 31 | A |
| eWAT | 100 ± 15 | 155 ± 57 | 145 ± 12 | 60 ± 5 | |

Four-week-old B6 mice were fed for 18 weeks a normal fat normal vitamin A diet (NF group), a normal fat high vitamin A diet (NF + A group), a high fat normal vitamin A diet (HF group) or a high fat high vitamin A diet (HF + A group). Details of the diets are given in the materials and methods section. Data are the means ± s.e.m. of 4 animals per group and are expressed relative to the mean value of the NF group, which was set at 100%. Ratios of each mRNA to 18S rRNA were calculated to correct for RNA quantity. ANOVA significances, $P < 0.05$: F, effect of high fat; A, effect of dietary vitamin A supplementation. eWAT, epididymal white adipose tissue; iWAT, inguinal white adipose tissue.

The effect of the experimental diets on the expression of all three UCPs in BAT was analysed at the mRNA level (Table 3). Long-term high fat diet resulted in increased BAT UCP1, UCP2 and UCP3 mRNA levels (by +124%, +66% and +70%, respectively), while long-term vitamin A supplementation only had a significant effect on the UCP1 mRNA levels, which increased by +44%. There was a negative interaction between the two diet variables resulting in lower BAT UCP1 mRNA levels in the HF + A group than in the HF group.

### Acute RA-treatment up-regulated muscle UCP3 expression in NMRI mice

To confirm the stimulatory effect of retinoids on muscle UCP3 expression and the differential regulation of UCP3 in muscle and BAT by retinoids, we analysed muscle and BAT UCP3 mRNA levels in NMRI mice that had been acutely treated with all-trans-RA, compared with control mice receiving vehicle only. This treatment has previously been shown to result in body weight loss, reduced adiposity, and

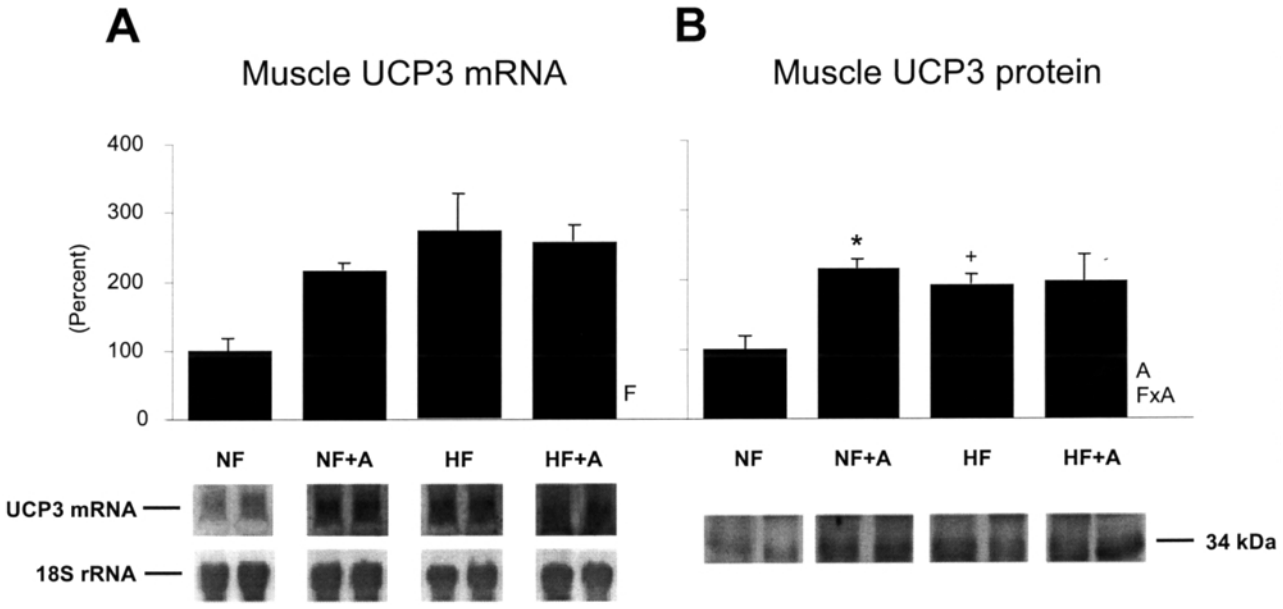


**Figure 2** Effects of high fat diet and vitamin A supplementation on UCP3 mRNA (A) and UCP3 protein (B) expression in skeletal muscle of B6 mice. Four-week-old B6 mice were fed for 18 weeks a normal fat normal vitamin A diet (NF group), a normal fat high vitamin A diet (NF + A group), a high fat normal vitamin A diet (HF group) or a high fat high vitamin A diet (HF + A group). Details of the diets are given in the materials and methods section. Data are the means ± s.e.m. of 4 animals per group and are expressed relative to the mean value of the NF group, which was set at 100%. Ratios of each mRNA to 18S rRNA were calculated to correct for RNA quantity. Representative northern and western blots are shown at the bottom (A, 20 μg total RNA/lane; B, 50 μg total protein/lane). ANOVA significances, $P < 0.05$: F, effect of high fat; A, effect of dietary vitamin A supplementation; FxA, interaction of high fat and vitamin A supplementation. Student's *t*-test significances, $P < 0.05$: *, NF + A *vs* NF; †, HF *vs* NF.

enhanced thermogenic capacity, with increased mRNA expression levels of both UCP1 and UCP2 (but not UCP3) in BAT.[22,28,29] As shown in Figure 3, all-trans-RA-treatment led to a significant increment of UCP3 mRNA in skeletal muscle but, in accordance with our previous results,[28] it did not affect UCP3 mRNA levels in BAT. Serum NEFA levels were unaffected by RA-treatment (control mice: 1.77 ± 0.12 mEq/l; RA-treated mice: 1.66 ± 0.17 mEq/l; $n = 9$ for both groups).

**Table 3** Effects of 18 weeks of high fat diet and dietary vitamin A supplementation on the relative UCP1, UCP2 and UCP3 mRNA levels in interscapular brown adipose tissue of B6 mice

| | *NF* | *NF + A* | *HF* | *HF + A* | *ANOVA* |
|---|---|---|---|---|---|
| BAT UCP1 mRNA | 100 ± 9 | 144 ± 7* | 224 ± 12† | 158 ± 14‡ | F, FxA |
| BAT UCP2 mRNA | 100 ± 9 | 124 ± 14 | 166 ± 10 | 151 ± 7 | F |
| BAT UCP3 mRNA | 100 ± 24 | 89 ± 10 | 170 ± 12 | 176 ± 24 | F |

Four-week-old B6 mice were fed for 18 weeks a normal fat normal vitamin A diet (NF group), a normal fat high vitamin A diet (NF + A group), a high fat normal vitamin A diet (HF group) or a high fat high vitamin A diet (HF + A group). Details of the diets are given in the materials and methods section. Data are the means ± s.e.m. of four animals per group and are expressed relative to the mean value of the NF group, which was set at 100%. Ratios of each mRNA to 18S rRNA were calculated to correct for RNA quantity. ANOVA significances, $P < 0.05$: F, effect of high fat; FxA, interaction of high fat and vitamin A supplementation. Student's *t*-test significances, $P < 0.05$: *, NF + A *vs* NF; †, HF *vs* NF; ‡, HF + A *vs* HF.

## Discussion

To our knowledge, this work constitutes the first evidence of up-regulation of muscle UCP3 expression by retinoids *in vivo*. We found this up-regulation in response to both acute RA-treatment and dietary vitamin A supplementation, in two strains of mice (B6 and NMRI), and both at the mRNA level and the protein level. These results are in agreement with studies *in vitro* showing induction of UCP3 expression by RA in differentiated myotubes in cell culture.[24,25] Notwithstanding the possibility of post-transcriptional effects, the effect of retinoids on UCP3 expression may reflect enhanced activity of the retinoic acid receptor: retinoid X receptor (RAR:RXR) heterodimers and/or of peroxisome proliferator-activated receptor: RXR (PPAR:RXR) heterodimers, for both of which response elements have been described in the UCP3 gene promoter,[25,39] as well as in the UCP1 gene promoter (see ref. 1). The former heterodimers transactivate transcription upon binding of all-trans-RA or 9-cis-RA to the RAR moiety, while the latter require binding of 9-cis-RA to the RXR moiety, in addition to a PPAR ligand, for maximal transcriptional activity.[40,41]

The up-regulating effect of dietary vitamin A supplementation and acute RA-treatment on UCP3 mRNA levels was

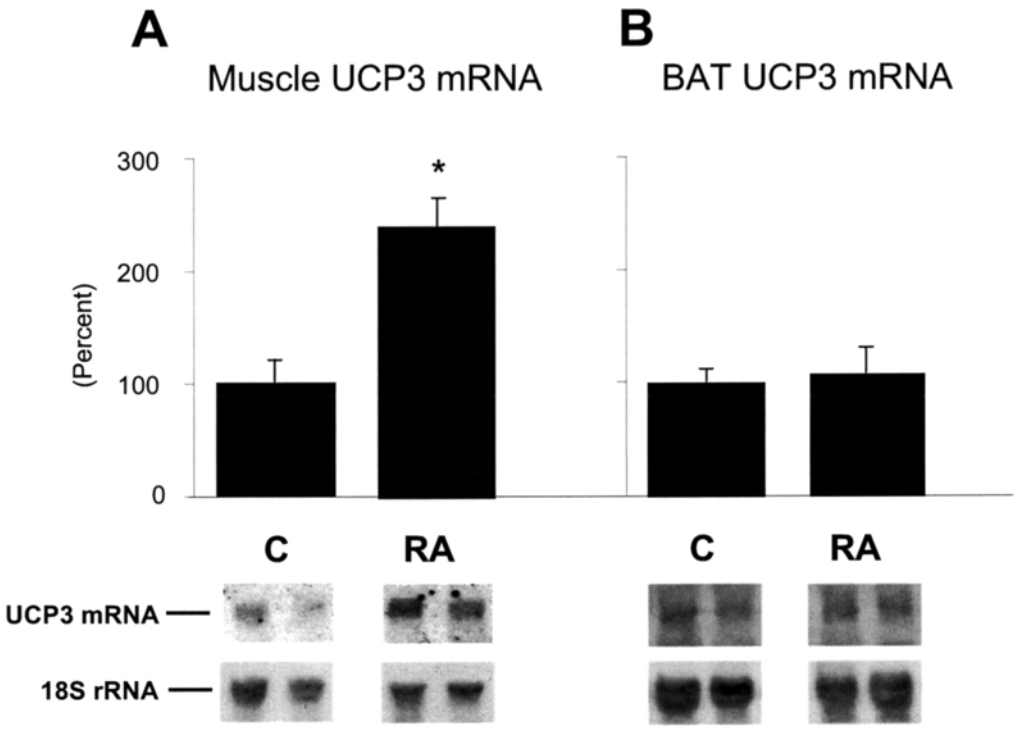


**Figure 3** Effect of retinoic acid-treatment on UCP3 mRNA expression in skeletal muscle (A) and BAT (B) of NMRI mice. Retinoic acid-treated animals (RA group) received a daily subcutaneous injection of 100 mg/kg of all-trans-retinoic acid during the 4 days preceding death; control animals (C group), were injected the vehicle (olive oil). Data are the means ± s.e.m. of nine animals per group, distributed in two independent experiments, and are expressed relative to the mean value of the C group, which was set at 100%. Ratios of each mRNA to 18S rRNA were calculated to correct for RNA quantity. Representative northern blots are shown at the bottom (15 μg total RNA/lane). Student's *t*-test significance: *, $P < 0.001$.

found in muscle but not in BAT. Retinoids should therefore be added to the list of hormones and conditions that differentially affect UCP3 gene expression in the two tissues, which include T3 and fasting.[42] Several hypotheses can be suggested to explain a muscle-specific effect of retinoids. First, RA-stimulated UCP3 gene expression could require the interaction of RAR:RXR heterodimers with a muscle-specific transcription factor. In fact, it has been shown in transfection experiments that the RAR:RXR-mediated stimulatory effect of RA on UCP3 gene expression is completely dependent on co-transfection of MyoD,[25] a master regulator of muscle cell differentiation that is selectively expressed in cells of the myogenic lineage. Another possibility is that RAR and/or RXR isoforms differentially expressed in muscle and BAT mediate the effect of retinoids on the UCP3 gene. In this context, it was reported that, of the three known RXR isoforms, RXR$\gamma$ displays a restricted expression in the myogenic lineage.[43]

In the last years, it has become increasingly clear that RA is a modulator of fat metabolism that promotes fat mobilization (and/or apoptosis) *in vivo* and the transformation of mature adipocytes into cells less capable to store triglycerides and more capable of using fatty acids. Mice acutely treated with RA lose body weight and adiposity[22,28,29] and, overall, the effects of acute RA-treatment on the expression of UCPs — the previously reported induction of BAT UCP1 and UCP2 levels[22,27,28] and the induction of muscle UCP3 reported here — fit well in a framework of enhanced fat metabolism triggered by RA. In this framework, RA induction of UCPs may serve to facilitate the handling of lipids as a fuel, either for heat production or ATP production. The role of the UCPs, in particular UCP3, in lipid metabolism may be related to their capability to export fatty acid anions from the mitochondria:[44] the hypotheses have been proposed that, in the face of an oversupply of fatty acids, such an export may favor CoASH delivery from the cytosol to the mitochondrial matrix, where it is needed for the beta-oxidation cycle and the tricarboxylic acid cycle,[45] and/or protect against toxic accumulation of non-metabolizable nonesterified fatty acids inside the mitochondrial matrix.[46] Induction of the UCPs may also help reducing ROS production in the fatty acid-oxidizing mitochondria.

Chronic dietary vitamin A supplementation also resulted in increased expression levels of UCPs, thus preparing the organism for an eventual fat mobilization such as that triggered by acute doses of RA or other lipolytic agents. However, in our hands it had no impact on body weight or adiposity of B6 mice fed a normal fat diet. In F-344 × BN rats,

vitamin A supplementation was previously reported to cause a 31% increase of BAT UCP1 mRNA expression levels (comparable to the 44% increase found here), that correlated with a modest 9% decrease of adiposity;[30] skeletal muscle UCP3 expression was not analysed in that report. From the comparison of acute RA-treatment experiments with chronic vitamin A supplementation experiments it may be suggested that the effects of retinoids on fat mobilization need high acute doses of RA to show up. Vitamin A supplementation had, nevertheless, some counterbalancing effect on the development of diet-induced obesity in B6 mice, with a trend to lower body weight gain in the HF + A group compared with the HF group not attributable to differences in energy intake. This is in line with increased body weight and adiposity previously reported in NMRI mice chronically fed a diet deficient in vitamin A.[28,29] From our results, it seems unlikely that the trend to lower body weight is related to an increased overall uncoupling potential in the HF + A group relative to the HF group, because the up-regulating effects of high fat diet and dietary vitamin A supplementation on muscle UCP3 were not additive, and we even found a negative interaction between the two diet variables concerning BAT UCP1 mRNA levels; only iWAT UCP2 mRNA levels were higher in the HF + A group than in the HF group, but this was not accompanied by a parallel increase of the UCP2 protein content of the tissue. Experiments are under development in our laboratory to further study the impact of vitamin A supplementation on diet-induced obesity and the possible influence of genetic background on it.

Enhanced expression of UCPs after high fat diet feeding is well known and may be related to an increased need to handle lipids as a fuel substrate under this condition. Up-regulation of muscle UCP3 mRNA levels by high fat diet was previously reported both in humans[47] and rodents,[15,17] although results are conflicting;[16] we confirmed this effect of high fat diet on muscle UCP3 both at the mRNA and the protein level. We also found increased levels of the mRNAs for all three UCPs in the BAT of high fat diet-fed B6 mice: previous reports in this strain found BAT UCP1 mRNA levels already increased[16] but BAT UCP2 and UCP3 mRNA levels unchanged[15,16] after experimental periods shorter than the one used by us (2 or 8 weeks *vs* 18 weeks). Concerning UCP2 mRNA levels in WAT depots, they were previously reported to remain unchanged in the WAT of B6 mice challenged with high fat diet for 2 to 8 weeks[13,15,16]—the suggestion was made that lack of early induction could contribute to the obesity proneness of the strain[16]—but to be markedly elevated in these animals after 18 weeks of high fat diet feeding.[13] In our hands, UCP2 expression in WAT depots of B6 mice, studied both at the mRNA and the protein level, remained unchanged even after 18 weeks of high fat diet. Discrepancy between our results and the latter report may be related to differences in the composition of the high fat diets used, particularly in the fat fraction. The high fat diet used in reference 13[48] was richer in fat than our high fat diets (in gravimetric percentage, 35.8% *vs* 23.6%); in addition, differences in the quality of the fat, which may be a factor of special relevance, cannot be discarded (fat quality was not stated for the diet used in reference 13).[48]

Forced elevation of circulating NEFA levels was shown to stimulate UCP3 expression in rat muscle[49] and a positive correlation was reported between circulating NEFA concentrations and muscle UCP3 mRNA levels in humans.[50] However, the up-regulation of UCP3 in skeletal muscle in this study, whether triggered by high fat diet feeding, dietary vitamin A supplementation or acute RA-treatment, could not be explained by increases of circulating NEFA levels. This is in agreement with a direct effect of RA on the UCP3 gene and with the concept that regulation of UCP3 expression by fat could be more related to actual increases in the rate of fat oxidation than to high NEFA levels *per se*. In fact, studies in humans under high fat diets showed increases of fat oxidation and UCP3 expression in muscle that were not accompanied by increases of circulating NEFA levels.[47,51]

Lower levels of adipose leptin mRNA in B6 mice fed vitamin A-supplemented diets is in agreement with down-regulation of adipose leptin mRNA levels following acute RA-treatment in rats and mice[27,28] and reduction of circulating leptin levels following dietary vitamin A supplementation in rats.[30] Our results further suggest that retinoids may by themselves exert an inhibitory effect on leptin expression that is not secondary to a reduction of fat content, because the depressed adipose leptin mRNA levels found did not correlate with comparable reductions of the adipose depots mass.

In summary, our results do not sustain a role of muscle UCP3 as a major determinant of metabolic efficiency and energy balance, in accordance with results from UCP3 deficient mice,[3,8] but they show that muscle UCP3 expression responds to both the fat and the vitamin A load of the diet, and evidence a differential regulation of UCP3 by retinoids in muscle and BAT of rodents.

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