The FASEB Journal express article 10.1096/fj.00-0517fje. Published online February 5, 2001.

Protein expression of UCP3 differs between human type 1, type 2a, and type 2b fibers

Hesselink, M.K.C.,* Keizer, H. A.,* Borghouts, L. B.,* Schaart, G.,* Kornips, C.F.P.,[†] Slieker, L. J.,[‡] Sloop, K. W.,[‡] Saris, W.H.M.,[†] and Schrauwen, P.[†]

Nutrition and Toxicology Research Institute NUTRIM *Department of Movement Sciences; [†]Department of Human Biology, Maastricht University, P.O. Box 616, 6200 MD Maastricht, The Netherlands; [‡]Eli Lilly & Company, Lilly Corporate Center, Indianapolis, Ind.

Corresponding author: M.K.C. Hesselink, Department of Movement Sciences, Maastricht University, P.O. Box 616, 6200 MD Maastricht, The Netherlands. E-mail: matthijs.hesselink@bw.unimaas.nl

ABSTRACT

Uncoupling protein 3 (UCP3) dissipates energy as heat in human skeletal muscle, thus enhancing energy expenditure and affecting body mass. UCP3 has been associated with insulin resistance, obesity, sleeping metabolic rate, and transmitochondrial membrane transport of fatty acid anions. Factors affecting UCP3 gene-expression include fatty acids and GLUT4 content (up-regulation) and endurance exercise (equivocal reports). Fatty acid-induced up-regulation of UCP3 is most prominent in glycolytic fibers, whereas endurance training down-regulates UCP3 in slow fibers. A direct comparison between muscle fiber type and UCP3 protein expression in human muscle is essential to explore the physiological role of UCP3 further. We combined immunofluorescence with conventional fiber typing to study concurrently fiber type and UCP3 expression in human *vastus lateralis* muscle of healthy controls and type 2 diabetics. Here, we report for the first time that, in controls, as in diabetics, UCP3 protein expression is most abundant in type 2b fibers, less in type 2a fibers, and only moderately expressed in type 1 fibers. Of the potential roles of UCP3 or factors regulating UCP3 expression, only a role of UCP3 in preventing oxidative stress completely matches the reported fiber-type specific UCP3 expression. UCP3-induced dissipation of energy may therefore serve multiple fiber-type specific goals.

Key words: fiber type • skeletal muscle • type 2 diabetes • UCP3 • protein

The recently discovered uncoupling protein 2 (UCP2) and uncoupling protein 3 (UCP3) can both uncouple mitochondrial respiration from oxidative phosphorylation, thereby dissipating energy as heat. In contrast to UCP2, which is ubiquitously expressed in virtually all tissues tested (1, 2), human UCP3 is known to be selectively expressed in skeletal muscle (3). Because skeletal muscle is a major contributor to human energy expenditure, UCP3 is thought to play an important role in the dissipation of energy in human energy metabolism and is thus linked to obesity (4, 5) and type 2 diabetes (6–8). We

previously reported that, in Pima Indians, body mass index (BMI) correlates negatively with UCP3 mRNA, while sleeping metabolic rate was positively related to UCP3 mRNA (9) This suggests a role forUCP3 in obesity. The postulated role for UCP3 in type 2 diabetes relies on observations in rat gastrocnemius, which shows a positive relation between UCP3 mRNA and insulin resistance (10), and on linkage studies indicating that the UCP3 gene is located in the same chromosomal region as UCP2. These regions are also associated with hyperinsulinemia and obesity (1, 11). Moreover, when using a chemical uncoupling agent in L6 muscle cells, it was shown that, in concert with a declined ATP/ADP ratio, glucose uptake rose twofold with a concomitant up-regulation of GLUT1 (12). Therefore, expression of the endogenous uncoupler UCP3 might be associated with glucose homeostasis and thus type 2 diabetes.

We know several factors that induce gene expression of UCP3. Incompatible with the concept of energy dissipation, increased UCP3 expression in muscle was reported during fasting (10, 13, 14), an energy expenditure attenuating condition. Because UCP3 also increases upon high-fat feeding (15), it is now believed that increased UCP3 expression during fasting and high-fat feeding is induced by increased free fatty acid levels and that UCP3 is involved in transport of fatty acid anions across the mitochondrial membrane (16). Free fatty acid-induced up-regulation of UCP3 is more pronounced in muscles comprising type 2 muscle fibers than in muscles comprising type 1 fibers (14). Because fast oxidative glycolytic muscles (comprising mainly type 2 fibers) switch rapidly from carbohydrate metabolism toward fat oxidation, expression of UCP3 may facilitate mitochondrial fatty acid uptake (in addition to carnitine-dependent uptake). This finding suggests a role for UCP3 in lipid metabolism and underscores the importance of studying the expression of UCP3 in different muscle fiber types in obesity and type 2 diabetes, because impairments in lipid utilization have been reported in both populations (17, 18).

Previously, we have shown that endurance-trained athletes have lower UCP3 mRNA expression than untrained controls and that UCP3 gene expression correlates negatively with maximal aerobic power (19). Endurance training prominently down-regulates UCP3 in rat fast glycolytic (type 2) muscle and, to a lesser extent, in slow oxidative (type 1) muscle (20). Because endurance athletes have a high percentage of type 1 muscle fibers and endurance exercise heavily relies on these fibers, these findings are indicative for fiber-type specific expression of UCP3.

To date, fiber-type specific expression of UCP3 has been examined only indirectly at the mRNA level by studying homogenates of whole rat muscles classically qualified as FG, FOG, or SO muscles (3). Extrapolation of these findings to humans is most likely invalid, because fiber-type distribution within a human muscle is much more heterogeneous (21, 22), and recruiting the distinct fibers within a muscle (as occurs in humans) will affect energy metabolism differentially (23, 24).

Note that all reports available on UCP3 expression in skeletal muscle show data on the mRNA level. Due to mRNA instability and posttranslational modifications, alterations at the mRNA level only poorly reflect changes at the protein level, the actual parameter of interest with regard to the functional role of UCP3.

To explore the physiological role of UCP3 in human skeletal muscle further, it is essential to study the expression of UCP3 in the distinct human muscle fiber types in a direct manner and at the protein level. Therefore, the objective of the present study is to examine—for the first time directly, at the protein level and in human muscle—expression of UCP3 in the distinct muscle fibers in *vastus lateralis* muscle of healthy controls and type 2 diabetics.

METHODS

Subjects and biopsy procedure

After approval of the Institutional Ethical Review board, 10 middle-aged (age 46±5 yr, BMI 27.8±2.7; mean±SD) males with type 2 diabetes were included together with 3 healthy male controls (age 25±6 yr., BMI 22.2±0.5; mean± SD). All gave their written, informed consent before entering the study. Subjects were instructed to fast overnight and refrain from any vigorous exercise for 24 h prior to the needle biopsy (*vastus lateralis* muscle). Skin and muscle fascia were anesthetized with xylocaine, and an incision of ~0.7–1.0 cm was made through skin and fascia. All biopsies were taken ~20 cm proximally from the patella, and muscle tissue was immediately frozen in melting isopentane. UCP3 expression was studied by immunofluorescence on 4-μm cryosections, while muscle fiber typing (myosin ATP-ase staining (25)) was performed on serial transverse, 6-μm-thick cryosections.

Antibodies

The antibody against UCP3 was raised in a rabbit against a 20 amino acid peptide that comprised amino acids 147–166 of human UCP3 (hUCP3). Upon affinity chromatography, the purified serum was tested for specificity in immunofluorescence and immunoblots. Immunofluorescence was performed on rat H9C2 myoblasts (lacking endogenous UCP3 expression), which were stably transfected with human UCP3, as well as on sections of adult human skeletal muscle. Cells or sections were incubated with the UCP3 antibody. To test whether the signal raised by the UCP3 antibody was consistent with mitochondria, double immunofluorescence was performed with UCP3 and cytochrome *c*. Cytochrome *c* was labeled by a commercially available (Pharmingen) monoclonal mouse IgG_1 against cytochrome *c*. The primary antibodies were visualized by the following secondary antibodies: goat-anti-rabbit (GAR) Ig conjugated with Texas Red (UCP3) or goat-anti-mouse (GAM) IgG_1 conjugated with FITC (cytochrome *c*). Preincubation of the primary antibody with the peptide and omission of the primary antibody was performed in order to test cross-reactivity of the secondary antibodies used in immunofluorescence.

Negative specificity checks that were performed included immunoblotting on homogenates of human kidney, endogenously expressing UCP2 (2) while lacking UCP3 (3). Immunoblots on adult human skeletal tissue were run in order to check for cross-reactivity with proteins of other molecular mass. For immunoblotting, tissue was homogenized in ice-cold TRIS-EDTA buffer at pH 7.4. The homogenates were subsequently sonicated for 4 × 15 s. Routine polyacrylamide SDS-gel electrophoresis was performed essentially according to Laemmli (26). Nitrocellulose sheets were preincubated with blocking buffer for 60 min; it contained 5% nonfat dry milk (BioRad, Hercules, Calif.) and 0.05% Tween20 (Sigma, St. Louis, Mo.) in PBS. Incubation with anti-UCP3, diluted 1:1.000 in blocking buffer, was carried out

overnight at room temperature with gentle shaking. After washing, blots were incubated for 60 min at room temperature with horseradish peroxidase-conjugated swine anti-rabbit Ig (SWARPO, DAKO, Glostrup, Denmark), diluted 1:10.000 in blocking buffer. Blots were subsequently washed for 90 min in 0.05% Tween20/PBS, and 10 min in PBS. Chemiluminescence was performed by using a Super Signal West Dura Extended kit (Pierce, Rockford, Ill.). The molecular weight of the band visualized by chemiluminescence was compared with reference values.

Muscle sectioning and staining

Frozen muscle tissue was cryosectioned at –20ºC (CM3050, Leica, Nussloch, Germany). Two successive sections were sequentially thaw-mounted on one of two uncoated glass slides. Routine immunofluorescence was performed after methanol acetone fixation. Sections were incubated overnight at 4°C with both primary antibodies (1:10 in 1% BSA/PBS) and for 30 min at room temperature with goat-anti-rabbit (GAR) Ig conjugated with Texas Red (GAR-TxRd, SBA Inc., Birmingham, Ala.) or goat-anti-mouse (GAM) IgG_1 conjugated with fluorescein isothiocyanate-conjugated (GAM-FITC, SBA Inc., Birmingham, Ala.) and mounted in Mowiol (Hoechst, Frankfurt, Germany) containing 0.5 µg/ml 4'-6'-diamino-2-phenylindole (DAPI, Merck, The Netherlands) for staining nuclear DNA.

The second slide, with corresponding serial sections, was subjected to muscle fiber typing by using the ATP-ase staining method (25). Sections were immersed in acetate buffer at either pH 4.3 to distinguish type 1 and type 2 fibers, or pH 4.5 to subdivide the type 2 fibers into type 2a and type 2b fibers. Nuclei were stained for 1 min with hematoxylin solution according to Mayer (Fluka Chemie AG, Buchs, Switzerland). After the slides were rinsed for 20 min in tap water, they were dehydrated in an ethanol series of 50%–100% and 2× xylol 100%, and embedded in Entallan (Merck).

Of all subjects, a minimum of three sections were examined in duplicate and photographed by using a Leica DM fluorescence microscope, coupled to a CCD camera and image processing system (MetaSystem IV, Heidelberg, Germany). Sections stained with ATP-ase were arbitrarily photographed, after which matching fields were traced in the corresponding fluorescent sections at identical magnification. On all sections examined, at least four corresponding fields, comprising at least 150 different fibers, were matched offline by visual inspection. Subsequently, these fields were examined by comparing the relative abundance of UCP3 protein expression within the distinguishable fiber types (type 1, 2a, and 2b).

RESULTS

Immunofluorescence of the H9C2 myoblasts, transfected with hUCP3, by using the affinity purified UCP3 antiserum revealed a punctate mitochondrial staining pattern (Fig. 1a). Double staining of UCP3 with cytochrome *c* indicates that the UCP3 stain was restricted to mitochondria as is clearly indicated by the yellow to orange stain at sites where UCP3 and cytochrome *c* co-exist (H9C2 cells not shown; a representative muscle section is shown in Fig. 1b). Preincubation with the peptide yielded no detectable labeling (data not shown). In

addition, omission of the UCP3 antibody completely abolished the fluorescent signal (data not shown).

Western blotting of human *vastus lateralis* muscle against UCP3 raised one single band at ~34 kDa (Fig. 1c), corresponding to UCP3L. Cross-reaction of the primary antibody with other molecular weight proteins is negligible in the 5 to 60 kDa range in human *vastus lateralis* samples (Fig. 1c). The UCP3 antibody did not cross-react with UCP2, as judged by the absence of bands in the Western blot of human kidney (a UCP3 negative but UCP2 positive tissue) (Fig. 1d).

Abundance of UCP3 expression in distinct fiber types

Myosin ATP-ase staining with pH 4.3 (Figs. 2a and c) yielded the chessboard pattern of two distinguishable fiber types (type 1 and type 2), classically observed in human *vastus lateralis.* Differentiation between type 1, 2a, and 2b fibers was obtained by staining at pH 4.5 (Fig. 2e). Immunofluorescence against UCP3 showed an abundant signal in fibers classified as type 2 in ATP-ase staining, both in healthy controls (Fig. 2b) as in type 2 diabetics (Fig. 2d), whereas UCP3 was less abundantly expressed in type 1 fibers (Figs. 2b and d). Subclassifying type 2 fibers in 2a and 2b (Fig. 2e) revealed that UCP3 was more abundantly expressed in type 2b fibers than in type 2a fibers (Fig. 2f). No differences were observed between healthy controls and type 2 diabetic with regard to their relative expression of UCP3 in distinct muscle fibers (type 1, 2a, and 2b). Staining of cytochrome C displayed the reverse pattern than that observed after UCP3 labeling; that is, intense staining in type 1 fibers, less in type 2a, and least in type 2b fibers, which is consistent with the mitochondrial density in the respective fiber types.

DISCUSSION

Skeletal muscle plays a major role in energy expenditure, under resting conditions as well as during exercise. UCP3 is considered to be an important mediator of the metabolic rate and therefore has a putative role in the pathophysiology of obesity and type 2 diabetes. However, the studies targeting the functional role of UCP3 in human energy and substrate metabolism are all at the mRNA level, and convincing reports on protein expression of UCP3 are scarce due to the lack of specific antibodies (5). Here, we present an antibody that successfully passed all specificity checks and has been proven to recognize UCP3 in human skeletal muscle by Western blotting and by immunofluorescence. Therefore, we conclude that the antibody presented here does not cross-react with any of the other mitochondrial proton carriers presently known in skeletal muscle and is therefore the first antibody that specifically recognizes UCP3 in human skeletal muscle. This cardinal step opens the possibility to further explore the role of UCP3 in human energy and substrate metabolism and its putative role in the pathophysiology of metabolic disorders like type 2 diabetes and obesity. Very recently, studies in transgenic mice overexpressing UCP3 show an increased food intake, declined body mass, a striking reduction in adipose tissue mass, and improved glucose tolerance (27), which indicates that UCP3 indeed may play a role in metabolic disorders.

In human skeletal muscle, UCP3 mRNA exists as a short and a long transcript (UCP3S and UCP3L, respectively), both generated from the same gene. It is remarkable that the antibody raised one single band at 34 kDa, whereas the peptide used to raise the antibody comprises amino acids 147–166, which are common to UCP3S and UCP3L. This finding raises the possibility that UCP3S, which lacks its sixth membrane-spanning domain (3), is not expressed at the protein level in human skeletal muscle. The mitochondrial ADP/ATP translocator, a protein closely related to UCP3, requires the sixth membrane-spanning domain for insertion of the protein into the mitochondrial inner membrane (28). It could therefore be argued that UCP3S is not(or only loosely) inserted into mitochondria and may thus be lost during homogenization. However, in liver mitochondria, it has recently been shown *in vitro* that UCP3S and UCP3L are both imported and inserted into the mitochondrial inner membrane (29). Whether this finding also holds for human skeletal muscle mitochondria is unknown.

Our main objective here was to examine the expression of UCP3, directly and at the protein level, in the prevailing three fiber types of human skeletal muscle. After examining three duplicate (serially cut) sections per subject with a minimum of four microscopical fields (comprising ~150 muscle fibers), we consistently observed that fibers classified as type 1 by conventional ATP-ase staining showed the lowest expression of UCP3, whereas UCP3 was more abundantly expressed in human type 2 fibers. Upon subclassification of type 2 fibers into type 2a and 2b, it was shown that type 2b fibers exhibited the highest expression of UCP3. This relationship was observed in all sections examined and agrees with observations on the mRNA level in whole muscle homogenates of selected rat muscles (3, 10, 14, 30).

Moreover, we observed that the relation between muscle fiber typology and UCP3 expression in sections from type 2 diabetics did not deviate from the relationship reported in healthy controls. Because type 2 diabetics are characterized by more type 2b fibers than healthy controls (31), the observation that UCP3 is expressed more abundantly in type 2b fibers would support increased UCP3 mRNA levels in type 2 diabetics. We are aware of two studies that report higher UCP3 mRNA expression in type 2 diabetics (6, 8), while one report shows a decrease in UCP3 mRNA expression (7). Transgenic mice overexpressing GLUT4 also up-regulate UCP3 and possess increased glucose uptake in gastrocnemius muscle (type 2 fibers) (32). Conversely, transgenic mice overexpressing UCP3 have lower fasting glucose levels and blunted response to an oral glucose load (improved glucose tolerance) (27). This finding could imply that UCP3 is involved directly in glucose uptake in glycolytic (type 2) fibers. However, a plausible alternative would be that increased glucose uptake is secondary to a declined ATP/ADP ratio because of the uncoupling nature of UCP3. Indeed, it has been shown before that addition of a chemical uncoupler to L6 muscle cells results in an increased uptake of glucose. The abundant expression of UCP3 in glycolytic type 2 fibers compared with type 1 fibers matches a role for UCP3 in glucose uptake in the glycolytic fibers. However, it has been shown that contraction and insulin-induced glucose uptake is highest in oxidative (type 1) muscles. Clearly, comparison of UCP3 protein content between type 2 diabetics and controls, matched for age and BMI, is needed to assess the expression and role of UCP3 in type 2 diabetic subjects. Analysis of these groups is currently underway in our lab.

In people with high free fatty acid levels (starvation and high-fat feeding), UCP3 mRNA is most prominently up-regulated in glycolytic muscles (with a preponderance of type 2 fibers), which is unexpected considering the fasting-induced decline in energy expenditure. However, it has been hypothesized that UCP3, as it is a mitochondrial anion carrier, is involved in cycling of fatty acid anions across the mitochondrial membrane (16). In this respect, the high expression in type 2a fibers during fasting can be seen as an alternative route to force fatty acid anions into the mitochondria and, finally, to use fatty acids as a substrate. However, type 2b fibers express the highest levels of UCP3 while having the lowest capacity to oxidize fatty acids. In contrast with type 2a fibers, which can switch rapidly from glucose to fatty acid oxidation, the abundant expression of UCP3 in type 2b fibers does not match with a role for UCP3 in fatty acid handling.

Even before UCP3 was discovered by Boss et al. (3), it was hypothesized that mitochondria could possess a special mechanism, referred to as "mild uncoupling", to prevent excessive production of oxygen free radicals during and immediately after physical exercise (33). Excessive production of reactive oxygen species (ROS) occurs when the mitochondrial membrane potential is raised above a certain threshold (34). Thus, mild uncoupling, lowering the mitochondrial membrane potential, would prevent excessive ROS production. Indeed, it was shown recently that after ablation of the UCP3 gene, more ROS were produced (35); in transgenic mice, overexpressing UCP3 a 12% decline in membrane potential was observed (27). It was concluded that UCP3 has a role in minimizing the production of ROS, thus preventing excessive oxidative stress (35). With increasing exercise intensity, type 2a and 2b fibers are progressively recruited (for example, see ref 36), which is paralleled by increased oxidative stress (37). Moreover, it has been shown that upon acute exercise, fast-twitch muscles (comprising predominantly type 2 fibers) produce more malondialdehyde (MDA), a marker for oxidative stress, than do slow muscles (comprising type 1 fibers) (37, 38). Also, in type 2 fibers, and especially type 2b fibers, oxidative stress occurs during reoxygenation after anaerobic exercise, which increases the need for an effective ROS defense system (34). Hence, the profile of expression of UCP3 in type 1, 2a, and 2b fibers is compatible with a role of UCP3 in minimizing oxidative stress.

Data on UCP3 expression in relation to endurance training are equivocal: reports range from declined expression (20), no effect (39), to increased expression (40). Based on reports showing an inverse relationship between UCP3 mRNA and gross mechanical efficiency and maximal oxygen uptake (19), endurance training would be expected to lower UCP3 expression. This finding is consistent with the low expression of UCP3 in type 1 fibers reported here, because endurance-trained athletes have a preponderance of type 1 fibers. During exercise of high intensity among subjects, however, type 2b fibers are progressively recruited. The abundance of UCP3 in these fibers would indicate that ATP formation becomes less efficient with increasing exercise intensity, which could be considered remarkable.

We proved that the antibody presented here specifically recognizes UCP3 in human skeletal muscle, both in tissue sections as in muscle homogenates. Moreover, the present study is the first to show—in a direct manner and at the protein level—that UCP3 is most abundantly

expressed in type 2b (fast glycolytic) fibers, to a lesser extent in type 2a (fast oxidative glycolytic) fibers, and is only moderately expressed in type 1 (slow oxidative) fibers of human *vastus lateralis* muscle. We observed no differences in this relationship between healthy controls and type 2 diabetics.

However, of the postulated roles of UCP3 or factors known so far to induce the UCP3 gene, only a role of UCP3 in preventing oxidative stress appears to be fully compatible with the fiber-type specific expression of UCP3. It could therefore be hypothesized that UCP3-induced dissipation of energy serves multiple goals, which are muscle fiber-type specific.

REFERENCES

1. Fleury, C., Neverova, M., Collins, S., Raimbault, S., Champigny, O., Levi-Meyrueis, C., Bouillaud, F., Seldin, M. F., Surwit, R. S., Ricquier, D., and Warden, C. H. (1997) Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia. *Nat. Genet.* **15,** 269–272

2. Gimeno, R. E., Dembski, M., Weng, X., Deng, N., Shyjan, A. W., Gimeno, C. J., Iris, F., Ellis, S. J., Woolf, E. A., and Tartaglia, L. A. (1997) Cloning and characterization of an uncoupling protein homolog: a potential molecular mediator of human thermogenesis. *Diabetes* **46,** 900–906

3. Boss, O., Samec, S., Paoloni-Giacobino, A., Rossier, C., Dulloo, A., Seydoux, J., Muzzin, P., and Giacobino, J. P. (1997) Uncoupling protein-3: a new member of the mitochondrial carrier family with tissue-specific expression. *FEBS Lett.* **408,** 39–42

4. Schrauwen, P., Walder, K., and Ravussin, E. (1999) Human uncoupling proteins and obesity. *Obes. Res.* **7,** 97–105

5. Ricquier, D., and Bouillaud, F. (2000) The uncoupling protein homologues: UCP1, UCP2, UCP3, StUCP and AtUCP. *Biochem J.* **345 Pt 2,** 161–179

6. Bao, S., Kennedy, A., Wojciechowski, B., Wallace, P., Ganaway, E., and Garvey, W. T. (1998) Expression of mRNAs encoding uncoupling proteins in human skeletal muscle: effects of obesity and diabetes. *Diabetes* **47,** 1935–1940

7. Krook, A., Digby, J., O'Rahilly, S., Zierath, J. R., and Wallberg-Henriksson, H. (1998) Uncoupling protein 3 is reduced in skeletal muscle of NIDDM patients. *Diabetes* **47,** 1528–1531

8. Vidal, H., Langin, D., Andreelli, F., Millet, L., Larrouy, D., and Laville, M. (1999) Lack of skeletal muscle uncoupling protein 2 and 3 mRNA induction during fasting in type-2 diabetic subjects. *Am. J. Physiol.* **277,** E830–837

9. Schrauwen, P., Xia, J., Bogardus, C., Pratley, R. E., and Ravussin, E. (1999) Skeletal muscle uncoupling protein 3 expression is a determinant of energy expenditure in Pima Indians. *Diabetes* **48,** 146–149

10. Samec, S., Seydoux, J., and Dulloo, A. G. (1999) Post-starvation gene expression of skeletal muscle uncoupling protein 2 and uncoupling protein 3 in response to dietary fat levels and fatty acid composition: a link with insulin resistance. *Diabetes* **48,** 436–441

11. Gong, D. W., He, Y., Karas, M., and Reitman, M. (1997) Uncoupling protein-3 is a mediator of thermogenesis regulated by thyroid hormone, beta3-adrenergic agonists, and leptin. *J. Biol. Chem.* **272,** 24129–24132

12. Bashan, N., Burdett, E., Guma, A., Sargeant, R., Tumiati, L., Liu, Z., and Klip, A. (1993) Mechanisms of adaptation of glucose transporters to changes in the oxidative chain of muscle and fat cells. *Am. J. Physiol.* **264,** C430–440

13. Millet, L., Vidal, H., Andreelli, F., Larrouy, D., Riou, J. P., Ricquier, D., Laville, M., and Langin, D. (1997) Increased uncoupling protein-2 and -3 mRNA expression during fasting in obese and lean humans. *J. Clin. Invest.* **100,** 2665–2670

14. Samec, S., Seydoux, J., and Dulloo, A. G. (1998) Interorgan signaling between adipose tissue metabolism and skeletal muscle uncoupling protein homologs: Is there a role for circulating free fatty acids? *Diabetes* **47,** 1693–1698

15. Weigle, D. S., Selfridge, L. E., Schwartz, M. W., Seeley, R. J., Cummings, D. E., Havel, P. J., Kuijper, J. L., and Beltrandel Rio, H. (1998) Elevated free fatty acids induce uncoupling protein 3 expression in muscle: a potential explanation for the effect of fasting. *Diabetes* **47,** 298–302

16. Jezek, P., and Garlid, K. D. (1998) Mammalian mitochondrial uncoupling proteins. *Int. J. Biochem. Cell Biol.* **30,** 1163–1168

17. Blaak, E. E., Van Baak, M. A., Kemerink, G. J., Pakbiers, M. T., Heidendal, G. A., and Saris, W. H. (1994) Beta-adrenergic stimulation of energy expenditure and forearm skeletal muscle metabolism in lean and obese men. *Am. J. Physiol.* **267,** E306–315

18. Colberg, S. R., Simoneau, J. A., Thaete, F. L., and Kelley, D. E. (1995) Skeletal muscle utilization of free fatty acids in women with visceral obesity [see comments]. *J. Clin. Invest.* **95,** 1846–1853

19. Schrauwen, P., Troost, F. J., Xia, J., Ravussin, E., and Saris, W. H. (1999) Skeletal muscle UCP2 and UCP3 expression in trained and untrained male subjects. *Int. J. Obes. Relat. Metab. Disord.* **23,** 966–972

20. Boss, O., Samec, S., Desplanches, D., Mayet, M. H., Seydoux, J., Muzzin, P., and Giacobino, J. P. (1998) Effect of endurance training on mRNA expression of uncoupling proteins 1, 2, and 3 in the rat. *FASEB J.* **12,** 335–339

21. Staron, R. S. (1997) Human skeletal muscle fiber types: delineation, development, and distribution. *Can. J. Appl. Physiol.* **22,** 307–327

22. Simoneau, J. A., and Bouchard, C. (1989) Human variation in skeletal muscle fiber-type proportion and enzyme activities. *Am. J. Physiol.* **257,** E567–572

23. Achten, E., Van Cauteren, M., Willem, R., Luypaert, R., Malaisse, W. J., Van Bosch, G., Delanghe, G., De Meirleir, K., and Osteaux, M. (1990) 31P-NMR spectroscopy and the metabolic properties of different muscle fibers. *J. Appl. Physiol.* **68,** 644–649

24. Park, J. H., Brown, R. L., Park, C. R., McCully, K., Cohn, M., Haselgrove, J., and Chance, B. (1987) Functional pools of oxidative and glycolytic fibers in human muscle observed by 31P magnetic resonance spectroscopy during exercise. *Proc. Natl. Acad. Sci. USA* **84,** 8976–8980

25. Brooke, M. H., and Kaiser, K. K. (1970) Muscle fiber types: how many and what kind? *Arch. Neurol.* **23,** 369–379

26. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685

27. Clapham, J. C., Arch, J. R., Chapman, H., Haynes, A., Lister, C., Moore, G. B., Piercy, V., Carter, S. A., Lehner, I., Smith, S. A., Beeley, L. J., Godden, R. J., Herrity, N., Skehel, M., Changani, K. K., Hockings, P. D., Reid, D. G., Squires, S. M., Hatcher, J., Trail, B., Latcham, J., Rastan, S., Harper, A. J., Cadenas, S., Buckingham, J. A., Brand, M. D., and Abuin, A. (2000) Mice overexpressing human uncoupling protein-3 in skeletal muscle are hyperphagic and lean. *Nature (London)* **406,** 415–418

28. Endres, M., Neupert, W., and Brunner, M. (1999) Transport of the ADP/ATP carrier of mitochondria from the TOM complex to the TIM22.54 complex. *Embo. J.* **18,** 3214–3221

29. Renold, A., Koehler, C. M., and Murphy, M. P. (2000) Mitochondrial import of the long and short isoforms of human uncoupling protein 3. *FEBS Lett.* **465,** 135–140

30. Samec, S., Seydoux, J., and Dulloo, A. G. (1999) Skeletal muscle UCP3 and UCP2 gene expression in response to inhibition of free fatty acid flux through mitochondrial beta-oxidation. *Pflugers Arch.* **438,** 452–457

31. Marin, P., Andersson, B., Krotkiewski, M., and Bjorntorp, P. (1994) Muscle fiber composition and capillary density in women and men with NIDDM. *Diabetes Care* **17,** 382–386

32. Tsuboyama-Kasaoka, N., Tsunoda, N., Maruyama, K., Takahashi, M., Kim, H., Cooke, D. W., Lane, M. D., and Ezaki, O. (1999) Overexpression of GLUT4 in mice causes up-regulation of UCP3 mRNA in skeletal muscle. *Biochem. Biophys. Res. Commun.* **258,** 187–193

33. Skulachev, V. P. (1996) Role of uncoupled and non-coupled oxidations in maintenance of safely low levels of oxygen and its one-electron reductants. *Q. Rev. Biophys.* **29,** 169–202

34. Skulachev, V. P. (1998) Uncoupling: new approaches to an old problem of bioenergetics. *Biochim. Biophys. Acta* **1363,** 100–124

35. Vidal-Puig, A. J., Grujic, D., Zhang, C. Y., Hagen, T., Boss, O., Ido, Y., Szczepanik, A., Wade, J., Mootha, V., Cortright, R., Muoio, D. M., and Lowell, B. B. (2000) Energy metabolism in uncoupling protein 3 gene knockout mice. *J. Biol. Chem.* **275,** 16258–16266

36. Coyle, E. F. (2000) Physical activity as a metabolic stressor. *Am. J. Clin. Nutr.* **72,** 512S–520S

37. Alessio, H. M., Goldfarb, A. H., and Cutler, R. G. (1988) MDA content increases in fast- and slow-twitch skeletal muscle with intensity of exercise in a rat. *Am. J. Physiol.* **255,** C874–877

38. Liu, J., Yeo, H. C., Overvik-Douki, E., Hagen, T., Doniger, S. J., Chu, D. W., Brooks, G. A., and Ames, B. N. (2000) Chronically and acutely exercised rats: biomarkers of oxidative stress and endogenous antioxidants. *J. Appl. Physiol.* **89,** 21–28

39. Tonkonogi, M., Krook, A., Walsh, B., and Sahlin, K. (2000) Endurance training increases stimulation of uncoupling of skeletal muscle mitochondria in humans by non-esterified fatty acids: an uncoupling-protein-mediated effect? *Biochem. J.* **351,** 805–810

40. Pilegaard, H., Ordway, G. A., Saltin, B., and Neufer, P. D. (2000) Transcriptional regulation of gene expression in human skeletal muscle during recovery from exercise. *Am. J. Physiol. Endocrinol. Metab.* **279,** E806–814

Received August 8, 2000; revised November 7, 2000.

Fig. 1

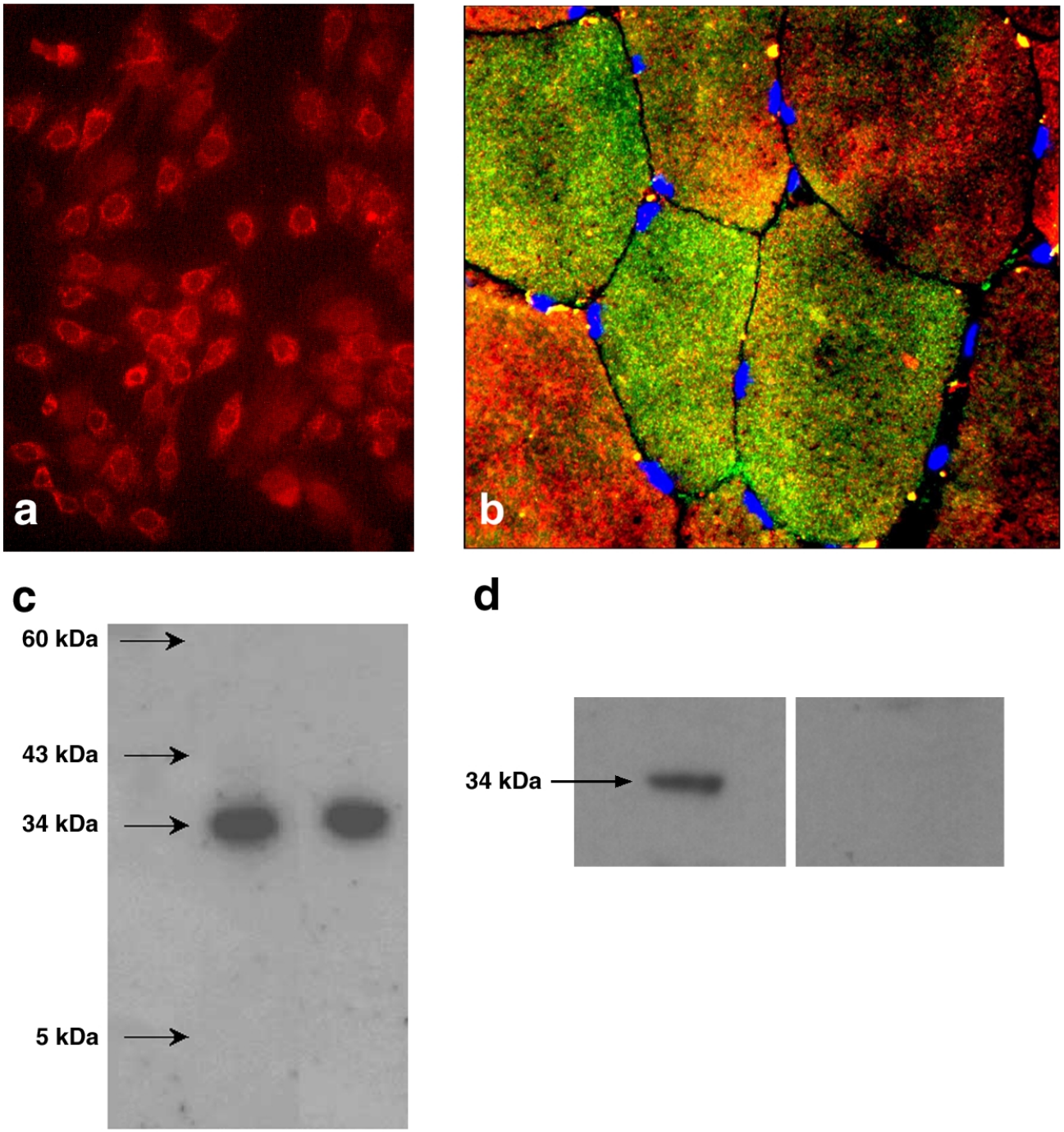

Figure 1. **(a)** Immunofluorescence of UCP3 in rat H9C2 myoblasts, stably transfected with hUCP3. Note the punctate mitochondrial staining. **(b)** Representative section of human vastus lateralis muscle obtained from a healthy control. The green stain represents cytochrome C, the red signal represents UCP3; nuclei are stained blue. At sites where the stain turns yellow–orange cytochrome C and UCP3 coexist, which indicates that the signal yielded by the UCP3 antibody is consistent with mitochondria. **(c)** Typical autoradiograph after Western blotting by using the antibody described here, note that a single band representing hUCP3 was detected at 34 kDa in human muscle samples (left lane healthy control, right lane type 2 diabetic). On each lane of the gel, ~15 mg of protein was loaded. No cross-reaction was observed in the 5 to 60 kDa region. **(d)** Autoradiograph after Western blotting using the antibody described here, in human skeletal muscle (left lane) and human kidney (right lane). The lack of signal in the human kidney sample, known to express UCP2 but not UCP3, shows that the present antibody does not cross-react with UCP2.

Fig. 2

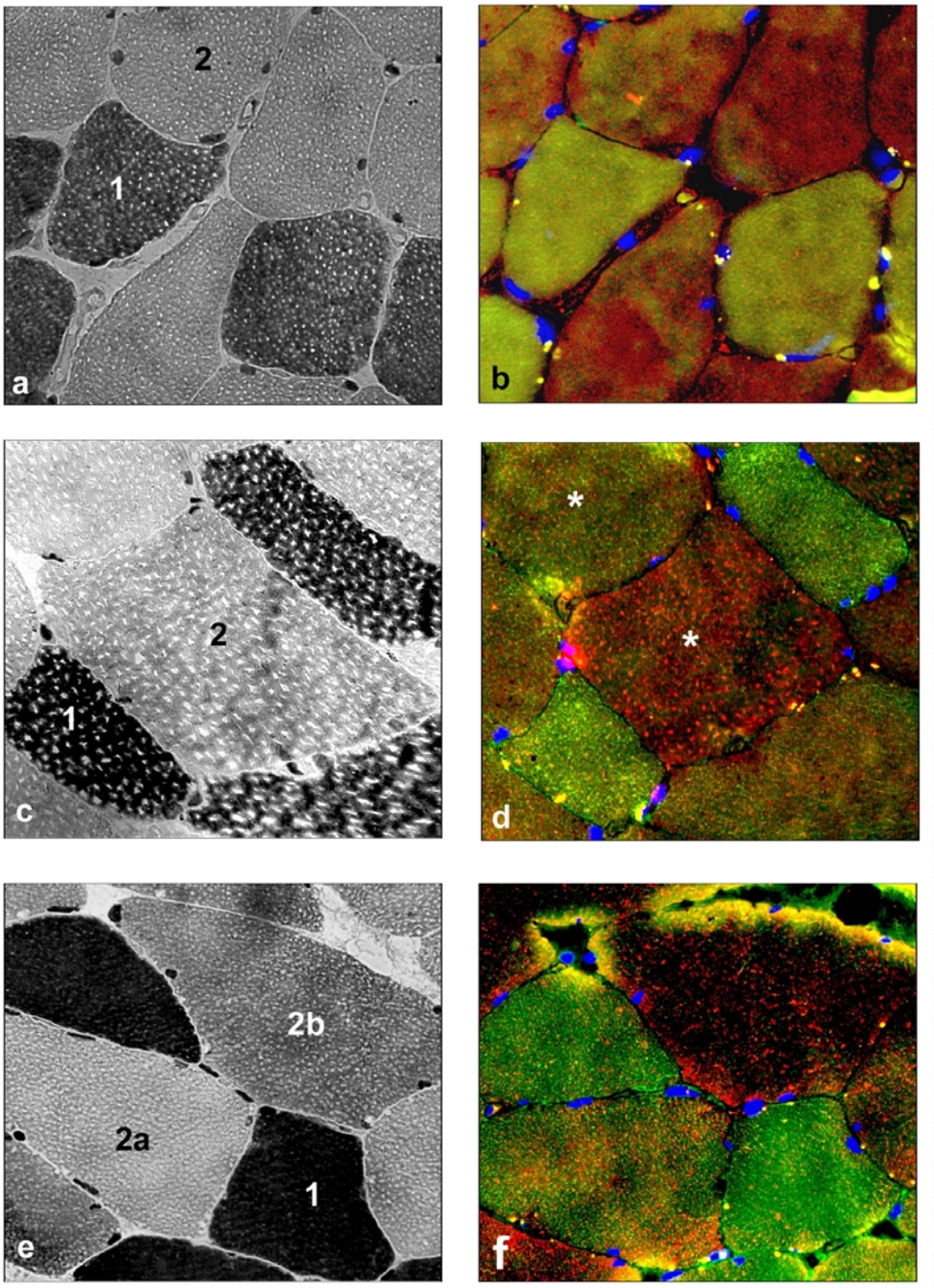

Figure 2. (a) ATP-ase staining (pH 4.3) of vastus lateralis obtained from a healthy control, type 1 fibers stain black, type 2 fibers stain pale. **(b)** Double fluorescence of the corresponding serial section, again cytochrome C is stained green, UCP3 stains red, en nuclei stain blue. Obviously, type 1 fibers express cytochrome C abundantly while possessing only a weak stain for UCP3, in the type 2 fibers the reverse is observed. **(c)** ATP-ase staining (pH 4.3) of vastus lateralis obtained from a type 2 diabetic, type 1 fibers stain black, type 2 fibers stain pale. **(d)** Double fluorescence of the corresponding serial section, the same fluorochromes were used as in **Figure 2b**. Note the difference in intensity of staining of UCP3 in the two type 2 fibers marked with an asterix. **(e)** ATP-ase stain of a section obtained from a type 2 diabetic at pH 4.5, which, in human muscle, permits differentiation between type 1 fibers (black), type 2a fibers (pale), and type 2b fibers (intermediate), in the corresponding serial section **(f)** it can be seen that expression of UCP3 is most abundant in fibers classified as type 2b, lower in type 2a and least in type 1 fibers. All pictures shown are representative and were obtained after randomly imaging the ATP-ase-stained sections with off-line matching with corresponding field in fluorescence.