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Decreased Fatty Acid β-Oxidation in Riboflavin-Responsive, Multiple Acylcoenzyme A Dehydrogenase-Deficient Patients Is Associated with an Increase in Uncoupling Protein-3

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Riboflavin-responsive, multiple acylcoenzyme A dehydrogenase deficiency (RR-MAD), a lipid storage myopathy, is characterized by, among others, a decrease in fatty acid (FA) β-oxidation capacity. Muscle uncoupling protein 3 (UCP3) is up-regulated under conditions that either increase the levels of circulating free FA and/or decrease FA β-oxidation. Using a relatively large cohort of seven RR-MAD patients, we aimed to better characterize the metabolic disturbances of this disease and to explore the possibility that it might increase UCP3 expression. A battery of biochemical and molecular tests were performed, which demonstrated decreases in FA β-oxidation and in the activities of respiratory chain complexes I and II. These metabolic alterations were associated with increases of 3.1- and 1.7-fold in UCP3 mRNA and protein expression, respectively. All parameters were restored to control values after riboflavin treatment. We postulate that the up-regulation of UCP3 in RR-MAD is due to the accumulation of muscle FA/acylCoA. RR-MAD is an optimal model to support the hypothesis that UCP3 is involved in the outward translocation of an excess of FA from the mitochondria and to show that, in humans, the effects of FA on UCP3 expression are direct and independent of fatty acid β-oxidation. (*J Clin Endocrinol Metab* 88: 5921–5926, 2003)

UNCOUPLING PROTEIN 3 (UCP3) is a member of the mitochondrial carrier family expressed predominantly in the skeletal muscle of rodents and humans (1). UCP3 does not seem to play a role in thermoregulation in rodents, and its uncoupling activity might, in fact, be a consequence of some until now unknown primary function (2–4). The observation that UCP3 expression is increased in situations where fatty acid (FA) entry into the mitochondria may exceed the β-oxidation capacity suggests that this protein is involved in the outward translocation of FA from the mitochondria matrix. This would prevent a deleterious intramitochondrial accumulation of FA (5, 6).

UCP3 mRNA expression was found to be dramatically increased in rodents by fasting (7) and this phenomenon was found to be mediated by increased levels of circulating free FA (FFA) (8). In obese humans, a positive correlation was observed between the level of circulating FFA and muscle UCP3 mRNA expression (9). Furthermore, in humans an increased level of circulating FFA induced by lipid infusion stimulated UCP3 mRNA expression in muscle (10). These experiments confirmed the pivotal role of FA in the control of muscle UCP3 expression in humans, but did not allow determination of whether this effect was direct or indirect.

Etomoxir, an inhibitor of carnitine palmitoyltransferase I, was used *in vivo* in rats (11) and humans (12) to investigate the relationship between FFA and UCP3 expression. Fasting combined with etomoxir treatment increased UCP3 mRNA in rat soleus slow oxidative (type I) muscle, strongly suggesting a direct effect of FA. In contrast, under the same conditions, UCP3 was reduced in tibialus anterior fast gylcolytic (type IIa/b) muscle, suggesting a dependency on fatty acid β-oxidation (11). In humans, etomoxir treatment resulted in an increase in muscle UCP3 protein content, suggesting a direct effect of FA (12). Whether this increase in UCP3 was fiber type dependent, as in rodents, was not determined. Furthermore, the physiological importance of this pharmacological intervention remained to be assessed.

Riboflavin-responsive, multiple acyl-coenzyme A (CoA) dehydrogenase deficiency (RR-MAD) is a muscle disease belonging to the group of lipid storage myopathies. All patients demonstrate a urinary organic acids profile compatible with either glutaric aciduria type II or ethymalonic-adipic aciduria (13, 14). Biochemically, the disease is characterized by the reduced activity of flavin-dependent acyl-CoA dehydrogenases, which could be associated with a deficiency of two flavin-dependent respiratory chain complexes: complexes I and II (15, 16). It is thought that the pathogenetic

Abbreviations: CoA, Coenzyme A; FA, fatty acid; FFA, free fatty acids; IMCL, intramyocellular lipid; RR-MAD, riboflavin-responsive, multiple acylcoenzyme A dehydrogenase deficiency; UCP, uncoupling protein.

mechanism underlying the biochemical defect in RR-MAD is a reduced amount of intramitochondrial flavin adenine dinucleotide (FAD), causing an accelerated breakdown of flavin-dependent enzyme proteins (14–16). As a consequence, among others, this deficit decreases fatty acid β-oxidation activity, with a concomitant increase in intramyocellular lipid content. It can be spectacularly cured by the administration of riboflavin, the precursor of FAD and flavin mononucleotide (FMN), which normalizes the activities of flavin-dependent mitochondrial enzymes (14–17).

With this in mind, it was interesting to study UCP3 expression in RR-MAD patients. This myopathy is a well characterized model in which fatty acid supply exceeds β-oxidation capacity. An increase in UCP3 expression in this model would support the hypothesis that UCP3 is involved in fatty acid exit from the mitochondria.

In the present study we measured the level of expression of UCP3 mRNA and protein and intramyocellular lipid (IMCL) in the vastus lateralis of seven patients suffering from well characterized RR-MAD before and after riboflavin therapy. Moreover, as human skeletal muscle fiber types display different oxidative capacities, UCP3 protein expression levels (18, 19), and IMCL contents (20), these parameters were measured in individual fiber types.

Subjects and Methods

Subjects

Seven patients with RR-MAD (mean ± SEM age, 34 ± 16 yr) and 6 healthy, age-matched controls (age, 40 ± 26 yr) participated in this study, which was approved by the local medical ethical committee. All subjects gave their informed consent, and the study was conducted according to the principles of the Declaration of Helsinki. RR-MAD was characterized by skeletal muscle weakness and exercise intolerance and by IMCL accumulation diagnosed by histochemistry.

Muscle biopsy and isolation of mitochondria

Open muscle biopsies of the vastus lateralis were performed on patients before and after riboflavin therapy and on subjects undergoing orthopedic surgery. The biopsies weighed between 1.5–7.0 g. The pretherapy biopsies were taken after 4–6 months of riboflavin therapy. Mitochondrial fractions were prepared from fresh muscle as previously described (21). The research protocol was approved by the institutional ethics committee, and a signed informed consent was obtained in each case.

Muscle homogenate fatty acid β-oxidation and carnitine content

In vitro β-oxidation of [1-^{14}C]butyrate, [1-^{14}C]octanoate, and [1-^{14}C]palmitate was measured in fresh muscle homogenate as previously reported (22). Briefly, muscle homogenization was performed in 50 mM Tris, 100 mM KCl, 5 mM $MgSO_4$, 1 mM EDTA, and 1 mM ATP, pH 7.2. The β-oxidation was measured in a buffer containing 65 mM KCl, 40 mM NaH_2PO_4, 1 mM EDTA, 2 mM glutathione, 6.5 mM $MgSO_4$, 14.2 μM cytochrome *c*, 32.5 μM coenzyme A, and 400 μM buyrate (specific activity, 125 μCi/mmol), 100 μM octanoate (specific activity, 200 μCi/mmol), and 20 μM palmitate (specific activity, 50 μCi/mmol) in a total volume of 2 ml at 37 C for 1 h and was stopped by the addition of 0.6 ml 5 M HCl. The $^{14}CO_2$ produced was trapped by 250 μl hyamine in a well. The well was placed in a vial containing 10 ml Instagel, and radioactivity was measured in a 1900 CA-Tri-Carb liquid scintillator analyzer (Camberra-Packard, Meriden, CT). Muscle carnitine content was measured in fresh homogenate using a radiometric method (23). Protein concentrations were determined using the Bradford method (24).

Muscle respiratory complex I and II activities

The activities of complex I, complex II, and citrate synthase were assayed spectrophotometrically in 600 g supernatant of tissue homogenate that was taken after homogenizing 30–50 mg muscle (50 mM Tris-HCl and 150 mM KCl, pH 7.4) (21). Complex I activity was determined after the oxidation of NADH at 340 nm in the presence of 34 mM potassium phosphate buffer, 1.7 mM ferricyanide, and 200 μM NADH. Complex II activity was measured at 600 nm in the presence of 5 mM potassium phosphate buffer (pH 7.0), 16 mM succinate, 0.1 mM 2,6-dichlorophenolindophenol, and 1.5 mM KCN. Citrate synthase was determined according to the method described by Srere *et al.* (25).

RNA extraction and RT-quantitative PCR

Total RNA was extracted and oligo-(deoxythymidine) primed first strand cDNA synthesized as previously reported (26). Real-Time PCR was performed using a LightCycler rapid thermal cycler system with the LightCycler-DNA Master SYBR-Green I mix (Roche, Rotkreuz, Switzerland) according to the manufacturer's instructions. The PCR conditions for the amplification of UCP3 and β-actin mRNA consisted of 2 min of denaturing at 95 C, followed by 30 cycles with denaturing at 95 C for 0 sec, annealing at 56 C for 6 sec, and extension at 72 C for 25 sec. For all genes, detection of the fluorescent product was carried out at the end of the 72 C extension period. β-Actin was used as a control to account for any variations due to efficiencies of the RT-PCR.

Western blotting and immunofluorescence

Western blotting and immunofluorescence were performed as reported previously (18, 19). A rabbit antihuman UCP3 antibody (code 1331, provided by L. J. Slieker, Eli Lilly & Co., Indianapolis, IN) prepared against a 20-amino acid peptide (human sequence amino acids 147–166), which recognizes both the long and short UCP3 isoforms and was previously shown not to recognize UCP2, was used (18, 27). For the detection of type I and IIa muscle fibers, respectively, a monoclonal antibody (A4.840, immunoglobulin M) raised against adult human slow myosin heavy chain at a dilution of 1:20 and a monoclonal antibody (N2.261, immunoglobulin G1) reactive with adult human fast IIa myosin heavy chain at a dilution of 1:20 were used. Both antibodies were obtained from Iowa Hybridoma Bank. The reaction product of each Western blot was analyzed by densitometry using Imagemaster (Amersham Pharmacia Biotech, Piscataway, NJ) (18, 27), and immunofluorescence images were viewed and photographed using a Axiophot I microscope mounted with an Axiocam color CCD camera (Carl Zeiss, Inc., New York, NY). The specific fluorescence within each fiber was quantified using the Zeiss KS400 V3.0 program (19).

Intramyocellular lipid determination

Lipid accumulation was determined using an Oil Red O stain as previously described (28). In brief, muscle sections were incubated in formalin for 10 min, then washed three times for 30 sec each time in deionized water before staining for 7 min with the Oil Red O working solution. After washing again three times for 30 sec each time, the sections were counterstained with Harris's hematoxylin for 4 min and then rinsed under running tap water for 3 min. Finally the sections were covered with a coverslip. The stained lipids were viewed and quantified using the same system as that used for immunofluorescence.

Statistical analysis

A one-way ANOVA was used to compare subject characteristics, β-oxidation rates, carnitine levels, respiratory chain complex activities, and muscle UCP3 expression among pretreatment patients, posttreatment patients, and healthy controls. A three (health status as the between factor) × three (fiber types as the within factor) factor ANOVA was used to compare the influence of health status and fiber type on UCP3 protein expression and lipid content. When a significant interaction between health status and fiber type was observed, stratified analysis was used to locate the significant differences. To be specific, one-way repeated measures ANOVA followed by contrasts were used to compare the percent change in UCP3 between type I, IIa, and IIx muscle fibers, for each of the treatment and control groups. Although the overall α level

for the ANOVA was set at 0.05, the sharpened Bonferroni method was used to adjust the individual α level to a significance of $P < 0.0167$ when multiple testings were performed in the stratified analysis. The statistical power was greater than 0.75 for all analyses, indicating that any nonsignificant results were signs of no differences rather than a consequence of small sample size. All values are reported as the mean ± SD.

Results

Clinical data

As shown in Table 1, all of the patients presented exercise intolerance and muscle weakness with degrees of severity ranging from light (+) to severe tetraplegic myopathy or respiratory insufficiency (++++). Muscle biopsies from all patients were characterized by a large lipid accumulation, and these symptoms were reversed to levels of controls by the administration of riboflavin with or without carnitine, referred to as riboflavin treatment.

Acyl-CoA dehydrogenase deficiency and biochemical consequences

Figure 1A shows that the β-oxidation of labeled short-chain (butyrate, C2), medium-chain (octanoate, C8), and long-chain (palmitate, C16) FA were decreased in the RR-MAD patients before compared with after treatment by 70%, 86%, and 89%, respectively ($P < 0.05$). The inhibition of fatty acid β-oxidation in muscle resulted in an upstream accumulation of substrates such as acyl-CoA, acylcarnitine, and FFA in the mitochondria and cytoplasm of the cells. Carnitine accumulation was evidenced by an increase in the circulating level of this compound (Table 2). Excretion of acylcarnitine into the urine resulted in a deficit of carnitine. This is illustrated in Fig. 1B, which shows that the level of muscle carnitine is decreased by 82% in the RR-MAD patients before riboflavin treatment compared with healthy subjects and is restored to only 51% of control values after treatment. The decreased activity of different acyl-CoA dehydrogenases should also impair branched chain amino acid degradation, with a consequent accumulation and excretion in the urine of glutaric acid and α-hydroxyglutaric acid. It should also decrease short-chain acyl-CoA dehydrogenases with a consequent accumulation and excretion into the urine of ethylmalonic adipic acid. Therefore, RR-MAD is also referred to as glutaric aciduria type II and ethylmalonic adipic aciduria (13). As shown in Table 2, all of the patients in this study had pathological values for at least one of these parameters.

Muscle complex I and II deficiency

The activities of the respiratory chain complexes I and II, which are FMN and FAD dependent, respectively, should also be affected in RR-MADD patients. This was found to be the case in this study, as shown in Fig. 2, A and B. Indeed, the mean values for complex I (Fig. 2A) and complex II (Fig. 2B) were decreased by 44% and 54%, respectively, in the RR-MAD patients before treatment compared with levels in

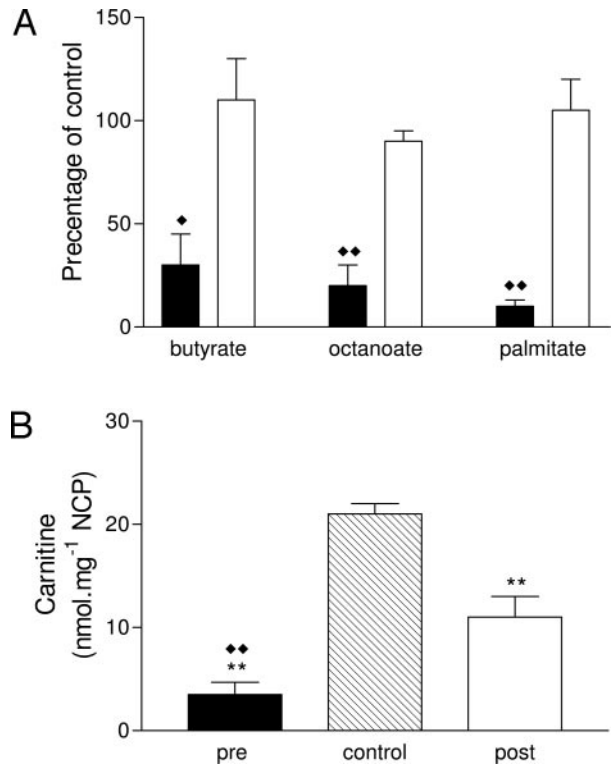

FIG. 1. Muscle fatty acid β-oxidation activity and carnitine content. A, Oxidation of labeled substrates in muscle homogenate, expressed as a percentage of the control values. The amounts of $^{14}CO_2$ produced were 956 ± 107 (butyrate), 304 ± 48 (octanoate), and 2142 ± 299 (palmitate) pmol/h·mg noncollagen protein (NCP). ■, Patients before treatment; □, patients after riboflavin treatment. n = 3 patients and 9 healthy controls. B, Amount of total carnitine expressed as nanomoles per milligram of NCP. pre, Patients before treatment; controls, healthy age-matched subjects; post, patients after riboflavin treatment. All values are the mean ± SE (n = 7 patients and 6 healthy controls). **, $P < 0.001$ (*vs.* controls). ♦, $P < 0.05$; ♦♦, $P < 0.01$ (*vs.* posttreatment).

TABLE 1. Clinical data

Subject no.	Sex	Age (yr)	Exercise intolerance	Muscle weakness	Clinical feature	Riboflavin therapy (mg/d)	Carnitine therapy (mg/d)
1	M	31	++	+	Sister with LSM, after pregnancy, died	200	
2	M	55	+	+	IDDM	200	
3	M	15	+++	+++	Protein C deficiency	100	4
4	M	25	++	++	Riboflavin homeostasis defect	100	2
5	F	35	++	+++	Epilepsy	100	2
6	M	36	++++	++++	Increased FAD pyrophosphatase	100	4
7	F	41	++++	++++		200	2

Exercise intolerance and muscle weakness with degrees of severity ranging from light (+) to severe tetraplegic myopathy or respiratory insufficiency (++++). LSM, Lipid storage myopathy; IDDM, insulin-dependent diabetes mellitus.

TABLE 2. Accumulated substrates linked to inhibited β-oxidation

Subject no.	Blood acylcarnitine	Urinary GA II	Urinary EMA
1	+	N	N
2	+		+
3	NT		+
4	+	+	
5	+	+	
6	+	N	N
7	NT	+	

GA II, Glutaric acid; EMA, ethyl malonic acid; NT, not tested; N, in the normal range of control levels; +, increased *vs.* control levels.

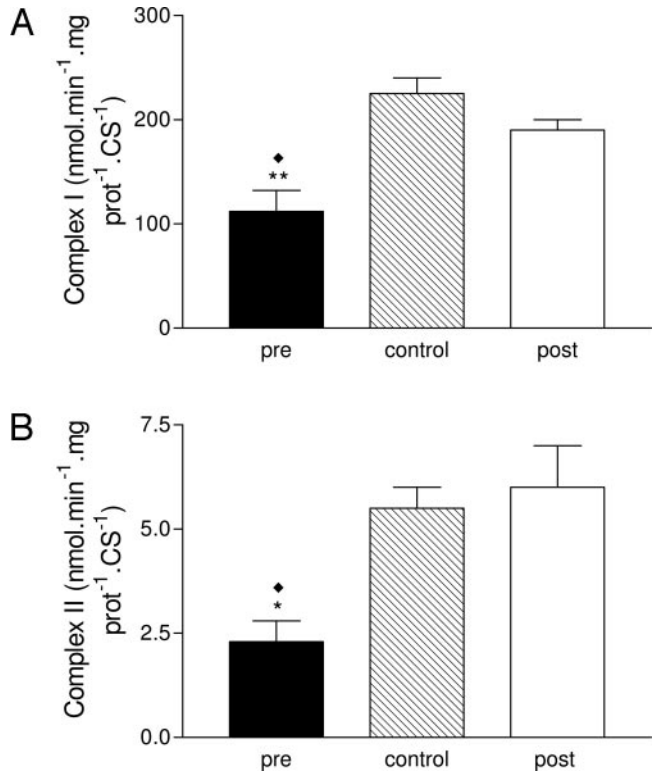

FIG. 2. Activities of respiratory complexes I and II. pre, Patients before treatment; controls, healthy age-matched subjects; post, patients after riboflavin treatment. Activities of NADH H^+ dehydrogenase (A; complex I; cofactor FMN) and activities of succinate dehydrogenase (B; complex II; cofactor FAD) are expressed as nanomoles per minute per milligram of muscle protein normalized for citrate synthase activity. All values are expressed as the mean ± SE (n = 7 for patients and 14 for healthy controls). *, $P < 0.05$; **, $P < 0.001$ (*vs.* controls). ◆, $P < 0.05$ (*vs.* posttreatment).

the healthy subjects and returned to control values after riboflavin treatment ($P < 0.05$).

All of the above data confirm that the lipid accumulation observed in the muscles of the patients in this study is due to a reversible deficit in fatty acid β-oxidation. Together, the clinical and biochemical data strongly suggest that our seven RR-MAD patients are affected by RR-MAD.

UCP3 expression and IMCL content

As shown in Fig. 3, the expressions of UCP3 mRNA (A) and UCP3 protein (B) were increased by 3.1- and 1.7-fold, respectively, in RR-MAD patients before riboflavin treatment compared with those in healthy subjects and were restored to control values by the treatment ($P < 0.0167$).

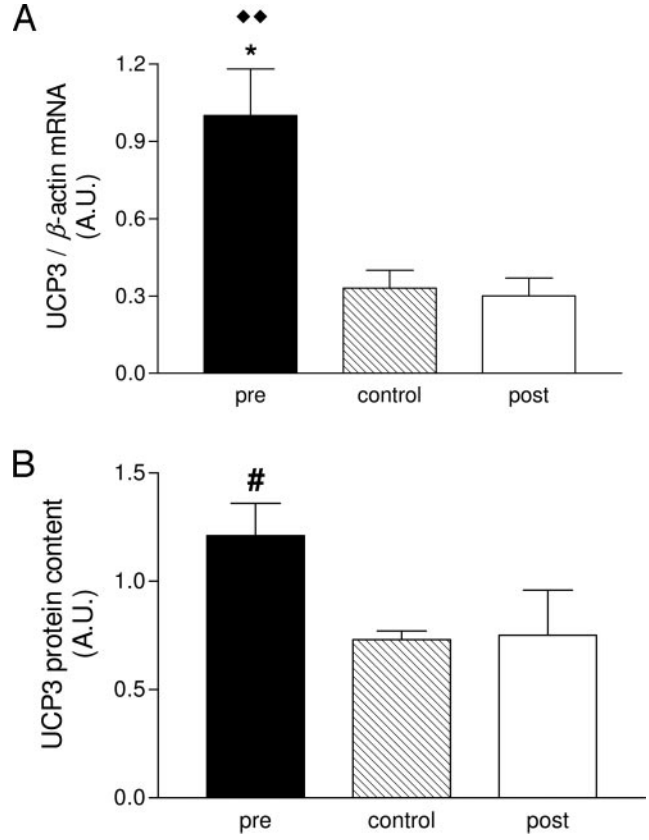

FIG. 3. UCP3 mRNA and protein levels in muscle biopsies. pre, Patients before treatment; controls, healthy age-matched subjects; post, patients after riboflavin treatment. All values are expressed as the mean ± SE (n = 7 patients and 6 healthy controls). #, $P < 0.05$; *, $P < 0.005$ (*vs.* controls). ◆◆, $P < 0.01$ (*vs.* posttreatment).

As shown in Fig. 4A, the IMCL content in healthy control subjects was 2.7-fold higher in type I than in type IIa muscle fibers and 2.4-fold higher in type IIa than in type IIx fibers ($P < 0.0167$). A greater IMCL content in type I than in type II fibers has previously been reported in obese and lean subjects (29). The preferred storage over oxidation of FA in RR-MAD patients was indicated by 2.0-, 1.7-, and 2.1-fold increases in IMCL compared with controls in type I, IIa, and IIx fibers, respectively ($P < 0.0167$). After riboflavin treatment and probably as a consequence of increased fatty acid β-oxidation, IMCL was decreased to control values in each fiber type.

As shown in Fig. 4B, the level of UCP3 protein in healthy control subjects was 2.4-fold higher in type IIx than in type IIa muscle fibers and 2.3-fold higher in type IIa than in type I fibers ($P < 0.0167$). This has previously been observed in healthy (18, 19) and endurance-trained subjects (19). In RR-MAD patients, UCP3 expression before treatment was 3.9-, 2.1-, and 1.7-fold higher than in healthy subjects in type I, IIa, and IIx muscle fibers, respectively. These increases in the different fiber types are significantly different from each other ($P < 0.0167$). UCP3 expression was decreased to control values in each fiber type by riboflavin treatment.

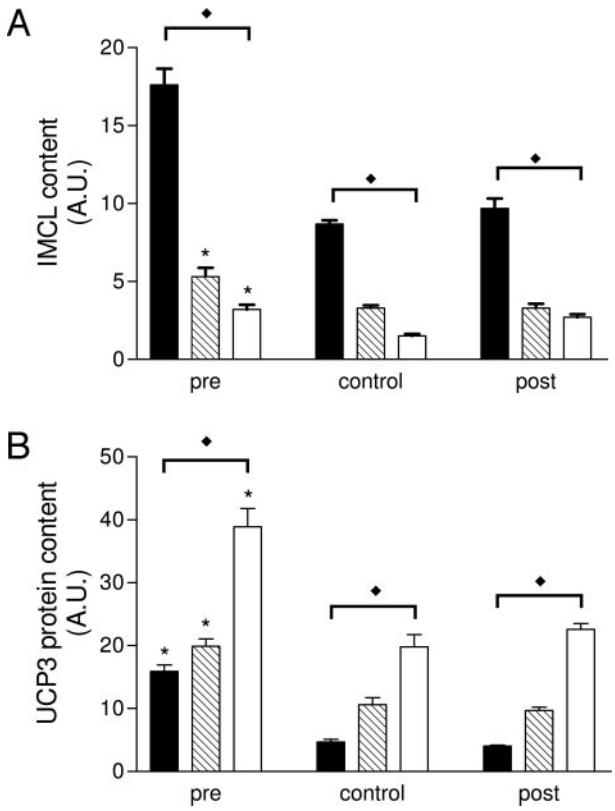

FIG. 4. IMCL content and UCP3 protein levels in the different skeletal muscle fiber types. pre, Patients before treatment; controls, healthy age-matched subjects; post, patients after riboflavin treatment. ■, Type I fibers: ▨, type IIa fibers; □, type IIx fibers. A, IMCL content in the different fiber types. B, UCP3 protein content in the different fiber types. IMCL content and UCP3 levels were determined using an Oil Red O stain and an immunofluorescence technique, respectively. All values are expressed as the mean ± SE (n = 7 patients and 6 healthy controls). ♦, $P < 0.0167$ (means for all fiber types within each group are significantly different); *, $P < 0.005$ (means are significantly different for each fiber type *vs.* control and posttreatment groups).

Discussion

Muscle metabolic disturbances

This study is the first thorough description, in a relatively large number of seven patients, of the metabolic disturbances observed in the muscle due to RR-MAD myopathy and of the effects of riboflavin treatment on the latter. It shows decreases in the β-oxidation of butyrate, octanoate, and palmitate and in the activities of the respiratory chain complexes I and II in RR-MAD patients, which were restored to control valves by riboflavin treatment. Decreases in β-oxidation of butyrate, octanoate, and palmitate; in carnitine content; and in respiratory chain complex I and II activities have also already been reported in the muscles of two RR-MAD patients (16, 30). The present study is the first to demonstrate a deficit in muscle β-oxidation and in complex I and II activities in a relatively large cohort of RR-MAD patients. Our study also shows in RR-MAD patients an increase in muscle UCP3 mRNA and protein that is reversed by riboflavin treatment. What are the causes and consequences of this striking increase in UCP3 expression?

Causes and consequences of the increase in UCP3 expression

It has been shown in rodents (8, 11) and humans (8, 10) that UCP3 expression in muscle is controlled by the levels of circulating and probably im FFA. The results of the present study suggest that it is the increase in intracellular fatty acid upstream of the inhibited β-oxidation that is responsible for the increase in UCP3 expression in RR-MAD patients. As the accumulation of FA is secondary to the inhibition of β-oxidation in these patients, the results of our study strongly suggest that the effect of FA on UCP3 expression in humans is direct and independent of fatty acid β-oxidation. In RR-MAD patients, the increase in UCP3 level was the highest in type I fibers. This might be explained by the fact that in the latter, which use more FA for metabolic fuel, a decrease in β-oxidation should induce a higher upstream accumulation of FA.

The present study is the first to show, in a longitudinal design, that a decrease in fat oxidative capacity increases UCP3 mRNA and protein levels, whereas the restoration of fat oxidative capacity is followed by a rapid decline in these parameters. These data fit the hypothesis that when fatty acid entry into the mitochondria exceeds fat oxidation capacity, UCP3 is involved in the outward translocation of nonesterified fatty acid anions out of the mitochondria matrix, preventing the deleterious effects of intramitochondrial fatty acid accumulation (5, 6). The protective function of UCP3 should be associated with a decrease in the proton gradient across the inner mitochondrial membrane and therefore with an uncoupling of oxidative phosphorylation (6). Endurance-trained subjects have a higher muscle efficiency and lower UCP3 mRNA (26, 31) and protein (19) contents compared with untrained subjects. The opposite pattern, *i.e.* decreased muscle efficiency and increased UCP3 expression, is observed in RR-MAD patients. The increased UCP3 expression would make the reduced capacity of RR-MAD patients to produce ATP worse by uncoupling the already low flux of electron through the respiratory chain. The results of the present study therefore show, for the first time in a myopathy, a phenomenon of adaptation that might make muscle weakness worse. We hypothesize that this would be the price to pay to fight intramitochondrial fatty acid accumulation.

Acknowledgments

The monoclonal antibodies directed against adult human and myosin heavy chain isoforms (referred to as A4.840 and N2.261, respectively) used in the present study were developed by Dr. Blau and were obtained from the Development Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences (Iowa City, IA). We thank Eli Lilly & Co. for providing us with the UCP3 antibody.

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