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Combined cDNA array/RT-PCR analysis of gene expression profile in rat gastrocnemius muscle: relation to its adaptive function in energy metabolism during fasting

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ABSTRACT

We evaluated the effects of fasting on the gene expression profile in rat gastrocnemius muscle using a combined cDNA array and RT-PCR approach. Of the 1176 distinct rat genes analyzed on the cDNA array, 114 were up-regulated more than twofold in response to fasting, including all 17 genes related to lipid metabolism present on the membranes and all 10 analyzed components of the proteasome machinery. Only 7 genes were down-regulated more than twofold. On the basis of our analysis of genes on the cDNA array plus the data from our RT-PCR assays, the metabolic adaptations shown by rat gastrocnemius muscle during fasting are reflected by i) increased transcription both of myosin heavy chain (MHC) Ib (associated with type I fibers) and of at least three factors involved in the shift toward type I fibers [p27kip1, muscle LIM protein (MLP), cystein rich protein-2], of which one (MLP) has been shown to enhance the activity of MyoD, which would explain the known increase in the expression of skeletal muscle uncoupling protein-3 (UCP3); ii) increased lipoprotein lipase (LPL) expression, known to trigger UCP3 transcription, which tends, together with the first point, to underline the suggested role of UCP3 in mitochondrial lipid handling (the variations under the first point and this one have not been observed in mice, indicating a species-specific regulation of these mechanisms); iii) reduced expression of the muscle-specific coenzyme Q (CoQ)7 gene, which is necessary for mitochondrial CoQ synthesis, together with an increased expression of mitochondrial adenylate kinase 3, which inactivates the resident key enzyme for CoQ synthesis, 3-hydroxy-3-methylglutaryl CoA reductase (HMGR), the mRNA level for which fell during fasting; and iv) increased transcription of components of the proteasomal pathways involved in protein degradation/turnover.

Key words: differentiation • energy expenditure • protein degradation • muscle fibers • uncoupling protein 3

Adaptations to fasting have been evolved by mammals in order to survive the stress of energy deprivation. The major part of the metabolic response to fasting is represented by the shift in fuel utilization from carbohydrates and lipids (in the fed state) to almost exclusively lipids (during fasting). Skeletal muscle, by virtue of its mass and total energy requirement, is a primary tissue in the clearance of dietary glucose and lipids from the circulation, thus it plays an important role in maintaining overall metabolic homeostasis (1). Skeletal muscle also possesses a remarkable capacity to adapt to changes in metabolic demand, thus contributing to the modulation of energy expenditure. The recognition that subtle changes in energy expenditure represent a significant risk factor for the development of metabolic diseases such as obesity, insulin-resistance, diabetes, and cardiopathies has intensified the search for specific cell signaling and regulatory proteins that may be involved in the modulation of energy expenditure in skeletal muscle.

Among the various skeletal muscles, the gastrocnemius muscle is particularly responsive to fasting in shifting toward the use of lipids as metabolic fuel, thus allowing the sparing of glucose for those organs and/or tissues with an obligatory requirement for glucose, notably the brain (2). Although several investigators have reported altered levels of certain mRNAs in rat muscle upon fasting (3, 4) the full extent and importance of the alterations in gene transcription in muscles during fasting remain unknown, and no systematic analysis of the changes in muscle mRNA in fasted rats has been reported. Studying the key transcriptional events related to fiber differentation, intermediary metabolism, hormonal transduction, and protein turnover (including transcriptional effects on the proteasome machinery) would be expected to shed light on the adaptative role of skeletal muscle in higher mammals during fasting, in which the absence of glucose has to be compensated for by recruiting alternative metabolic pathways.

With regard to intermediary metabolism, lipid metabolism is of particular relevance, and it has been proposed to have an important connection with energy metabolism, and in particular energy expenditure, through possible interactions in the mitochondrial matrix between mitochondrial acyl-CoA thioesterase I (MTE I) and uncoupling protein 3 (UCP3) (5, 6). In addition to lipid metabolism, protein breakdown is known to be increased during fasting. In muscle, and indeed most tissues, several intracellular proteolytic systems can contribute to the degradation/turnover of proteins (7–9).

To obtain a clear insight into the molecular events that accompany the transition from the fed to the fasted state, we examined the effect of a 48-h fast on the expression of genes in rat gastrocnemius muscle using a combined cDNA array and RT-PCR approach. The obtained data were divided in four groups containing i) genes involved in the structural and functional shift toward lipid metabolism and reduced energy expenditure in fasted gastrocnemius muscle; ii) hormone receptors; iii) free-radical scavengers, kinases, heat shock proteins, and transcription factors; and iv) factors involved in protein degradation/turnover. In addition, to try to strengthen the hypotheses made concerning an active role for UCP3 in lipid metabolism, we correlated the obtained expression data, wherever possible, with the known elevations in the levels of UCP3 observed during fasting. Furthermore, we compared our data with those previously obtained (using a similar microarray) in mouse gastrocnemius muscle (relating to fiber composition, lipid

metabolism, and protein degradation/turnover) because of possible differences in the mechanisms involved in the adaptation to fasting between smaller and larger animals.

MATERIALS AND METHODS

Antibodies

A polyclonal antibody raised against the C-terminal region of the human UCP3 protein (AB3046) and a monoclonal antibody raised against the human MHCIb protein (MAB1628) were purchased from Chemicon International (Temecula, CA).

Rat treatments

Male Wistar rats (250–300 g) (Charles River, Lecco, Italy) were housed individually in a temperature- and light-controlled room (28°C and 12 h light, 12 h dark cycle, respectively). Two different groups were used throughout: control rats (N), which had free access to food (commercial mash) and water, and fasted rats (Fa), exposed to 48 h of fasting but with free access to water. At the end of the treatments, rats were anesthetized by an intraperitoneal injection of chloral hydrate (40 mg/100 g body weight), then killed by decapitation. Tissues were excised, weighed, and immediately frozen in liquid nitrogen, and then stored at –80°C for later processing. All experiments were performed in accordance with general guidelines regarding animal experiments.

Microarray expression analysis

Experiments were performed using ATLAS RAT 1.2 cDNA arrays (Clontech Laboratories, Palo Alto, CA). First, total RNA extraction from the gastrocnemius muscles of three fed and three fasted rats was performed using the ATLAS pure total RNA labeling system according to the manufacturer's instructions (Clontech). Note that <100 mg of tissue was ground to powder in liquid nitrogen, after which at least 5 ml lysis buffer was added and the mixture homogenized further using a polytron, ensuring the viscosity of the solution was kept to a minimum for effective inactivation of endogenous RNase activity during the subsequent phenol extractions. Three animal samples per group were pooled and used for subsequent preparation of total RNA. Forty-eight micrograms of pooled total RNA (16 μg/animal) were used for the synthesis of the cDNA probes. In brief, after DNase digestion, polyA+ RNA was purified and labeled with [α-^{33}P] dATP with a specific activity of 10 μCi/μl (Amersham Pharmacia, Milano, Italy) to obtain a high-resolution signal. The radioactivity of the cDNA probes (~2×10^6 cpm) was verified by scintillation counting. Subsequently, ATLAS RAT 1.2 cDNA array membranes were hybridized according to the manufacturer's instructions (Clontech). Hybridization signals were quantified by means of a Bio-Rad (Hercules, CA) Molecular Imager FX using the supplied software. Array exposures were normalized with respect to the signal intensity for 40S ribosomal protein S12 (RPS12), the expression of which did not vary in fasted gatrocnemius muscle, as verified by semiquantitative PCR (see Fig. 1). The quantified signals were expressed in arbitrary units, and the ratio (fasted/control) was calculated. Only genes whose expression was changed at least twofold are reported, smaller changes being considered physiologically irrelevant. The values in arbitrary units are included because they provide information on relative mRNA abundance among the individual genes in each condition.

RT-PCR assays

Individual samples of total RNA, prepared as described above, from three animals per group, were analyzed by semiquantitative PCR. One microgram of total RNA was reverse-transcribed using 100 pmol random hexamers (Invitrogen Life Technologies, Groningen, The Netherlands), 2.0 units Superscript reverse-transcriptase, 0.5 units RNase inhibitor, and 1 mM deoxynucleotide triphosphates (dNTPs) in reverse-transcriptase buffer (all from HT Biotechnology, Cambridge, UK), the total volume being adjusted to 20 μl with distilled water. The reaction was carried out for 1 h at 40°C. One quarter of the RT-reaction mixture was used directly for the PCR reaction in a total volume of 25 μl, containing 0.25 units of SuperTaq polymerase, 0.25 mM dNTPs, SuperTaq PCR buffer (all from HT Biotechnology), 5% (v/v) DMSO (Sigma-Aldrich, Milano, Italy), and 0.38 pmol of the relevant oligonucleotide primers (Sigma Genosys, Cambridge, UK). Table 1 shows the primers used in this study except those for myosin heavy chain isotype oligonucleotides, which were taken from ref. 10. Parallel amplifications (20, 25, and 30 cycles) of a given cDNA were used to determine the optimum number of cycles. For each gene under study, a readily detectable signal within the linear range was observed after 30 cycles. For the actual analysis, samples were heated for 5 min at 94°C, then 30 cycles were carried out, each consisting of 1 min at 94°C, 1.5 min at 61°C, and 1.5 min at 72°C. This was followed by a final 10-min extension at 72°C. In this study, 40S ribosomal protein S12 (RPS12) was used as the expression control. One-half of the PCR reaction products was separated on a 2% agarose gel containing EtBr, and the products were readily visualized.

Preparation of gastrocnemius muscle mitochondria and lysates

Mitochondria were isolated after homogenization in an isolation medium consisting of 220 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl, 1 mM EDTA, 5 mM EGTA, and 5 mM $MgCl_2$, pH 7.4, supplemented with the following protease inhibitors: 1 mM benzamidine, 4 μg/ml aprotinin, 1 μg/ml pepstatin, 2 μg/ml leupeptin, 5 μg/ml bestatin, 50 μg/ml N-tosyl-L-phenylalanine-chloromethyl ketone, and 0.1 mM phenylmethylsulfonylfluoride (all from Sigma Aldrich). After brief homogenization, samples were centrifuged at 700*g*, and supernatants were collected and transferred into new tubes with subsequent centrifugation at 10,000*g*. The final mitochondrial pellet was resuspended in a minimal volume of supplemented isolation medium and was stored at –80°C or immediately used for Western immunoblot analysis.

To obtain whole-cell lysates, we homogenized gastrocnemius muscle cell lysis buffer consisting of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonylfluoride (all from Sigma Aldrich). To remove cell debris, we centrifuged lysates at 16.000*g* for 10 min at 4°C. The supernatant was stored –80°C or immediately used for Western immunoblot analysis.

Western immunoblot analysis

Analyses were performed using either mitochondrial protein (for UCP3 measurements) or total cell lysate (for MHCIb measurements). Samples were prepared for loading by adding SDS loading buffer, as described by Laemmli (11), followed by heating for 3 min at 95°C. Samples containing 30 μg of protein were loaded in each lane and were electrophoresed on a 13% SDS-PAGE gel (for detection of the 32 kDa UCP3 protein) or a 8% SDS-PAGE gel (for detection of

the 200 kDa MHCIb protein). A polyclonal antibody against UCP3 and a monoclonal antibody against MHCIb (see Materials and Methods) were used as primary antibodies in a chemiluminescence protein-detection method. (NEN Life Sciences Products, Boston, MA). The protein concentration was determined by the method of Hartree (12).

RESULTS

General features of the response to fasting in rat gastrocnemius muscle

We observed that of the 1176 distinct rat genes analyzed on the cDNA array, 114 were up-regulated more than twofold in response to fasting, including all 17 genes related to lipid metabolism present on the membranes and all 10 analyzed components of the proteasome machinery (Table 2). Only 7 genes were down-regulated more than twofold (Table 3). To confirm the microarray data and to obtain a more detailed understanding of the metabolic adaptations taking place in the fasting rat, we used RT-PCR to measure the expression levels of several genes present on the cDNA array as well as those of additional genes absent on the cDNA array [uncoupling protein (UCP) 3, myosin heavy chain (MHC)Ib**,** IIa, IId(x)and IIb, coenzyme Q (CoQ)7, 3-hydroxy-3-methylglutaryl CoA reductase (HMGR), peroxisome proliferator activator receptors (PPAR)-α, -δ, -γ1, and -γ2] (see Fig. 1*A*).

Genes involved in the structural and functional shift toward lipid metabolism and reduced energy expenditure in fasted gastrocnemius muscle

Our microarray data revealed a marked fasting-induced up-regulation of muscle factors involved in changing fibers toward type I. To confirm the data obtained from the cDNA array, we used RT-PCR to analyze the mRNA levels of two factors involved in muscle differentiation toward type I fibers, termed p27kip1 and muscle lim protein (MLP), (Fig. 1*A*). The expression of p27kip1 was elevated by ~15-fold (microarray result: 21-fold) and that of MLP by about ninefold (microarray result: sixfold). To investigate this aspect in more detail, we used RT-PCR to measure the mRNA levels of the four different skeletal muscle myosin heavy chain (MHC) genes after a 48-h fast. In agreement with the well-known shift toward lipid metabolism during fasting in rat gastrocnemius muscle, we observed that the expression of MHCIb (also termed slow myosin, which directs the formation of slow oxidative fibers) was elevated by ~12-fold (Fig. 1*A*). The mRNA levels of the fast oxidative-glycolytic myosins (MHCIIa and MHCIId(x) and that of the fast glycolytic myosin MHCIIb did not differ significantly between the fasted and the control rats (data not shown). The threefold increase of MHCIb protein was in line with that of the MHCIb mRNA (Fig. 1*B*).

The microarray data revealed that the mRNA levels of all genes involved in lipid metabolism increased during fasting, including those of the very long, long, and medium acyl-CoA dehydrogenases (VLCAD, LCAD, and MCAD; by 4.6-, 7.3-, and 3.8-fold, respectively). To confirm the expression data for several of the genes involved in lipid metabolism present on the array, we used RT-PCR (Fig. 1*A*). The expression of lipoprotein lipase (LPL), which increases the uptake of nonesterified fatty acids (FFA) into muscle cells, increased by about threefold (microarray result: 765 units in the fasted rats compared with 0 units in the controls). The enzymes involved in β-oxidation all act on CoA esters, so an important event before β-oxidation is the ATP-dependent formation of fatty acyl-CoA esters, catalyzed by acyl-CoA synthetase

(ACS). During fasting, ACS mRNA levels increased in rat gastrocnemius muscle (RT-PCR,:2.4-fold; microarray: 2.7-fold). Furthermore, the mRNAs of two proteins involved in the uptake of acyl-CoA into the mitochondrion, carnitine palmitoyl transferase 1 and 2 (CPT1β and CPT2), were up-regulated during fasting by seven- and fivefold, respectively (Table 2 and Fig. 1). CPT1β catalyzes the formation of long-chain acyl-carnitine from activated fatty acids and free carnitine, thus committing them to oxidation as they are subsequently translocated into the mitochondrion. CPT2 reconverts the carnitine esters to their respective CoAs once they are inside the mitochondrial matrix, thus completing the carnitine-dependent uptake of activated fatty acids. We also observed a marked up-regulation of cytosolic acyl-CoA thioesterase (CTE; Table 2).

Fasting increased the mRNA levels of all the genes involved in energy metabolism present on the microarray (see Table 2). This increase in gene expression was accompanied by increased UCP3 mRNA (by 2.1-fold, Fig. 1*A*), and protein levels (1.9-fold, Fig. 1*B*). The expression of carbonic anhydrase III (CAIII), which facilitates the transport of the CO_2 produced by oxidative phosphorylation, was up-regulated 2.4-fold. Furthermore, a confirmatory RT-PCR analysis revealed an increase of about threefold in adenine nucleotide translocator (ANT), a gene involved in mitochondrial nucleotide transport (microarray results: 915 units after fasting compared with 0 in the controls). To investigate the fasting-induced changes in ubiquinone synthesis in the gastrocnemius muscle, the mRNA levels of mitochondrial membrane protein CoQ7 and HMGR were analyzed by RT-PCR. The mRNA level for CoQ7 (predominantly present in skeletal muscle and heart, and necessary for mitochondrial ubiquinone synthesis) was reduced 2.5-fold, whereas the mRNA level for HMGR, the key enzyme in the cholesterol-CoQ synthesis pathway, was reduced by threefold (see Fig. 1*A*). This would result in a lowered production of CoQ during fasting, both systemically and at the mitochondrial level.

Hormone receptors

Our microarray data revealed that after 48 h of fasting, retinoic acid receptor α (RXRα) transcript levels were elevated about sevenfold (Table 2), whereas the mRNA for the tumor necrosis factor α (TNF-α) receptor was increased by ~11-fold (Table 2). In addition, the insulin receptor (INSR) was markedly up-regulated (Table 2). In addition to the above receptors, PPAR expression was measured by RT-PCR. Surprisingly, fasting lowered the mRNA levels of both PPAR-α and -δ (by about three- and twofold, respectively, see Fig. 1*A*). In contrast, mRNA levels of PPAR-γ1 and -γ2 were undetectable in gastrocnemius muscles from both fasted rats and the controls (data not shown).

Free radical scavengers, kinases, heat shock proteins, and transcription factors

Fasting markedly increased the mRNA levels of various transcription factors, among which were subunit p105 of nuclear factor (NF)-κB and activator protein (AP)-1 (both by about fivefold, Table 2). In addition, our data demonstrate that heat shock proteins (among which is mitochondrial hsp60) were also increased by fasting (Table 2), as were scavengers of free radicals, such as cytosolic superoxide dismutase and phospholipid hydroperoxide glutathione peroxidase (2.1- and 8.6-fold, respectively, Table 2). The expression of adenylate kinase 3 (AK3), which is located within the mitochondrial matrix, was elevated ~3.6-fold (Table 2).

Protein degradation/turnover

As a result of the fasting state, there were increases in all the proteasome genes analyzed (Table 2), although our cDNA array results revealed unaltered mRNA levels for ubiquitin and ubiquitin conjugating enzyme ($E2_{14k}$) (data not shown). In rat gastrocnemius muscle, both normal and fasted, polyubiquitin was one of the most abundant mRNAs analyzed on the array (arbitrary units: 8683 in the controls and 7882 in the fasted rats). With regard to the lysosomal pathway of protein breakdown, we observed a marked elevation of the mRNA levels of two cysteine proteases, cathepsin L and cathepsin C/J, by 2.1- and 2.9-fold, respectively (Table 2). Note that cathepsin L was preferentially expressed in rat gastrocnemius muscle (Table 2).

DISCUSSION

Genes involved in the structural and functional shift toward lipid metabolism and reduced energy expenditure in fasted gastrocnemius muscle

Our data show that in the rat gastrocnemius muscle, the metabolic shift toward lipid metabolism is accompanied by a physical shift in fiber composition from type II (fast twitch) to type I (slow twitch) through a change in MHC expression induced by numerous factors. The increase in MHCIb expression, defining the formation of type I fibers (13), and observed here for the first time in muscle from fasted rats, on the mRNA as well as the protein level, correlated well with the increased mRNA levels of various factors involved in muscle differentiation toward the "slow" phenotype, including p27kip1 (14, 15); muscle LIM protein (MLP) (16); and its analog, cystein-rich protein 2 (17). MLP has been shown to interact with the muscle basic helix-loop-helix (bHLH) transcription factor MyoD, enhancing its activity (18). The human UCP3 promoter contains a considerable abundance of the MyoD family binding motif (E box), so increased MLP expression most likely results in an increased transcription of the UCP3 gene, because it has been shown that the human UCP3 gene is strictly dependent on the binding of MyoD to its promoter for transcription (19). In accordance with the need to increase lipid oxidation in the fasting state, we observed a threefold increase in lipoprotein lipase (LPL), an enzyme that positively affects UCP3 transcription (20). These results allow us to suggest that during fasting, the composition of gastrocnemius muscle shifts from type II to type I fibers, accompanied by increased lipid oxidation, and point toward a role for UCP3 in this metabolic shift. The increases in LPL, VLCAD, LCAD, and MCAD mRNA levels we observed are in accordance with previously reported data indicating that fasting augments the transcription rate of these genes in gastrocnemius muscle (21). Interestingly, Jagoe et al., who used a similar cDNA-array approach to analyze gene expression patterns in gastrocnemius muscle from fasted mice, observed no significant changes in the expression of the myosin genes or for the aforementioned genes involved in lipid metabolism (22). These are important observations underlining the presence of species-specific metabolic adaptations. In accordance, it has been reported that low-frequency stimulation of muscle provokes a pronounced induction of "slow" MHCIb in rabbit, but only a minor up-regulation of MHCIb in the rat (10).

In conditions involving ATP depletion/ADP accumulation (such as fasting or exercise), AMP levels are elevated. One of the enzymes responsible for this elevation is adenylate kinase 3 (AK3), located within the mitochondrial matrix, the expression of which was increased fourfold during fasting (Table 2). AK3 catalyzes the formation of AMP from GDP and ADP because it

maintains the reaction (GDP + ADP ↔ GTP + AMP) close to equilibrium under all physiological conditions (23). The resulting elevated AMP level triggers the activity of AMP-activated kinase (AMPK), known to be involved in increased fatty acid oxidation (24, 25). Zhou et al. (25) have shown that administration of 5′-amino-4-imidazolecarboxamide (AICAR), which upon monophosphorylation is converted to the AMP analog ZMP (24), results in activation of AMP-activated kinase, accompanied by a rapid increase in UCP3 expression, in skeletal muscle (25). Taken together, these data indicate good correlations between fasting and increased expression of AK3, increased activity of AMPK, increased fatty acid oxidation, and increased UCP3 expression.

Apart from the increased fat oxidation, the fasting state is accompanied by a lowered basal metabolic rate and a lower mitochondrial respiration rate. In view of the effects of UCP3 on metabolic rate and thermogenesis in vivo (6, 26–28), the observed elevation of UCP3 protein levels during fasting seems to be in contrast to what one might expect. In line with the need for the suppression of energy dissipation during fasting, UCP3 does not produce any uncoupling (29), despite the elevated levels of free fatty acids (FFA), essential cofactors for UCP3-mediated uncoupling (30, 31). Another essential cofactor for the uncoupling activity of UCP3 is CoQ (28). Recently, we showed that CoQ is indispensable for the in vivo uncoupling activity of UCP3 in rat gastrocnemius muscle (6), and that endogenous CoQ levels are low after fasting, thus explaining the observed lack of uncoupling activity despite the increased UCP3 expression. In addition, in the presence of an elevated lipid metabolism, it is conceivable that the formation of peroxidated fatty acids is increased, which is in accordance with the hypothesis that UCPs may serve as antioxidant agents by translocating FFA peroxides from the inner to the outer leaflet of the mitochondrial inner membrane (32).

Our data indicate a pathway leading to a down-regulation of CoQ during fasting. AMPK, as well as triggering fatty acid oxidation and UCP3 expression (25), rapidly blocks the activity of the residing HMGR, the key enzyme in cholesterol and CoQ synthesis (33–36), via phosphorylation (36). The reduced HMGR mRNA levels we saw after a 48-h fast point toward long-term systemic repression of CoQ synthesis. In addition, the reduced mRNA levels for CoQ7 (the gene being predominantly expressed in skeletal muscle and heart, and encoding a mitochondrial protein necessary for CoQ synthesis [37]) point toward a local reduction in muscle CoQ synthesis. The expression of CoQ7 has also been shown to be decreased in gastrocnemius muscle in fasted mice (22). Decreased activity and expression levels of HMGR and CoQ7 would explain the low intramitochondrial CoQ levels we previously observed in the gastrocnemius muscle of the fasted rat (6).

ATPase subunit levels were up-regulated during fasting (Table 2), which suggests a compensatory effect to keep ATP production at basal levels in spite of decreased respiration. In addition, the expression of carbonic anhydrase III (CAIII) was up-regulated 2.4-fold (Table 2), which contrasts with the results reported in mice, in which this gene was found to be down-regulated two- to threefold (22). It is well known that CAIII, which facilitates CO_2 transport during oxidative phosphorylation, is present at high concentration in the cytoplasm of mammalian slow oxidative (type I) skeletal muscle fibers (38), which explains its increase in fasted rat gastrocnemius muscle and once again points toward a difference in the metabolic adaptation to fasting between rat and mouse. Interestingly, the few genes found to be down-regulated during fasting included 14-3-3 gamma, the muscle isoform of the 14-3-3 superfamily

(Table 3). This was the first muscle protein for which a direct physical interaction with UCP3 was shown by use of a yeast two-hybrid system (39). It has been suggested that this interaction may facilitate mitochondrial uptake, accelerating uncoupling activity (39). As yet, however, this idea remains hypothetical.

Hormone receptors

FFAs regulate the expression of numerous lipid-metabolism genes through the action of the PPARs (40). Increased intramuscular LPL activity, resulting in an increased uptake of nonesterified FFA into the tissue itself, may thus be an important trigger for PPAR-mediated transcription in fasted gastrocnemius muscle. Induction of UCP3 transcription by increased LPL activity (20), for instance, can be explained by the presence of PPAR response elements (PPREs) in the human UCP3 promoter (41, 42). The promoters of CTE (43) and CPT1β (44) also harbor PPREs. By up-regulating CPT1β and CPT2 mRNA levels, FFA regulate their increased uptake into the mitochondrion.

Despite the central role played by PPARs in the stimulation of lipid metabolism, our RT-PCR data revealed decreased mRNA levels of PPAR-α and -δ, which is in contrast to an earlier report where skeletal muscle PPAR-α and -δ did not significantly change after an overnight fast (45). It is unclear, however, which particular muscle type it regarded. However, in accordance with our findings, lowered PPAR-α and -δ levels have also been reported in the fasted rat heart (46). Addition of specific PPAR agonists to L6 myotubes revealed that only activation of PPAR-δ (not of PPAR-α or PPAR-γ) caused increased UCP3 expression, which suggests that PPAR-δ mediates at least part of the increased UCP3 expression induced by fatty acids in skeletal muscle in vivo (47). In the present study, PPAR-γ mRNA levels were below the detection limit in the fed as well as in the fasted state, which makes a regulatory role for PPAR-γ in fasted gastrocnemius muscle, as recently suggested by Samec et al. (48), seem unlikely. Despite their lowered mRNA levels after fasting, note that the activity of the PPARs is strongly influenced by changes in their phosphorylation state, which may be mediated by, among others, their ligands and protein kinase A (49). A different phosphorylation state may explain the apparently contrasting findings for PPAR-δ down-regulation and enhanced UCP3 expression after fasting.

The increased RXRα and TNFR1 mRNA levels observed on the array correlate well with increased UCP3 expression because (i) retinoids and TNF-α have been shown to stimulate UCP3 expression in muscle cells (19, 50) and (ii) RXRα may form heterodimers with THR and PPAR to bind to specific regulatory motifs in the UCP3 promoter (19, 41, 42). Of significant interest is the increased expression of the INSR, which could imply that upon refeeding, the fasted muscle would be able to shift back rapidly to glucose metabolism.

Free-radical scavengers, kinases, heat shock proteins, and transcription factors

The increased mRNA levels of the free-radical scavengers cytosolic superoxide dismutase (SOD) and phospholipid hydroperoxide glutathione peroxidase indicate that during fasting, free-radical concentrations are maintained at low levels. Maintenance of the byproducts of increased lipid β-oxidation at low levels is also reflected by the up-regulation we observed of glucose 6-phosphate dehydrogenase as this enzyme reduces $NADP^+$ to NADPH, which maintains

gluthatione in its reduced and active form as a scavenger of harmful oxidative metabolites. Another way of protecting the cell against the damaging effects of the accumulation of free radicals during fasting would be mild uncoupling. FFA have been shown to act as natural uncouplers by way of the adenine nucleotide translocator (ANT), a mechanism that reduces free-radical formation (51). The increase in ANT mRNA observed in this study could indicate activation of mild uncoupling during fasting.

The elevation of nearly all the investigated kinases, in addition to mitochondrial adenylate kinase 3 (AK3), explains the increased activation of enzyme systems and receptors we observed in this study. In analogy to the data of this study, increased expression of heat shock proteins has also been observed during exercise (52), in which a similar increased transcription of genes involved in lipid metabolism occurs (21).

Protein degradation/turnover

The fasting-induced up-regulation of the mRNA levels of all the analyzed components of the proteasome machinery was accompanied by unaltered polyubiquitin mRNA levels. This is in contrast to the data reported by Jagoe et al. (22), who reported an approximately fourfold increase in polyubiquitin levels in fasted mouse gastrocnemius muscle. To our knowledge, our study is the first to look for regulation of polyubiquitin mRNA levels in fasted rat gastrocnemius muscle. In other skeletal muscles from fasted rats, polyubiquitin mRNA levels have been reported to rise by two- to fourfold (53–55). In rat gastrocnemius muscle, both normal and fasted, polyubiquitin is one of the most abundant mRNAs, being comparable in abundance to the mRNAs for lipid metabolism genes (see the arbitrary units in Table 2). This indicates that the ubiquitin proteins are not in short supply during fasting. In line with results obtained in the fasting mouse (22), we did not observe a significant change in the mRNA level for the ubiquitin-conjugating enzyme $E2_{14k}$ (arbitrary units: 788 in the controls and 690 in the fasted rats). $E2_{14k}$ is a component of the complex that adds ubiquitin to target proteins, marking them for destruction by the proteasome machinery (7, 56). There seems to be no absolute correlation between the steady-state levels of the mRNAs for the components of proteolytic pathways and the actual proteolytic activity within that pathway (57). This would imply that the unaltered polyubiquitin and $E2_{14k}$ mRNA levels we observed in fasted rat gastrocnemius muscle do not hinder fasting-induced protein breakdown. The powerful increases in the expression levels seen in fasted rat gastrocnemius muscle of proteasome components, as well as of IGFBPs, which, by forming dimers with RXRα (58), inhibit ubiquitin and $E2_{14k}$ mRNA breakdown through inhibition of IGF-1 (59, 60), nevertheless favor increased protein breakdown through increased proteasome action. Regarding the lysosomal pathway of protein degradation, the elevated mRNA levels of cathepsin L and cathepsin C/J indicate activation of this pathway during fasting. Cathepsin L, which was found to be highly expressed in fasted gastrocnemius muscle (Table 2), has been identified as an early marker of muscle wasting (59). One of the potential activators of cathepsin L transcription is TNF-α (59), of which the receptor was strongly up-regulated by fasting (Table 2). Cathepsin K expression levels, however, were decreased by fasting (Table 3). However, both cathepsin K and cathepsins C/J are expressed at much lower levels than cathepsin L, pointing toward cathepsin L as the trigger for the lysosomal pathway of protein degradation in fasted gastrocnemius muscle.

In conclusion, the transcriptional data obtained in this study allow us to shed light on different aspects of the complex biochemical, molecular, and physiological mechanisms of adaptation to fasting in skeletal muscle to counteract the stress of energy deprivation. In addition, the notable differences between mouse and rat concerning the mechanisms recruited for maintenance of overall metabolic homeostasis suggest that considerable caution needs to be applied when using the results from animals of different species as comparative models, especially with regard to humans.

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Table 1

Oligonucleotide primers used for RT-PCR experiments[a]

Gene (GenBank #)	Sense oligonucleotide	Antisense oligonucleotide
p27 kip 1(D83792)	5'-GGGGAGGAAGATGTCAAAGC-3,	5'-GCCTGAGACCCAATTGAAGG-3'
MLP(X81193))	5'-TGGAGGTGCAAAATGTGGAG-3'	5'-CCTCCCATGACCTTCTCAGC-3'
LPL (NM_012598)	5'-TCATCAACTGGCTGGAGGAAG-3' (21)	5'-TATGCCTTGCTGGGGTTTTCT-3' (21)
ACS (D90109)	5'-AACACGTCAGTGAAGCGATG-3'	5'-AACACATTTGCCCCTTTCAC-3'
CPT1β (NM_013200)	5'-CTCAGCCTCTACGGCAAATC-3'	5'-CTTCTTGATCAGGCCTTTGC-3'
CPT2 (J05470)	5'-GTGGCAAGGAGTTCCTGAAG-3'	5'-TGGTTCATCTGCTGGTATGC-3'
PPARα (NM_013196)	5'-TCCACGAAGCCTACCTGAAG-3'	5'-GAACTCTCGGGTGATGAAGC-3'
PPARδ (U40064)	5'-AACATCCCCAACTTCAGCAG-3'	5'-GGAAGAGGTACTGGCTGTCG-3'
ANT (D12770)	5'-TGCTCAAGTTCACAGGTTCAC-3' (21)	5'-CTTCTGTTTGCTGTGGAATC-3' (21)
HMGR (X55286)	5'-GAAGCTATGGTTGACGTAAAC-3' (21)	5'-GCTTGCTGAGGTAGAAGGTTG-3' (21)
RPS12 (M18547)	5'-GCTGCTGGAGGTGTAATGGA -3'	5'-CTACAACGCAACTGCAACCA -3'

[a]See Materials and Methods for further details. Primer length is 20 nt unless otherwise indicated (between brackets).

Table 2

Genes up-regulated during fasting[a]

Gene/protein name	Fasted/ Control (Arbitrary Units)	Ratio Fasted/ Control	GenBank Accession #
Genes involved in the structural and functional shift toward lipid metabolism and reduced energy expenditure in fasted gastrocnemius muscle			
p27Kip1	1035/50	20.7	D83792
LIM protein (MLP), muscle	4204/696	6.1	X81193
cysteine-rich protein 2 (CRP2)	811/9	90.1	D17512
carbonic anhydrase III (CA3)	4817/1981	2.4	M22413
adenine nucleotide translocator (ANT)	915/0		D12771
long chain-specific acyl-CoA dehydrogenase precursor (LCAD)	2499/344	7.3	J05029
mitochondrial muscle carnitine palmitoyltransferase (CPT1β)	13419/2070	6.3	D43623
mitochondrial carnitine palmitoyltransferase II (CPT II)	2622/530	5	J05470
very long chain acyl-CoA dehydrogenase precursor (VLCAD)	2427/532	4.6	D30647
medium chain acyl-CoA dehydrogenase precursor (MCAD)	3976/1040	3.8	U43175
long chain acyl-CoA synthetase 2 (LACS2)	1704/628	2.7	D90109
Lipoprotein lipase (LPL)	765/0		L03294
Cytosolic acyl-CoA thioesterase (ACT/CTE)	272/0		D88890/Y09332
cytochrome P-450 2C23	431/106	4.2	X55446
adipocyte lipid-binding protein (ALBP)	7598/2072	3.7	U75581
epidermal fatty acid-binding protein (E-FABP)	2673/1010	2.6	U13253
fatty acid-binding protein (FABP)	8663/3459	2.5	J02773
Apolipoprotein D (APOD)	726/0		X55572
acyl-CoA oxidase (ACO)	193/0		J02752
annexin I (ANXI)	972/0		M19967
annexin III (ANX3)	638/0		M20559
annexin IV (ANX4)	1270/0		D38224
calmodulin	3814/1289	2.9	X13817
neuronal acetylcholine receptor protein alpha 5 subunit precursor	2432/320	7.6	J05231
Na,K-ATPase beta 3 subunit	1398/340	4.1	D84450
ATP synthase, subunit c	5282/1802	2.9	D13124
urate transporter/channel	793/285	2.8	U67958
ATPase, sodium/potassium, alpha(+) isoform catalytic subunit	1906/866	2.2	M14512
anion exchange protein 2 (AE2)	413/0		J05166
glutathione S-transferase P subunit	3259/544	6	X02904
glutathione S-transferase subunit 13	463/83	5.6	S83436
cytochrome c oxidase, subunit VIIa	3393/904	3.7	X54080
ATPase, subunit F	2695/1033	2.6	U43175
mitochondrial ATP synthase D subunit	3120/1301	2.4	D10021
Hormone receptors			
tumor necrosis factor receptor 1 (TNFR1)	634/59	11	M63122
retinoid X receptor alpha (RXR)	957/139	6.9	L06482
Nur 77 nerve growth factor induced protein I-B (NGFI-B)	682/336	2.1	U17254
insulin receptor (INSR)	1503/0		M29014
Free radical scavengers, kinases, heat shock proteins, and transcription factors			
phospholipid hydroperoxide glutathione peroxidase	2917/340	8.6	X82679

copper-zinc-containing superoxide dismutase 1 (Cu-Zn SOD1)	4802/2265	2.1	Y00404
glucose 6-phosphate dehydrogenase	358/0		X07647
mitochondrial adenylate kinase 3 (AK3)	1596/440	3.6	D13062
rac-alpha serine/threonine kinase (RAC-PK-alpha)	2625/312	8.4	D30040
casein kinase II (CKII) beta subunit	1938/348	5.6	L15619
casein kinase I (CKI) delta	1493/296	5	L07578
PCTAIRE1 cdc2-related serine/threonine kinase	624/138	4.5	U36444
glycogen synthase kinase-3 alpha	434/154	2.8	X53427
Mak; male germ cell-associated kinase	1677/600	2.8	M35862
Janus tyrosine protein kinase 1 (JAK1)	495/226	2.2	AJ000556
mitochondrial matrix protein P1(HSP60)	1292/89	14.5	X54793
heat shock 70-kDa protein (HSP70)	332/43	7.7	Z27118
heat shock 27-kDa protein (HSP27)	4141/600	7	M86389
heat shock 90kD protein (HSP90-beta)	3648/1571	2.3	S4539
transcription factor AP-1(c-jun)	2158/429	5	X17163
NF-kappa-B transcription factor p105 subunit (NFKB p105)	360/81	4.6	L26267
interferon regulatory factor 1 (IRF1)	602/5	120.4	M34253
rac-alpha serine/threonine kinase (RAC-PK-alpha)	2625/312	8.4	D30040
c-H-ras proto-oncogene	1927/530	3.6	M13011
rab8, ras-related GTPase	1084/313	3.5	M83675
B-cell receptor-associated protein 32 (BAP32)	2742/797	3.4	M61219
signal transducer & activator of transcription 3 (STAT3)	1193/350	3.4	X91810
proto-oncogene c-crk	313/96	3.3	D44481
A-raf proto-oncogene	2409/876	2.7	X06942
elongation factor SIII P15 subunit	685/251	2.7	L29259
Myc-Max-interacting tumor suppressor (MXI1)	1647/669	2.5	AF003008
Max; c-myc dimerization partner & coactivator	1103/527	2.1	D14447
Protein degradation/turnover			
proteasome subunit RC6-1	3345/246	13.6	D30804
proteasome component C2	2261/201	11.2	M29859
proteasome iota subunit	2140/237	9.1	D10755
proteasome component C3	1978/252	7.8	J02897
proteasome beta subunit precursor	2481/376	6.6	L17127
proteasome component C8	946/151	6.3	M58593
proteasome activator rPA28 subunit alpha	1505/254	5.6	D45249
26S protease regulatory subunit 4	916/194	4.7	D50696
ATPase, proteasomal	1872/461	4.1	U77918
proteasome subunit C5	2657/887	3	X52783
cathepsin L	4377/2163	2.1	Y00697
cathepsin C/J	492/170	2.9	D90404
insulin-like growth factor-binding protein 3 precursor	2148/394	5.5	M31837
insulin-like growth factor-binding protein (rIGFBP-6)	2278/546	4.2	M69055
Other genes			
SR13 myelin protein	1140/10	114	M69139
ADP-ribosyl cyclase 1	199/10	20	D30795
CD4 homologue, W3/25 antigen	955/59	16	M15768
tissue inhibitor of metalloproteinase 2 (TIMP2)	5188/2167	2.4	L31884
tissue inhibitor of metalloproteinase 3 (TIMP3)	1246/141	8.8	U27201
protein phosphatase 2C alpha	857/107	8	J04503
ras-related protein Rab2	907/116	7.8	J02999
14-3-3 protein zeta/delta	2067/337	6.1	D17615
14-3-3 protein theta	340/0		D17614
cyclin-dependent kinase 4 (CDK4)	1438/250	5.7	L11007
clusterin (CLU)	3263/603	5.4	M64723

guanine nucleotide-binding protein G(I) alpha 2 subunit (GNAI2)	1526/304	5	M17528
dC-stretch binding protein (CSBP)	1437/321	4.5	D17711
gelatinase A	1720/379	4.5	U65656
PDGF-associated protein	1678/371	4.5	U41744
mast cell protease 1 precursor (RMCP-1)	1780/415	4.3	U67915
GTP-binding protein (G-alpha-8)	2199/499	4.3	M17525
interleukin 13 precursor (IL-13)	1364/330	4.2	L26913
interferon induced protein	3372/888	3.8	X61381
mast cell protease-3 precursor	1874/527	3.6	D38495
macrophage migration inhibitory factor (MIF)	1827/561	3.3	U62326
lipocortin 2	1453/449	3.2	S73557
ras-related protein rab1A	538/172	3.1	J02998
apurinic/apyrimidinic endonuclease	1051/354	3	D44495
ATPase, transitional endoplasmic reticulum	966/325	3	U11760
endoplasmic reticulum stress protein (ERP72)	685/230	2.9	M86870
ADP-ribosylation factor 5 (ARF5)	2781/1002	2.8	L12384
structure-specific recognition protein 1 (SSRP1)	788/320	2.5	L08814
rab13, ras-related GTPase	166/69	2.4	M83678
type I procollagen C proteinase enhancer protein	731/316	2.3	U94710
S19; 40S ribosomal protein S19	7623/3369	2.3	X51707
40S ribosomal protein S17 (RPS17)	5532/2452	2.3	K02933
yeast mitochondrial protein import homolog	4887/2136	2.3	M84716
urokinase-type plasminogen activator precursor (UPA)	1305/603	2.2	X63434
ribosomal protein S4	1896/878	2.2	X14210
presenilin 1	315/153	2.1	D82363

[a]Only genes up-regulated by at least twofold are shown. Novel genes analyzed in gastrocnemius muscle of fasted rats are depicted in bold. A complete gene list is at the www.clontech.com.

Table 3

Genes down-regulated during fasting[a]

Gene/protein name	Fasted/ Control (Arbitrary Units)	Ratio Fasted/ Control	GenBank Accession #
Genes involved in the structural and functional shift toward lipid metabolism and reduced energy expenditure in fasted gastrocnemius muscle			
14-3-3 protein gamma subtype (muscular)	0/85		S55305
sodium-dependent dopamine transporter	62/335	0.18	M80570
Free radical scavengers, kinases, heat shock proteins, and transcription factors			
mitogen-activated protein kinase 2	182/493	0.4	M64300
dual-specificity mitogen-activated protein kinase kinase 2	367/452	0.4	D14592
protein tyrosine phosphatase PTP-S	45/232	0.2	X58828
Pim-1 proto-oncogene	16/69	0.23	X63675
Protein degradation/turnover			
cathepsin K	175/373	0.5	AF010306

[a]Only genes down-regulated by at least twofold are shown. Novel genes analyzed in gastrocnemius muscle of fasted rats are depicted in bold. A complete gene list is at www.clontech.com.

Fig. 1

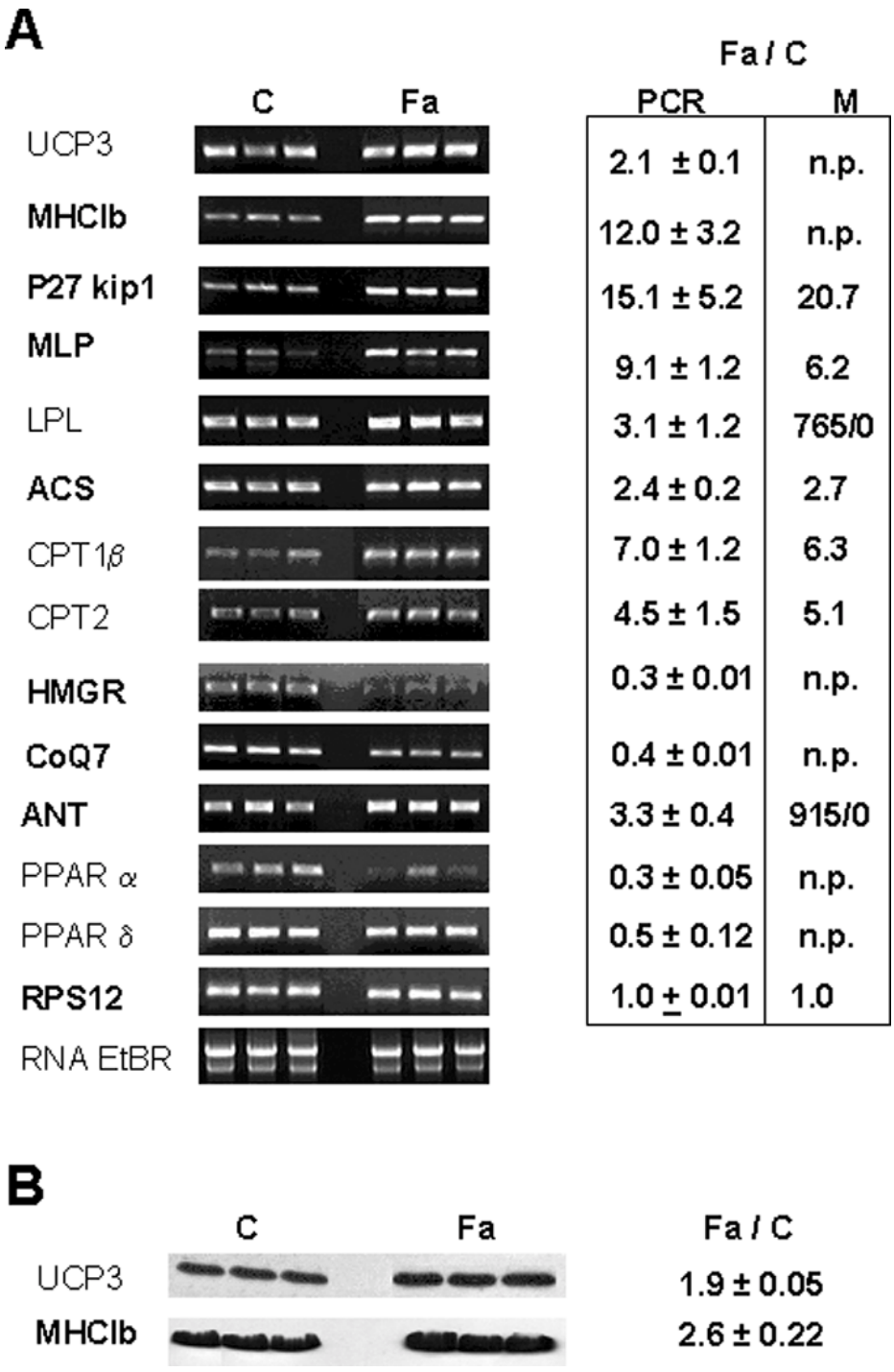

Figure 1. ***A)*** Combined cDNA array/RT-PCR analysis of the expression of genes involved in metabolic adaptations during fasting (including genes present on the cDNA array membrane). The signal ratios (fasted/control) for the PCR products and the membrane hybridization signals are shown next to the PCR data. RNA concentration and quality were verified by loading 5 μg of total RNA from the control and fasted rats entered into the analysis. Novel genes analyzed in gastrocnemius muscle of fasted rats are depicted in bold. M, microarray data; F/C, fasted/control (ratio or arbitrary units); EtBR, ethidium bromide; n.p., not present on the cDNA array membrane. The signal ratios shown for RT-PCR are each the means ± SE of three separate experiments. ***B)*** Western immunoblot analysis of UCP3 and MHCIb protein. Abbreviations are as in A. The ratios (fasted/control) for the protein signals are shown next to the protein data and are each the means ± SE of three separate experiments.