Changes in uncoupling protein-2 and -3 expression in aging rat skeletal muscle, liver, and heart

ROCCO BARAZZONI AND K. SREEKUMARAN NAIR
Endocrine Research Unit, Mayo Clinic and Foundation, Rochester, Minnesota 55905

Received 1 May 2000; accepted in final form 31 October 2000

Barazzoni, Rocco, and K. Sreekumaran Nair. Changes in uncoupling protein-2 and -3 expression in aging rat skeletal muscle, liver, and heart. Am J Physiol Endocrinol Metab 280: E413–E419, 2001—Uncoupling protein (UCP)-2 and -3 mediate mitochondrial (mt) proton leak in vitro and are potential regulators of energy expenditure and ATP production. Aging is associated with alteration of tissue functions, suggesting impaired mtATP production. To determine whether age-related changes in UCP expression occur, we measured the transcript levels of UCP-2 and -3 in skeletal muscle, liver, and heart in 6- and 27-mo-old rats. UCP-2 transcripts were higher in old animals in the white (+100%), red (+70%, both P < 0.04) gastrocnemius muscle and in the liver (+300%, P < 0.03), whereas they were comparable in the heart in both age groups. UCP-2 transcript levels correlated positively with mitochondrial-encoded cytochrome c oxidase transcripts normalized for mtDNA (P < 0.01) and negatively with mtDNA copy number (P < 0.001). UCP-3 transcripts were lower in the less oxidative white (−50%, P < 0.04) and unchanged in the more oxidative red (−15%, P = 0.41) gastrocnemius muscle in old animals. Similar changes at protein level were confirmed by UCP-2 protein in aging liver (+300%, P < 0.01) and UCP-2 (+85%, P < 0.05) and UCP-3 (−30%, P = 0.4) protein in aging mixed gastrocnemius muscle. Aging is thus associated with tissue-specific changes of UCP-2 and -3 gene expression. Increased UCP-2 expression may limit ATP production and is related to mitochondrial dysfunction in aging tissues. In vitro studies have shown reduced mitochondrial membrane potential in aging hepatocytes (21), also indicating impaired ability for ATP production and directly suggesting increased uncoupling in the aging liver. On the whole, the above observations suggest that ATP production may be limited by increased uncoupling of oxidative phosphorylation in aging tissues.

Uncoupling protein (UCP)-1 produces heat by mediating mitochondrial proton leak in brown adipose tissue and therefore dissipating the electrochemical proton gradient across the inner mitochondrial membrane (7). UCP-2 and -3 are recently discovered members of the mitochondrial uncoupling protein family (8, 17–19, 31, 47) with wider tissue distribution than UCP-1 (8, 17–19, 31, 47). In particular, in both rodents and humans, UCP-2 is expressed in most tissues and organs (17, 31), whereas UCP-3 is mainly expressed in skeletal muscle and adipose tissue (8, 18, 19, 31, 47). Several reports provide indirect (26, 29, 43) and direct (14, 20, 46) evidence of a role of UCP-2 and UCP-3 in the in vivo regulation of energy production and expenditure. Such a role has been elucidated in vitro, where they both mediate mitochondrial proton leak in an identical fashion (25), and their overexpression leads to reductions of mitochondrial membrane potential (18, 19).

Aging is generally associated with a moderate reduction of whole body metabolic rate and energy expenditure in humans (34, 44, 45, 50). Most studies however, agree that resting oxygen consumption is unchanged when normalized for reduced lean body mass (34, 44, 45, 50), and unchanged whole body oxygen consumption has been reported in aging rats (39). In spite of these observations, impaired mitochondrial function and energy production are hypothesized to be involved in age-related alterations of many body functions (22, 48). Aging is associated in both humans (15, 16, 30, 32, 33, 36) and rodents (5, 23) with a decline in skeletal muscle function, including reduced muscle mass and strength and possibly increased muscle fatigability, although available evidence for enhanced fatigability is not conclusive (30, 32, 33, 40). Age-related muscle dysfunction strongly supports reduced ATP availability in this tissue. In vitro studies have shown reduced mitochondrial membrane potential in aging hepatocytes (21), also indicating impaired ability for ATP production and directly suggesting increased uncoupling in the aging liver. On the whole, the above observations suggest that ATP production may be limited by increased uncoupling of oxidative phosphorylation in aging tissues.

In the current study, we have therefore measured UCP-2 and -3 expression in selected tissues in young and old rats. UCP-2 transcript levels were measured in the medial white and the lateral red portions (2) of the gastrocnemius muscle and in the liver, whereas UCP-3 transcripts were measured in both portions of the gastrocnemius. UCP-2 gene expression was also measured in the heart to include a tissue with no apparent age-related changes of in vivo ATP production (28). Liver UCP-2 and mixed gastrocnemius UCP-2 and UCP-3 protein levels were also determined to confirm...
age-related qualitative changes in gene expression at the protein level.

METHODS

Animals and experimental protocol. Young (n = 7, age = 6 mo) and old (n = 9, age = 27 mo) Fischer 344 male rats were purchased from the National Institute of Aging. Animal care and all experiments were carried out in keeping with institutional guidelines at Mayo Clinic and Foundation. Rats were kept in cages at 22°C under controlled conditions with a 12:12-h light-dark cycle and consumed a regular chow diet ad libitum. All animals were anesthetized with an intraperitoneal overdose of pentobarbital sodium. After achievement of adequate anesthetic effect, the lateral and medial heads of the gastrocnemius muscle, liver, and heart were removed quickly in this order. Tissues were immediately frozen in isopentane, kept in liquid nitrogen, and stored at −80°C until analysis. These experiments were performed as part of a protocol designed to study the effects of aging on mitochondrial gene expression (3).

RNA analysis. cDNA probes for UCP-2 and UCP-3 and 28S rRNA transcripts were generated by RT-PCR amplification from rat skeletal muscle total RNA. Primers for the UCP-2 probe corresponded to nucleotides 467–490 (forward) and 1196–1205 (reverse, PCR product of 746 bp) of the rat UCP-2 sequence (GenBank accession no. AB010743). Primers for the UCP-3 probe corresponded to nucleotides 235–254 (forward) and 980–1003 (reverse, PCR product of 768 bp) of the rat UCP-3 sequence (GenBank accession no. U92069). Primers for the 28S rRNA probe corresponded to nucleotides 4203–4222 (forward) and 4370–4389 (reverse, PCR product 186 bp) of the rat ribosomal RNA genome (GenBank accession no. V01270). Amplification products were cloned into the TA-plasmid vector (TA Cloning kit; Invitrogen), as previously described (3). Total RNA isolation, Northern blotting, and hybridization to UCP-3, UCP-2, and 28S probes (in this order) were performed as described previously (3). Resulting images were quantified by laser densitometry (Ultrascan; Pharmacia). UCP bands were normalized to the corresponding 28S rRNA band, and individual results were expressed as a percentage of the average value for young animals.

Western blot analysis. To confirm changes in UCP-2 and UCP-3 transcripts at the protein level in mitochondria, liver UCP-2 and mixed gastrocnemius UCP-2 and UCP-3 protein levels were measured by Western blot. Tissues for protein determination were selected based on sufficient availability for this additional determination. The mitochondrial protein fraction was isolated from −50 mg of liver and 100 mg of mixed gastrocnemius muscle by sequential centrifugation as previously described (36). Protein concentration in all samples was measured by a spectrophotometer (BCA Protein Assay Reagent; Pierce, Rockford, IL). Equal amounts of protein (150 μg for liver and 50 μg for each protein for muscle) were then separated on 12% acrylamide gels and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were hybridized to rabbit antibodies for UCP-2 or UCP-3. Liver samples were hybridized overnight at 4°C to the anti-UCP-2 antibody (AB3226; Chemicon International, Temecula, CA) at a 1:800 dilution. Gastrocnemius muscle samples were hybridized overnight at 4°C to the anti-UCP-2 antibody (Alpha Diagnostic International, San Antonio, TX) at a 1:2000 dilution for 1 h at room temperature. Membranes were then exposed to films for 30 min to 3 h (Kodak Biomax MR, Kodak, Rochester, NY), and resulting images were quantified by laser densitometry (Ultrascan; Pharmacia). To ascertain the lack of cross-reactivity of the anti-UCP-3 antibody to UCP-2, liver and skeletal muscle samples were transferred to the same blot in a preliminary experiment and incubated with the anti-UCP-3 antibody by use of the protocol described above. Although the expected signal was detected in skeletal muscle, no bands were detectable in the liver sample, where UCP-3 is not known to be expressed (data not shown). Individual results from all experiments were expressed as a percentage of the average value for young animals. Results were further normalized for mitochondrial protein yield and expressed as UCP-2 and UCP-3 per milligram of tissue to compare them with the transcript levels in the whole tissue and to account for the potential reduction of mitochondrial protein content in aging skeletal muscle and liver.

Statistical analysis. UCP-2 and UCP-3 mRNA or protein levels in young and old animals were compared using an unpaired Student’s t-test for the two skeletal muscles, the heart muscle, and liver protein. A nonparametric Wilcoxon test was used to compare UCP-2 transcript levels between young and old animals in the liver. The relationships between UCP mRNA and mitochondrial DNA copy number as well as normalized cytochrome c oxidase I and III mRNA expression from the same animals (3) were analyzed using linear regression analysis. The same analysis was used to study the relationships between UCP mRNA and protein levels in the liver and the gastrocnemius muscle. Results were considered statistically significant at P < 0.05.

RESULTS

UCP-2 and UCP-3 expression. UCP-2 transcript levels were higher in old animals in both the lateral more oxidative and the medial more glycolytic heads of the gastrocnemius muscle (P < 0.04, Fig. 1A). UCP-2 mRNA levels were also higher in old animals in the liver (P < 0.03, Fig. 2A), whereas they were comparable in both age groups in the heart (Fig. 3). At variance with UCP-2, UCP-3 mRNA levels were lower in the older animals in the white more glycolytic medial head (P < 0.04), whereas the decrease was smaller in magnitude (−15%) and not statistically significant in the more oxidative lateral red head of the gastrocnemius muscle (Fig. 4A).

UCP-2 protein levels were also substantially higher in old animals in both the mixed gastrocnemius muscle (+100%, P < 0.05 in the mitochondrial protein fraction; +85%, P < 0.05 per mg whole tissue protein, Fig. 1B) and the liver (+400%, P < 0.01 in the mitochondrial protein fraction; +300%, P < 0.01 per mg whole tissue, Fig. 2B). Protein and mRNA levels were significantly correlated in the liver (r = 0.53; P = 0.05), and UCP-2 protein levels were correlated with UCP-2 transcripts in both the medial (r = 0.57; P < 0.05) and the lateral (r = 0.41; P = 0.10) portions of the gastrocnemius muscle. UCP-3 protein measured in the mixed gastrocnemius muscle tended to be reduced, but the change was not statistically significant (−15%, P = 0.61 in the mitochondrial protein fraction; −30%, P = 0.36 per mg whole tissue, Fig. 4B). UCP-3 protein
levels in aging mixed gastrocnemius were thus intermediate between those of UCP-3 transcripts in the lateral more oxidative and the medial more glycolytic muscles and were also significantly related to UCP-3 transcripts in both groups (medial: $r = 0.53; P < 0.05$; lateral: $r = 0.56; P < 0.05$).

**Correlations.** Age-related changes in UCP-2 transcript levels mirrored those recently reported in mitochondrial DNA copy number and were similar to those observed in mitochondrial DNA-normalized cytochrome $c$ oxidase I and III transcripts in the same animals (3). We therefore investigated the relationships between these variables in the different age groups. UCP-2 transcript levels were positively related to both mitochondrial DNA-normalized cytochrome $c$ oxidase I ($r = 0.51; P < 0.01$) and III ($r = 0.50; P < 0.01$) in all tissues when all animals were considered together. UCP-2 mRNA expression was also inversely correlated to mitochondrial DNA copy number in all tissues ($r = 0.51; P < 0.001$). These correlations were confirmed in the aging animals alone (Fig. 5, A-C), whereas they were no longer present when the young animals were considered separately (Fig. 5, D-F). No correlation was observed between UCP-3 mRNA or protein and mitochondrial gene expression.

**DISCUSSION**

The current study demonstrates that aging is associated with profound tissue-specific alterations of UCP-2 and -3 gene expression in different tissues. UCP-2 mRNA expression is markedly increased in both white and red gastrocnemius muscle and in liver in aging rats. Substantially higher liver and skeletal muscle UCP-2 protein levels in old animals confirm the mRNA data and indicate that similar age-related changes occur for transcript and protein levels. UCP-2 promotes proton leak in vitro (25), and the age-associated increment of its expression therefore suggests increased uncoupling of oxidative phosphorylation in old animals. Increased UCP-2 expression may limit ATP production in aging skeletal muscle and liver, thus representing a novel potential molecular mediator of impaired energy production in the aging process. Although changes of UCP activity with aging are theoretically possible and might partially offset the effects of its increased expression, the current findings are
consistent with reduced potential for ATP production suggested by age-related muscle dysfunctions in both human (15, 16, 30, 32, 33, 36) and rodent (5, 23) models and by previous in vitro data directly demonstrating reduced mitochondrial membrane potential in aging hepatocytes (21). At variance with the above findings, no changes were detected in UCP-2 transcript levels in the aging heart. This observation is again in agreement with the preserved cardiac potential for in vivo energy production in aging rats (28), whereas it clearly demonstrates that UCP-2 expression is differentially regulated in aging tissues.

The association of UCP-2 transcripts with relative levels of mitochondrial templates and mitochondrial DNA-normalized cytochrome c oxidase I and III mRNA strongly indicates that the age-associated tissue-specific changes in mitochondrial gene expression (3) are related to and might contribute to changes in UCP-2 expression in these tissues. To our knowledge, these data represent the first observation supporting a coordinated expression of mitochondrial oxidative and nuclear uncoupling protein genes. Of note, parallel increments of uncoupling and mitochondrial oxidative metabolism are a well-described feature of energy metabolism (9, 10, 35, 42), and this obligatory proton leak may account for up to 20% of the basal metabolic rate in rats (35). The unchanged oxygen consumption (34, 39, 44, 45, 50) in the presence of reduced mitochondrial density in aging tissues, including muscle and liver (24, 41), suggests that oxidative metabolism may indeed be enhanced in aging mitochondria. The inverse correlation between UCP-2 and levels of mitochondrial DNA, a possible index of mitochondrial content (3, 24, 41), also suggests that increased UCP-2 expression is related to reduced mitochondrial density with potential increments of mitochondrial oxidative workload in aging tissues. These combined data therefore introduce the possibility that ubiquitously expressed (17, 30) UCP-2 may be a molecular mediator of obligatory proton leak (9, 10, 35, 42). Further in vitro studies are needed to directly address this hypothesis.

The reduced or unchanged UCP-3 transcript levels in the white and red gastrocnemius muscle indicate a differential regulation of UCP-2 and UCP-3 gene expression in aging skeletal muscle. Although average UCP-3 protein levels are not significantly altered by aging in the mixed gastrocnemius, differential changes in transcript levels in the white more glycolytic and red more oxidative portions further suggest that age-related changes in rat skeletal muscle UCP-3 expression may be influenced by muscle fiber composition and metabolic characteristics. Differential changes of UCP-2 and UCP-3 transcripts have previously been related to changes in free fatty acid utilization and turnover in rodents (7). In particular, UCP-3 but not UCP-2 gene expression was enhanced after short-term fasting and fatty acid infusion in rats (49). Muscle UCP-3 expression is not increased in newborn mice until dietary ingestion of fat occurs (11). In human polymorphism studies, UCP-3 mutations have been associated with as much as 50% reductions in basal fat oxidation rates (1), and reduced skeletal muscle UCP-3 mRNA levels...
but not UCP-2 expression has been reported in people with type 2 diabetes (27). These observations indicate that UCP-3 expression may be directly related to oxidative fuel levels and specifically to muscle mitochondrial fatty acid oxidation (7). The lower UCP-3 mRNA in the aging medial gastrocnemius may therefore suggest impaired fat oxidative disposal in this tissue, as previously reported in aging rat muscles with intermediate oxidative capacity (13, 37). In contrast, however, aging does not appear to affect UCP-3 transcript levels in more oxidative red muscle fibers, which indeed rely more heavily on fatty acid oxidation for energy production (2). Plasma free fatty acid levels were not altered with aging (young = 0.20 ± 0.02 meq/l; old = 0.20 ± 0.02 meq/l). This observation is in agreement with some (12, 38) but not all (4, 6) previous reports on this issue in both rodents and humans. Available data (4, 6, 12, 38) suggest that differential age-related changes of plasma free fatty acid may be related to feeding status, especially to duration of fasting, and to concomitant glucose intolerance in humans. In the current study, differential age effects on UCP-3 expression with preserved UCP-3 transcripts in more oxidative muscles that preferentially utilize free fatty acid are consistent with a lack of major age-related changes in circulating free fatty acid levels. Finally, the combined data from UCP-2 and UCP-3 suggest that net modifications of mitochondrial uncoupling with aging may also be influenced by muscle fiber type. Age-related uncoupling may be more profoundly increased in more oxidative muscle groups with preserved UCP-3 expression, whereas reduced UCP-3 might blunt at least in part the effects of the substantial increase of UCP-2 in highly glycolytic muscles.

Fig. 5. A-C: correlation in old animals between ratio of transcript levels of cytochrome c oxidase (Cox) subunit I normalized for mitochondrial (mt) DNA and log UCP-2 mRNA in all tissues (A), ratio of transcript levels of cytochrome c oxidase subunit III normalized for mtDNA and log UCP-2 mRNA in all tissues (B), and mtDNA and log UCP-2 mRNA levels in all tissues (C). D-F: correlation in young animals between ratio of transcript levels of cytochrome c oxidase subunit I normalized for mtDNA and log UCP-2 mRNA in all tissues (D), ratio of transcript levels of cytochrome c oxidase subunit III normalized for mtDNA and log UCP-2 mRNA in all tissues (E), and mtDNA and log UCP-2 mRNA levels in all tissues (F).
REFERENCES


