Impact of high-fat diet and antioxidant supplement on mitochondrial functions and gene transcripts in rat muscle

R. SREEKUMAR, J. UNNIKRISHNAN, A. FU, J. NYGREN, K. R. SHORT,
J. SCHIMKE, R. BARAZZONI, AND K. SREEKUMARAN NAIR
Endocrinology Division, Mayo Clinic, Rochester, Minnesota 55905

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A HIGH-FAT DIET HAS BEEN REPORTED to adversely affect the health of human and animal species (7, 23, 25, 26). It has been reported that high levels of unsaturated fat increase fat-mediated oxidative stress and decrease antioxidant enzyme activity (22). A high-fat diet has been reported to increase atherogenesis (23, 24) and impairs glucose metabolism in rat skeletal muscle, which is the major site of insulin-stimulated glucose disposal (10). Feeding a high-fat diet to rodents has been shown to cause whole body and skeletal muscle insulin resistance (9, 32), hyperinsulinemia, and hyperglycemia (12) and, if continued for a longer period, could lead to the development of diabetes (26). An increased incidence of cancer with high-fat diet has been reported in experimental animals (30). In contrast, there are various reports indicating the beneficial effects of antioxidant supplementation in preventing cancer (3) and cardiovascular disease (19). It implies that oxidative damage and its consequences may result in many chronic health problems that are attributed to a high-fat diet. It has been proposed that mitochondrial DNA is especially susceptible to reactive oxygen species (ROS) damage (29).

We hypothesized that a high-fat diet would alter the transcription of genes involved in many body functions, especially those involved in ROS scavenging and mitochondrial ATP production. The purpose of this study was to determine the impact of a high-fat diet on rat skeletal muscle gene expression and mitochondrial function. We also determined whether these changes could be prevented or attenuated by antioxidant (vitamins A and E and selenium) supplementation.

EXPERIMENTAL PROCEDURES

Animals and experimental protocol. Male Sprague-Dawley rats were purchased from Harlan (Indianapolis, IN) at ~10 wk of age and were maintained on a standard chow diet for 2 wk. The following experiments were started after the animals were in our facility for 2 wk and lasted for an additional 36 wk. Animals were randomly assigned to one of the following dietary groups (n = 6 animals/group). The control group (Control) diet consisted of AIN-93G (Dyets, Bethlehem, PA), with protein (15%), fat (25%), and carbohydrate (60%), methionine (3 g/kg), mineral mix (50 g/kg), and vitamin mix (1 g/kg). Adequate amounts of selenium (1.24 g/kg) and dl-α-tocopherol acetate (0.05 g/kg) were included. For the high-fat diet group (HFD), the high-fat diet consisted of the same antioxidant content as the control diet but was supplemented with additional calories in the form of whole-milk powder and sweetener. The final composition of this diet was protein antioxidants; gene expression; mitochondrial adenosine triphosphate production

Address for reprint requests and other correspondence: K. S. Nair, Mayo Clinic & Foundation, 200 1st St. SW, Rm 5–194 Joseph, Rochester, MN 55905 (E-mail: nair.sree@mayo.edu).

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(20%), fat (60%), and carbohydrate (20%), with the same amount of vitamins and minerals. For the group having a high-fat diet supplemented with antioxidants (HFD+AO), the diet received by the HFD group was supplemented with additional antioxidants (8,000 IU vitamin A, 300 IU vitamin E, and 0.5 mg/kg selenium).

Throughout the experiment, animals were individually housed in wire-bottom cages in a controlled environment (12:12 h light-dark cycle, 20–22°C, 50–60% relative humidity). At the end of the study period, rats were injected with an intraperitoneal overdose of pentobarbital sodium. The gastrocnemius muscle was then quickly removed. One portion (60–70 mg) was kept in saline-soaked gauze on ice for mitochondrial studies, whereas the remainder of the muscle was immediately frozen in isopentane, cooled to the temperature of liquid nitrogen, and stored at −80°C until analysis.

**Analysis of gene transcripts.** To determine the muscle gene transcript profile in HFD and HFD+AO groups, the relative abundance of mRNAs in these two groups was compared with that of the control group by use of high-density oligonucleotide microarrays containing probes for ~800 genes (U34 array; Affymetrix, Santa Clara, CA).

**GeneChip expression probe array.** GeneChip expression probe arrays contain collections of pairs of probes for each of the mRNAs being analyzed (16). Each probe pair consists of a 25-mer that is perfectly complementary (referred to as a perfect match, or PM) to a subsequence of a particular message and a companion 25-mer that is identical except for a single base difference in the central position. The mismatch (MM) probe of each pair serves as an internal control for hybridization specificity. The analysis of PM-MM pairs allows low-intensity hybridization patterns from mRNAs to be sensitively and accurately recognized in the presence of cross-hybridization signals.

**RNA isolation.** Total RNA was isolated from frozen muscle tissue (gastrocnemius) by using TRIzol reagent (Life Technologies, Gaithersburg, MD), which was further purified using an affinity resin column (RNeasy; Qiagen, Chatsworth, CA). Total RNA thus isolated was converted to cDNA by use of the Superscript cDNA synthesis kit (GIBCO-BRL, Gaithersburg, MD). Double-stranded cDNA was then purified by phase lock gel (Eppendorf, Westbury, NY) with phenol-chloroform extraction (17).

**Sample preparation, fragmentation, array hybridization, and scanning.** The purified cDNA was used as a template for the in vitro transcription reaction for the synthesis of biotinylated cRNA with the use of RNA transcript labeling reagent (Affymetrix). This labeled cRNA was fragmented and hybridized onto the U34 array as described (17). Briefly, appropriate amounts of fragmented cRNA and control oligonucleotide B2 were added along with control cRNA (BioB, BioC, BioD), herring sperm DNA, and BSA to the hybridization buffer. The hybridization mixture was heated at 99°C for 5 min following by incubation at 45°C for 5 min before the sample was injected into the microarray. Then, the hybridization was carried out at 45°C for 16 h with mixing on a rotisserie at 60 rpm. After hybridization, the solutions were removed, and the arrays were washed and stained with streptavidin-phycocerythrin (Molecular Probes, Eugene, OR). After washes, probe arrays were scanned using the Hewlett-Packard GeneChip system confocal scanner (17). The quality of the fragmented biotin-labeled cRNA in each experiment was evaluated before being hybridized onto the U34 expression array by both gel electrophoresis and hybridizing (fraction of the sample) onto a test-2 array and analysis as a measure of quality control. For the gene transcript analysis by the high-density microarrays, we used the pooled muscle samples (120 mg) from six rats in each group (~20 mg from each rat).

**Data analysis.** GeneChip 3.0 (Affymetrix) was used to scan and quantitatively analyze the scanned image. Once the probe array had been scanned, GeneChip software automatically calculated intensity values for each probe cell and made a presence or absence call for each mRNA. Algorithms in the software used probe cell intensities to calculate an average intensity for each set of probe pairs representing a gene that directly correlated with the amount of mRNA. Algorithms in the software used probe cell intensities to calculate an average intensity for each set of probe pairs representing a gene that directly correlated with the amount of mRNA. Algorithms in the software used probe cell intensities to calculate an average intensity for each set of probe pairs representing a gene that directly correlated with the amount of mRNA. Algorithms in the software used probe cell intensities to calculate an average intensity for each set of probe pairs representing a gene that directly correlated with the amount of mRNA.
Table 1. Comparison of gene transcript patterns between control and HFD groups

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>GADD45</td>
<td>DNA damage</td>
</tr>
<tr>
<td>MAPK1</td>
<td>Signal transduction/cell proliferation</td>
</tr>
<tr>
<td>Protein kinase</td>
<td>Stress response/chaperone</td>
</tr>
<tr>
<td>Heat shock protein 70</td>
<td></td>
</tr>
<tr>
<td>Stress-inducible chaperone mt-GrpE</td>
<td></td>
</tr>
<tr>
<td>DnaJ-like protein (RDJ1)</td>
<td></td>
</tr>
<tr>
<td>Hydroxysteroid sulfotransferase</td>
<td>Sulfur metabolism</td>
</tr>
<tr>
<td>Serum amyloid A protein</td>
<td>Unknown</td>
</tr>
<tr>
<td>Vascular endothelial cell growth factor</td>
<td></td>
</tr>
<tr>
<td>Vascular cell adhesion molecule-1</td>
<td></td>
</tr>
<tr>
<td>Heat shock protein (HSP-E7I)</td>
<td></td>
</tr>
<tr>
<td>Hydroxysteroid sulfotransferase</td>
<td></td>
</tr>
<tr>
<td>Serine amyloid A protein</td>
<td>Unknown</td>
</tr>
<tr>
<td>NADP dehydrogenase</td>
<td>Energy metabolism</td>
</tr>
<tr>
<td>NADH-ubiquinone oxidoreductase</td>
<td>Energy metabolism</td>
</tr>
<tr>
<td>Glucose regulated protein (GRP94)</td>
<td>Glucose metabolism</td>
</tr>
</tbody>
</table>

HFD, high-fat diet; HFD + AO, high-fat diet supplemented with antioxidants; GADD45, growth arrest and DNA damage-inducible gene 45; MAPK1, mitogen-activated protein kinase-1. Gene expression in HFD and HFD + AO groups were compared with the control group.

Northern blot analysis of uncoupling proteins-2 and -3. cDNA probes for uncoupling protein (UCP)-2, UCP-3, and 28S rRNA transcripts were generated by RT-PCR amplification from control and HFD rat 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. V01702). Amplification products were cloned into the TA-plasmid vector (TA Cloning kit; Invitrogen), as previously described (2). Total RNA isolation, Northern blotting, and hybridization to UCP-2, UCP-3, and 28S probes (in that order) were performed as described (2). Resulting images were quantified by laser densitometry (Ultrascan; Pharmacia), and UCP bands were normalized to the corresponding 28S rRNA band.

Mitochondrial ATP production rate. Mitochondria were purified from skeletal muscle, and ATP production was determined using a bioluminiscence technique as previously described (21, 31). Mitochondrial suspensions diluted in ATP-monitoring reagent (AMR; formula SI; BioThema, Dalarö, Finland) were added to cuvettes containing AMR, substrate, and ADP. The substrates added (in mM final concentration) were 1) 1 pyruvate plus 1 malate, 2) 1 palmitoyl-l-carnitine plus 1 malate, 3) 10 α-ketoglutarate, or 4) 1 pyruvate plus 0.05 palmitoyl-l-carnitine plus 1 malate plus 10 α-ketoglutarate, with additional blank tubes used for measuring background. ATP production for all reactions was monitored simultaneously at 25°C with an automated routine in a BioOrbit 1251 luminometer (BioOrbit Oy, Turku, Finland). Internal calibration of each reaction cuvette was performed by addition of an ATP standard. Citrate synthase activity was measured in mitochondria and tissue homogenates as previously described (21) and used to calculate the ATP production rate.

RESULTS

Body weight. We measured the body weight of 12-wk-old rats (395 ± 0.2 g) just before the start of the various feeding programs and randomized six rats each into three different groups (control, HFD, and HFD+AO). At the conclusion of the 36-wk study, the HFD (730 ± 21.6 g) and HFD+AO (769 ± 24.3 g) animals had similar body weights and were heavier than the control (626 ± 24.6 g) animals (P < 0.01).

Gene transcript levels. Comparisons were made between control animals and animals from the two intervention groups (HFD and HFD+AO). Alterations in gene transcripts involving several functions were identified (Table 1). Of 800 genes whose expression pattern we monitored using high-density oligonucleotide microarrays, 18 (↑ 8 and ↓ 10) and 19 (↑ 10 and ↓ 9) gene transcripts were altered at least twofold in HFD and HFD+AO rats, respectively, compared with the controls.

Of the 18 altered gene transcripts in the HFD group, 28% were mediators of DNA repair/damage/free-radical scavenger function, 17% each were involved in...
signal transduction/cell proliferation and stress response/chaperone function, and 10% were associated with cell growth/adhesion (HFD column, Table 1). The remaining 28% were involved in energy metabolism (NADH dehydrogenase), ion pump (Na\(^+\)/K\(^-\)-ATPase α\(_1\)-subunit), iron transport (transferrin receptor), sulfur metabolism (hydroxysteroid sulfotransferase), and unknown function (serum amyloid A).

Of the 18 gene transcripts found altered in HFD rats, 4 were completely normalized and 12 were partially normalized by antioxidant supplementation for 36 wk (Table 1). Stress-inducible chaperone mitochondrial GrpE, chaperonin 60, and DnaJ-like protein (RDJ1), all involved in stress response/chaperone function, and NADH dehydrogenase, involved in energy metabolism, are the four genes whose expression was normalized. Twelve transcripts partially normalized, including growth arrest and DNA damage-inducible gene (GADD45), DNA polymerase-α, Cu-Zn SOD, glutathione peroxidase I, Mn-SOD, Ras-related protein, protein kinase, hydroxysteroid sulfotransferase, vascular endothelial cell growth factor, vascular cell adhesion molecule-1, transferrin receptor, and Na\(^+\)/K\(^-\)-ATPase α\(_1\)-subunit. In addition, antioxidant supplementation increased the transcript levels of four additional genes [glutathione-S-transferase, NADH-ubiquinone oxidoreductase, glucose-regulated protein, and heat shock protein (HSP)E71], which were normal in the HFD group, whereas serum amyloid A protein showed a further increase with antioxidant supplementation.

We validated three of the gene transcript expression levels using the real-time PCR approach (Fig. 1). The real-time PCR analysis also showed that, in HFD animals, SOD-1 and SOD-2 mRNA levels were reduced compared with control animals, which was in agreement with the microarray data. In HFD+AO animals, SOD-1 and SOD-2 gene transcript levels by real-time PCR were higher than in HFD animals, as was also shown by microarray analysis.

**UCP-2 and UCP-3 expression.** UCP-2 transcript levels were higher in HFD rats (2.73 ± 0.35) than in controls (1.80 ± 0.2; \(P < 0.05\)), whereas no significant difference in UCP-3 transcripts was observed between HFD (4.72 ± 1.50) and the control (5.61 ± 0.99) rats. UCP-2 and UCP-3 transcripts were not measured in HFD+AO animals.

**Mitochondrial ATP production and citrate synthase activity.** As shown in Table 2, mitochondrial ATP production rates and citrate synthase activity were not different among the groups.

**DISCUSSION**

The focus of the current study was to determine the effect of high-fat diet on gene transcript profiles and mitochondrial function in skeletal (gastrocnemius) muscle of rats. To determine whether changes in gene transcript profiles could be attenuated or prevented by supplementation with antioxidants, we measured gene transcript profiles in skeletal muscle from animals that were kept on a high-fat diet and antioxidant supplements. Skeletal muscle is a major organ involved in oxidation of circulating fatty acids and a potential site of ROS damage. The microarray approach provides an opportunity to examine this important issue in a global manner. We also confirmed the findings of the microarray approach by measuring transcript levels of three genes (COX III, SOD-1, and SOD-2) from control, high-fat diet (HFD), and HFD supplemented with antioxidants (HFD+AO). Values are means ± SE. The relative levels of SOD-1 and SOD-2 transcripts were lower in HFD animals than in the controls (\(P < 0.01\)). SOD-1 and SOD-2 transcripts were higher in HFD+AO than in HFD (\(P < 0.05\)).
The most striking aspect of the data set is that ~28% of the genes that had altered expression in the HFD group are mediators of DNA damage/repair/free-radical scavenger function. There was a markedly increased expression of the GADD45 gene in the HFD group, and its expression declined with antioxidant supplementation. Hollander et al. (11) and Jackman et al. (14) have shown that GADD45a null mice generated by gene targeting exhibited several phenotypes characteristic of mice deficient in p53, including genomic instability, increased radiation carcinogenesis, and a low frequency of exencephaly. It has been shown previously that, in aging rat muscle, the transcript level of GADD45 is increased (15). Of interest, the magnitude of increase in GADD45 transcript level was attenuated by the addition of antioxidants to the high-fat diet, suggesting that the alteration induced by the high-fat diet is partly due to oxidative damage by ROS. Gene transcription of DOS scavengers such as SOD-1, SOD-2, and glutathione peroxidase were significantly decreased in HFD rats along with the DNA repair enzyme DNA polymerase-α. Both SOD and glutathione-S-transferase have been shown to play a protective role against oxidative damage in various tissues by neutralizing ROS (28, 33). In the present study, 36 wk of antioxidant supplementation actually improved the expression levels of several genes involved in free-radical scavenging function along with SOD and glutathione S-transferase. Removal of the ROS by SOD and of hydrogen peroxide by catalase and glutathione peroxidase prevent formation of the very reactive hydroxyl radical, which is postulated to be responsible for much of the cellular damage. Antioxidant supplementation for 36 wk partially prevented the alterations in expression of these genes.

The high-fat diet also resulted in alterations in the expression of genes involved in cell proliferation/signal transduction such as mitogen-activated protein kinase (MAPK)-1 (↑), Ras-related protein (↑), and protein kinase (↓). Antioxidant supplementation did not alter the transcript levels of MAPK and protein kinase, whereas Ras-related protein mRNA level was improved. The Ras-MAPK pathway transduces the mitogenic signals initiated by growth factors and has been implicated in mammary cancer promotion in rats fed a high-fat diet (30). Another interesting observation was the upregulation of several genes involved in stress response in HFD rats, suggesting an increased oxidative stress associated with these animals. This includes heat shock protein 70 (HSP70), stress-inducible protein GrpE, RDJ1, and chaperonin 60. The antioxidant supplement with the high-fat diet normalized all of these gene expression abnormalities except for HSP70 (which remained upregulated).

Analysis of gene transcript levels in HFD animals also revealed no alterations in genes involved in energy metabolism except NADH dehydrogenase, which was downregulated 2.1-fold in this group compared with the controls. NADH dehydrogenase is part of complex I in the mitochondrial electron transport system and is involved in oxidative phosphorylation. In HFD+AO rats, only one gene involved in energy metabolism, NADH-ubiquinone oxidoreductase, showed alteration (upregulation) among the 800 genes we surveyed. The lack of change in multiple genes of the mitochondrial oxidative phosphorylation pathways may explain why the diets had no effect on ATP production.

Several genes involved in tissue development/growth or cell adhesion function were altered in both of these groups of animals. Cell adhesion represents a process that is vital in immune function and inflammation, and it has been reported that antioxidants regulate cell adhesion by modulating specific signal transduction pathways (20). These include vascular endothelial cell growth factor and vascular cell adhesion molecule-1. The expression of vascular endothelial cell growth factor and vascular cell adhesion molecule-1 were 11.8 and 9.3-fold lower, respectively, in HFD rats compared with the controls. In the HFD+AO group, expression of both of these genes was also reduced compared with controls (Table 1). However, the magnitude of the reduction was less than one-half that in the HFD group, suggesting that antioxidants may have had a significant protective effect on the pathways leading to transcription of these genes.

Na⁺-K⁺-ATPase is an integral membrane protein responsible for establishing and maintaining the electrochemical gradients of Na⁺ and K⁺ ions across the plasma membrane. Because these gradients are essential for osmoregulation, for sodium-coupled transport of a variety of organic and inorganic molecules, and for electrical excitability of nerve and muscle, the enzyme plays an essential role in cellular physiology. It is composed of two subunits, a large catalytic subunit (α) and a smaller glycoprotein subunit (β) of unknown class/function.
function. In the HFD group (↓ 3.6), as well as in the HFD+AO group (↓ 2.0), rats showed a decline in Na+-K+-ATPase α1-subunit transcript level, with a slight improvement in the HFD+AO group.

To validate the observations in the microarray experiment, we measured the gene transcript levels of COX III, SOD-1, and SOD-2 using real-time PCR (Fig. 1). The gene transcript level of COX III, a mitochondrial-encoded subunit of cytochrome c oxidase, was decreased 23 and 15%, respectively, in HFD and HFD+AO rats compared with the controls. Similarly, the gene transcript levels of SOD-1 (↓ 63% HFD, ↓ 24% HFD+AO) and SOD-2 (↓ 67% HFD, ↓ 23% HFD+AO) also showed a decline in mRNA levels in the HFD groups. The decrease in transcript levels of SOD-1 and SOD-2 in HFD animals compared with the control animals (P < 0.01) and the increase in these transcript levels in HFD+AO animals compared with HFD animals (P < 0.05) were significant.

Of interest, it was also noted that mRNA levels of UCP-2 were higher in HFD rats, whereas UCP-3 mRNA levels were similar in HFD animals compared with the control animals. Four weeks of high-fat diet feeding have been shown to increase UCP-3 mRNA (18) and protein expression (5) in skeletal muscle. In contrast, Corbalan et al. (6) have shown that the gastrocnemius UCP-3 mRNA levels were significantly reduced in rats fed a cafeteria diet compared with lean animals. The combination of the low-fat diet received by the control group and the shorter duration of the study could very well explain the difference in result between our present study and these studies (6, 18). There is increasing experimental evidence to indicate that these UCPs are involved in proton leak and regulation (or modulation) of thermogenesis (2, 4, 8, 13, 27). High fat intake appears to promote uncoupling of oxidative phosphorylation, thus allowing possible proton leak. In HFD rats, muscle mitochondrial ATP production and enzyme activity (citrate synthase) were not different from those in control rats. The upregulation of UCP-2 mRNA in HFD rats, muscle mitochondrial ATP production and phosphorylation, thus allowing possible proton leak. In contrast, Corbalan et al. (6) have shown that the gastrocnemius transcript levels in HFD rats despite increased expression of UCP-2.

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