Time course of high-fat diet-induced reductions in adipose tissue mitochondrial proteins: potential mechanisms and the relationship to glucose intolerance

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Submitted 30 April 2008; accepted in final form 31 August 2008

Sutherland LN, Capozzi LC, Turchinsky NJ, Bell RC, Wright DC. Time course of high-fat diet-induced reductions in adipose tissue mitochondrial proteins: potential mechanisms and the relationship to glucose intolerance. Am J Physiol Endocrinol Metab 295: E1076–E1083, 2008. First published September 9, 2008; doi:10.1152/ajpendo.90408.2008.—Increasing evidence suggests that reduced adipose tissue mitochondrial content is associated with the pathogenesis of type 2 diabetes. These investigations have utilized severely insulin-resistant rodent models. Thus, it is difficult to ascertain the potential mechanisms that initiate these changes and whether reductions in adipose mitochondrial content are an initiating event in the development of impaired glucose homeostasis. Thus, we sought to determine the time course of high-fat diet-induced reductions of mitochondrial content in epididymal adipose tissue in relation to changes in purported mediators of mitochondrial biogenesis and the development of impaired glucose homeostasis. Male Wistar rats were fed a high-fat diet (~59% of kcals from fat) for 2, 4, or 6 wk. Six weeks of high-fat feeding resulted in reductions in CORE I, COX IV, cytochrome c, HSP60, relative mtDNA copy number, and PGC-1α expression. These changes were not associated with decreases in eNOS and AMPK or increases in markers of oxidative stress. Interestingly, ex vivo treatment of adipose tissue cultures with palmitate led to decreases in PGC-1α expression and COX IV and CORE I protein content as observed in vivo. Thus, the high-fat diet-induced reductions in adipose tissue mitochondrial proteins may be mediated by increases in plasma fatty acids. Importantly, reductions in adipose tissue mitochondrial content occurred after the development of impaired glucose homeostasis. Thus, reductions in adipose tissue mitochondrial proteins are most likely not a causal event in the development of impaired glucose homeostasis.

peroxisome proliferator-activated receptor-γ coactivator-1; insulin resistance; obesity; rat; type 2 diabetes

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meostasis. Within this context, the purpose of the present investigation was to determine the time course of high-fat diet-induced reductions in adipose tissue mitochondrial content in relation to changes in reputed mediators of mitochondrial biogenesis and the development of impaired glucose homeostasis. Given the close association between adipose tissue mito-

 MATERIALS AND METHODS

Materials. Reagents, molecular weight marker, and nitrocellulose membranes for SDS-PAGE were purchased from Bio-Rad (Mississauga, ON, Canada). ECL Plus was a product of Amersham Pharma-
cia Biotech (Arlington Heights, IL). Antibodies against COX IV and CORE I were purchased from Molecular Probes (Eugene, OR). Phosphospecific (Thr177) AMPKα antibodies were from Cell Signaling (Danvers, MA). An anti-eNOS antibody was purchased from BD Transduction Laboratories (Mississauga, ON, Canada). Antibodies against heat shock protein-60 (HSP60) and cytochrome c were from Stressgen (Victoria, BC, Canada) and Mitosciences (Eugene, OR), respectively. An antibody against β-actin was a product of Sigma (St. Louis, MO). Horseradish peroxidase-conjugated donkey anti-rabbit and goat anti-mouse IgG secondary antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Glucose standards were obtained from NERL Diagnostics (East Providence, RI) and glucose reagents from Diagnostic Chemicals Limited (PEI, Canada). Rat insulin enzyme-linked immunosorbent assay (ELISA) kits and adiponectin ELISA kits were purchased from Linco (St. Charles, MO). A nonesterified fatty acid (NEFA) assay kit was a product of Wako Diagnostics (Richmond, VA). RNaseasy and DNeasy extraction kits were purchased from Qiagen (Mississauga, ON, Canada).SuperScript II Reverse Transcriptase was a product of Invitrogen (Carlsbad, CA). An antibody against eNOS was obtained from Transduction Laboratories (Mississauga, ON, Canada). Antibodies against COX IV and PGC-1α were purchased from Santa Cruz Biotech (Arlington Heights, IL). Antibodies against COX IV and PGC-1α were purchased from Santa Cruz Biotech (Arlington Heights, IL). Antibodies against COX IV and PGC-1α were purchased from Santa Cruz Biotech (Arlington Heights, IL).

Treatment of rats. All protocols followed Canadian Council on Animal Care guidelines and were approved by the Animal Use and Welfare Committee at the University of Alberta. Male Wistar rats (Charles River, Wilmington, MA) weighing ~200 g were housed two per cage with a 12:12-h light-dark cycle and were provided with water and standard rat chow ad libitum. Rats were acclimated to the animal housing facility for 1 wk prior to the start of the diet manipulation.

Table 1. Time course of high-fat diet-induced changes in body weight, epididymal fat pad mass, and plasma hormones and metabolites

<table>
<thead>
<tr>
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<th>Chow</th>
<th>High Fat</th>
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<tr>
<td>2 Weeks</td>
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<tr>
<td>Body weight, g</td>
<td>298.2±3.9</td>
<td>318.0±4.9*</td>
</tr>
<tr>
<td>Fat pad, g</td>
<td>3.0±0.2</td>
<td>5.3±0.3*</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>112.2±2.0</td>
<td>113.6±3.4</td>
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<tr>
<td>Insulin, µg/l</td>
<td>0.33±0.1</td>
<td>0.53±0.1*</td>
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<tr>
<td>Adiponectin, µg/ml</td>
<td>10.3±0.7</td>
<td>11.2±0.7</td>
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<tr>
<td>Leptin, ng/ml</td>
<td>0.62±0.27</td>
<td>2.28±0.36*</td>
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<td>NEFA, mmol/l</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>4 Weeks</td>
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<tr>
<td>Body weight, g</td>
<td>367.1±6.2</td>
<td>410.7±8.6*</td>
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<tr>
<td>Fat pad, g</td>
<td>4.3±0.3</td>
<td>8.0±0.8*</td>
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<tr>
<td>Glucose, mg/dl</td>
<td>106.7±5.6</td>
<td>115.7±5.2</td>
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<tr>
<td>Insulin, µg/l</td>
<td>0.45±0.1</td>
<td>1.38±0.3*</td>
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<tr>
<td>Adiponectin, µg/ml</td>
<td>9.0±1.0</td>
<td>10.7±0.6</td>
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<tr>
<td>Leptin, ng/ml</td>
<td>0.67±0.17</td>
<td>4.38±0.73*</td>
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<tr>
<td>NEFA, mmol/l</td>
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<tr>
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<th>Chow</th>
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<td>6 Weeks</td>
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<tr>
<td>Body weight, g</td>
<td>441.6±0.05</td>
<td>522.6±0.02*</td>
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<tr>
<td>Fat pad, g</td>
<td>5.6±0.4</td>
<td>16.9±1.8*</td>
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<tr>
<td>Glucose, mg/dl</td>
<td>103.1±8.1</td>
<td>122.7±4.3*</td>
</tr>
<tr>
<td>Insulin, µg/l</td>
<td>0.66±0.4</td>
<td>1.67±0.2*</td>
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<tr>
<td>Adiponectin, µg/ml</td>
<td>14.9±1.2</td>
<td>12.5±1.3</td>
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<tr>
<td>Leptin, ng/ml</td>
<td>1.24±0.28</td>
<td>8.31±1.27*</td>
</tr>
<tr>
<td>NEFA, mmol/l</td>
<td>0.28±0.05</td>
<td>0.47±0.02*</td>
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Values are means ± SE for 8–14 rats per group. NEFA, nonesterified fatty acid; ND, no data available at this time point. Insulin, glucose, leptin, and adiponectin were measured after an overnight fast, whereas NEFA was measured in the fed state. *P < 0.05 vs. chow-fed group at the same time point.
Source) supplemented with protease inhibitor cocktail (Sigma) and phenylmethylsulfonyl fluoride. Homogenized samples were sonicated for 5 s and centrifuged for 15 min at 2,500 g at 4°C. The fat cake was carefully removed, and the protein concentration of the supernatant was determined using the BCA method. The CV for this assay is <5% in our laboratory. The protein content of CORE I, COX IV, HSP60, eNOS, p-AMPK, and cytochrome c were determined by Western blot analysis as described previously (31, 32). Briefly, equal amounts of protein were separated on either 10% (CORE I, HSP60, eNOS, p-AMPK) or 15% (COX IV, cytochrome c) gels. Proteins were wet-transferred to nitrocellulose membranes for 90 min at 200 mA/tank. Membranes were blocked in tris-buffered saline-0.01% Tween 20 (TBST) supplemented with 5% nonfat dry milk for 1 h at room temperature with gentle agitation. Membranes were incubated in TBST-5% nonfat dry milk supplemented with appropriate primary antibodies overnight at 4°C with gentle agitation. The following morning, blots were briefly washed in TBST and then incubated in TBST-1% nonfat dry milk supplemented with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Bands were visualized using ECL plus and captured using a Typhoon Imaging system (GE Health Care). Imagequant software was used to quantify relative band intensities. β-Actin was used as an internal control. In preliminary experiments we found that 6 wk of high-fat feeding had no effect on the protein content of β-actin in epididymal adipose tissue (3.21 ± 0.12 chow fed, 3.48 ± 0.23 high-fat fed, arbitrary densitometric units n = 6).

Real-time PCR. RNA was isolated from epididymal adipose tissue using an RNeasy kit according to the manufacturer’s instructions. One
microgram of RNA was used for the synthesis of complementary DNA (cDNA) using SuperScript II Reverse Transcriptase, oligo(dT), and dNTP. Real-time PCR was performed using a 7900HTFast Real-Time PCR system (Applied Biosystems). A Taqman Gene Expression Assay was used to determine the expression of β-actin. Primers and probes for PGC-1α were designed using Primer Express 3.0 software (sequences are available upon request). Samples were run in duplicate in a 96-well plate format. For the determination of β-actin, each well (20 μl total volume) contained 1 μl of gene expression assay, 1 μl of cDNA template, 10 μl of Taqman Fast Universal PCR Master Mix, and 8 μl of RNase-free water. For PGC-1α, each 20-μl reaction contained 10 μl of PCR Master mix, 0.225 μl each of forward and reverse primers, 0.05 μl of probe, and 5.55 μl of RNase-free water. β-Actin was used as the reference gene, as we had found in preliminary experiments that β-actin expression did not change with high-fat feeding (P = 0.43). Relative differences in gene expression between chow- and high-fat-fed rats were determined using the 2−ΔΔCT (cycle threshold) method (15). Standard curve assays were performed for beta actin and the genes of interest. The amplification efficiencies of the genes of interest and β-actin were equivalent as determined using the equation 10(1/slope) − 1. Likewise, when plotting logcDNA dilution vs. ΔCT (CT gene of interest − CT β-actin), the slope of this relationship was <0.1, indicating that the genes of interest were amplified with equal efficiency.

Mitochondrial DNA copy number. Total DNA was isolated from epididymal adipose tissue by use of a DNeasy kit. Genomic DNA samples were treated with RNase to remove contaminating RNA. Relative mitochondrial DNA (mtDNA) copy number was measured by determining the ratio of a mtDNA target sequence (mitochondrial D-loop) to the expression of a nuclear target sequence (β-actin) by real-time PCR. These target genes have previously been used to determine relative amounts of mtDNA in rat tissue (3, 17). Primers and probes for β-actin and the mitochondrial D-loop were designed using Primer Express 1.5 software (sequences are available upon request). Samples were run in duplicate using a 96-well plate format using a 7900HT Fast-Real-Time PCR system (Applied Biosystems). Each well contained 10 μl of master mix, 0.225 μl each of forward and reverse primers, 0.05 μl of probe, 1 μl of genomic DNA, and 8.5 μl of RNase-free water. Relative differences in mtDNA copy number between chow- and high-fat-fed rats were determined using 2−ΔΔCT, as described by Nicklas et al. (17). Briefly, the CT for β-actin was subtracted from the CT for the mitochondrial D-loop to generate ΔCT. Chow-fed rats acted as the control standard and ∆∆CT was determined as ΔCT from high-fat fed − ΔCT from Chow fed. Mitochondrial D-loop and β-actin were amplified with equal efficiency (slope of DNA dilution vs. ΔCT <0.1).

Determination of adipose tissue thiobarbituric acid-reactive substances and protein carbonyls. Epididymal adipose tissue samples (~500 mg) were homogenized in 2 volumes of cell extraction buffer followed by centrifugation for 10 min at 2,500 g at 4°C. The fat cake was carefully removed, and the protein concentration of the resulting supernatant was determined. Lipid peroxidation, a marker of oxidative damage to lipids, was determined by measuring the formation of malondialdehyde (MDA)-thiobarbituric acid (TBA) adducts using a commercially available kit (Cayman Chemicals, Ann Arbor, MI). Changes in thiobarbituric acid-reactive substance (TBAR) formation were determined colorimetrically at 532 nm using a 96-well format. Similarly, oxidative damage to proteins was determined through measuring the formation of protein carbonyls with an assay kit from Cayman Chemicals. Protein carbonyl formation was detected using a microplate reader at a wavelength of 370 nm. The CV for these assays is <10% in our laboratory.

Statistical analysis. Data are presented as means ± SE. Comparisons between the means of chow and high-fat groups at the same time point were made using an unpaired Student’s t-test. Statistical significance was set at P < 0.05.

RESULTS

Effect of high-fat feeding on body weight, fat pad mass, and blood measurements. Significant differences in body mass and epididymal fat pad mass between control and high-fat-fed rats were evident at all time points (table 1). Fasting glucose levels were slightly elevated following 6 wk of high-fat feeding. High-fat feeding lead to increases in fasting insulin and leptin levels at all time points examined while having no effect on plasma adiponectin concentrations. NEFA concentrations were elevated in high-fat-fed rats following 6 wk of feeding.

High-fat diet-induced reductions in adipose tissue mitochondrial content. High-fat feeding for 6 wk led to ~30–50% reductions in the protein content of various markers of mitochondrial content, CORE I, COX IV, and cytochrome c in epididymal adipose tissue (Fig. 1). No changes were seen following either 2 or 4 wk of high-fat feeding. In addition to reductions in constituents of the respiratory chain, we saw decreases in the mitochondrial chaperone protein HSP60. Similarly 6 wk of high-fat feeding led to reductions in relative mtDNA copy number and the expression of PGC-1α (Fig. 2).

High-fat diet-induced changes in AMPK signaling, eNOS protein content, and oxidative stress. Having determined the earliest time point in which reductions in adipose tissue mitochondrial content with high-fat feeding occurs, we wanted to determine whether these changes were associated with parallel decreases in AMPK and eNOS, reputed mediators of mitochondrial biogenesis in white adipose tissue. Six weeks of high-fat feeding did not result in decreases in AMPK phosphorylation or total protein content. Likewise, eNOS protein in epididymal adipose tissue was not decreased. High-fat feeding for 6 wk did not lead to increases in protein carbonyl formation or MDA, markers of oxidative stress (Fig. 3).

Palmitate decreases PGC-1α expression in adipose tissue. Epididymal adipose tissue minces were incubated in M-199 supplemented with or without 500 μM palmitate for 24 h. This treatment led to a ~50% reduction in PGC-1α expression. Hyperinsulinemia (24-h incubation with 2 mU/ml insulin) had no effect on PGC-1α expression (data not shown). The palmitate-induced reductions in PGC-1α expression was associated with decreases in COX IV and CORE I protein content (Fig. 4).

Fig. 2. Six weeks of high-fat feeding results in decreases in relative mitochondrial (mt) DNA copy number and PGC-1α mRNA expression. Data are presented as means ± SE for 6–8 samples per group are expressed relative to chow-fed values at the same time point. *P < 0.05.
Time course of high-fat diet-induced alterations in glucose homeostasis. High-fat feeding led to impairments in glucose clearance and an exaggerated insulin response to an intraperitoneal glucose challenge in rats fed a high-fat diet for 4 and 6 wk (Fig. 5). The glucose-insulin AUC index (integrated AUC) can be used as a surrogate of whole body insulin action (6, 26) with increases in this value representing a worsening of systemic insulin action. As seen in Fig.
DISCUSSION

Adipose tissue is increasingly being recognized as an important player in the regulation of whole body glucose metabolism. Of interest to the study of type 2 diabetes, a strong association between reductions in adipose tissue mitochondrial content and systemic insulin resistance has been reported. Data from these investigations have been generated primarily using adipose tissue or adipocytes from genetically modified rodents such as db/db mice and fa/fa rats (4, 13, 29, 30). Unfortunately, due to the marked insulin resistance and hyperglycemia present in these animals, it is difficult to identify the specific mechanisms that trigger reductions in adipose tissue mitochondrial content and whether decreases in adipose tissue mitochondria are a potential initiating event in the pathogenesis of systemic insulin resistance and glucose intolerance. With these points in mind, we sought to determine how quickly reductions in adipose tissue mitochondrial content occurs with high-fat feeding, a common approach used to cause abdominal obesity and insulin resistance in rodents.

Compared with chow-fed controls, 6 wk of high-fat feeding led to ~30–50% reductions in the content of mitochondrial respiratory-chain proteins CORE I, COX IV, and cytochrome c.
Likewise, we found similar reductions in mtHSP60, a chaperone protein involved in mitochondrial protein folding that has been previously shown to be decreased in epididymal adipose tissue from ob/ob (30) and db/db mice (19). Confirming our Western blot data, we found reductions in relative mtDNA copy number, a marker of the number of mitochondria per cell. Importantly, our data demonstrate that 6 wk of high-fat feeding is the earliest time point in which reductions in adipose tissue mitochondrial protein content is evident. The magnitude of this reduction is similar to what has been reported in db/db mice and fa/fa rats (4, 13, 29, 30), yet develops in the absence of prolonged hyperglycemia and hyperinsulinemia.

PGC1-α is a transcriptional coactivator and master regulator of mitochondrial biogenesis (8). Reductions in PGC-1α expression have been reported in insulin-resistant rodents (23, 29) and in obese and type 2 diabetic subjects (1, 25). Likewise, the induction of mitochondrial biogenesis in adipose tissue by thiazolidinediones is accompanied by increases in PGC-1α expression (1, 30). In accord with these findings, we found decreases in PGC-1α expression concurrent with reductions in adipose tissue mitochondrial marker proteins. These results are temporally consistent with the notion that reductions in PGC-1α expression could be involved in mediating high-fat diet-induced decreases in adipose tissue mitochondrial proteins.

The regulation of PGC-1α expression and mitochondrial biogenesis in adipose tissue is a complex process involving multiple factors. In models of severe insulin resistance, positive effectors of mitochondrial biogenesis in adipose tissue such as AMPK (21) and eNOS (29) are reduced. On the other hand negative modulators of mitochondrial content (2), such as oxidative stress, are increased in adipose tissue from diabetic mice (11). In the current investigation, we assessed whether changes in these variables occurred concurrently with the initial reductions in markers of mitochondrial content caused by high-fat feeding. Following 6 wk of high-fat feeding, a time point in which markers of mitochondrial content were reduced, AMPK phosphorylation and eNOS content were unchanged. Consistent with a recent report in high-fat-fed mice (16), we did not find any differences in protein carbonyl or MDA content in adipose tissue from high-fat-fed rats. Collectively our results suggest that alterations in AMPK, eNOS, and oxidative stress are not initiating events in the process mediating decreases in adipose tissue mitochondrial protein content.

Given these results, we sought to identify potential mechanisms that could help explain high-fat diet-induced reductions in adipose tissue mitochondrial content. In our high-fat feeding model rats maintain normal glucose levels but are both hyperinsulinemic and hyperlipidemic. Given the fact that palmitate has previously been shown to reduce the expression of PGC-1α in skeletal muscle cells (5, 7), we wanted to determine if the ex vivo treatment of rat adipose tissue with palmitate could reproduce the effects of high-fat feeding on adipose tissue PGC-1α expression and markers of mitochondrial content. We found that palmitate, but not high insulin levels, led to reductions in PGC-1α expression and COX IV and CORE I protein content. These findings, in combination with the observed increases in NEFA levels following high-fat feeding, suggest a potential causal role for fatty acids in the downregulation of adipose tissue PGC-1α expression and mitochondrial content.

To determine the relevance of the observed reductions in adipose tissue mitochondrial proteins to the development of insulin resistance, we measured changes in glucose homeostasis following 2, 4, and 6 wk of high-fat feeding. Contrary to our hypothesis, yet consistent with what has been reported in skeletal muscle (2), we found that reductions in adipose tissue mitochondrial content occurred after the development of high-fat diet-induced insulin resistance. These findings are temporally inconsistent with the notion that reductions in adipose tissue mitochondrial content are a causal, initiating event in the development of impaired glucose homeostasis. Rather, the present findings argue that reductions in adipose tissue mitochondrial content develop as a consequence of high-fat diet-induced insulin resistance. It should be pointed out that we did not measure mitochondrial function in the present study. Thus, it still remains a possibility that reductions in adipose tissue mitochondrial function, independent of changes in mitochondrial content, could precede and be linked to the development of systemic insulin resistance. Furthermore, our results do not rule out the possibility that reductions in adipose tissue mitochondrial content may be associated with a further worsening of systemic insulin resistance.

This investigation is the first to characterize the temporal relationships between reductions in adipose tissue mitochondrial content, the potential mediators of this process, and changes in whole body glucose homeostasis in a model of diet-induced obesity. We found that reductions in adipose tissue mitochondrial content are associated with reductions in PGC-1α expression and that these effects can be reproduced ex vivo by treating adipose tissue organ cultures with palmitate. The most important finding of this study is that alterations in PGC-1α and mitochondrial markers occurred after the onset of impaired glucose homeostasis. Although the present findings in rat epididymal fat pads cannot necessarily be extrapolated to visceral fat depots in humans, our results argue against reductions in adipose tissue mitochondrial content being an initiating event in the development of impaired glucose homeostasis.

ACKNOWLEDGMENTS

We thank Donna Taylor and Abha Hoedel for outstanding technical assistance with the glucose tolerance tests.

GRANTS

D. C. Wright is an Alberta Heritage Foundation for Medical Research Scholar, Canadian Institutes of Health Research (CIHR) New Investigator and Canadian Diabetes Association Scholar. This research was supported by a Discovery Grant from the Natural Sciences and Engineering Research Council of Canada to R. C. Bell and an Operating Grant to D. C. Wright from CIHR.

REFERENCES

Diet-Induced Reductions in Adipose Mitochondria


