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Influence of increased adiposity on mitochondrial-associated proteins of the rat colon: a proteomic and transcriptomic analysis.

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Abbreviations:

2DPAGE = two dimensional polyacrylamide gel electrophoresis

DIOS = Diet Induced Obesity Sensitive

DIOR = Diet Induced Obesity Resistant

ETC = Electron Transport Chain

GAPDH = Glyceraldehyde-3-phosphate dehydrogenase

LC/MS/MS = Liquid Chromatography Tandem Mass Spectrometry

MPEFs = multiple protein expression forms

Key words:

- **Real-time PCR array**
- **Mitochondrial dysfunction**
- **Obesity**
- **Colon**

Seven figures and two tables

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Abstract

Epidemiological studies report obesity to be an important risk factor influencing colon pathologies, yet mechanism(s) are unknown. Recent studies have shown significant elevation of insulin, leptin and triglycerides associated with increased adipose tissue. In situ hybridisation studies have located insulin, leptin and adiponectin receptor expression in the colon epithelia. The influence of increased adiposity and associated deregulation of insulin and adipokines on regulation of the colon epithelium is unknown. Altered adipokine and insulin signalling associated with obesity has an impact on mitochondrial function and mitochondrial dysfunction is increasingly recognised as a contributing factor in many diseases. Proteomics and transcriptomics are potentially powerful methods useful in elucidating the mechanisms whereby obesity increases risk of colon diseases as observed epidemiologically. This study investigated colon mitochondrial-associated protein profiles and corresponding gene expression in colon in response to increased adiposity in a rat model of diet induced obesity. Increased adiposity in diet-induced obese sensitive rats was found to be associated with altered protein expression of 69 mitochondrial-associated proteins involved in processes associated with calcium binding, protein folding, energy metabolism, electron transport chain, structural proteins, protein synthesis and degradation, redox regulation, and transport. The changes in these mitochondrial protein profiles were not correlated with changes at the gene expression level assessed using real-time PCR arrays.

Introduction

Epidemiological studies report obesity to be an important risk factor influencing gastrointestinal and colon pathologies (1-3) but the mechanism(s) are unknown. A recent report by the World Cancer Research Fund and the American Institute for Cancer Research has identified obesity as a primary risk factor in large bowel cancer (4). Recent studies have found obesity to be associated with colon pathologies, alterations in metabolism, insulin signalling and in adipokines levels (5-7) promoting speculation on the role of insulin and adipokine signalling in colon pathology. Recent studies in our lab have shown significant elevation of insulin, leptin and triglycerides associated with increased adipose tissue in a rat model of diet induced obesity (8). Furthermore, in situ hybridisation studies have located insulin, leptin and adiponectin receptor expression in the colon epithelia (8). The influence of increased adiposity and associated deregulation of insulin and adipokines on regulation of the colon epithelium is unknown. Obesity is also associated with a low level of chronic oxidative stress. Previously, proteomic analysis revealed altered regulation of redox signalling and mitochondrial functions in the colon in response to oxidative stress (9). Altered adipokine and insulin signalling associated with obesity has an impact on mitochondrial function (10) and mitochondrial dysfunction is increasingly recognised as a contributing factor in many human diseases including diabetes, metabolic disease and cancer (11).

Mitochondria are intracellular double membrane-bound structures that regulate energy metabolism, cell division and cell death (12). Consequently mitochondrial dysfunction, damage and mutations of mitochondrial proteins give rise to a range of ill understood patterns of disease (11). Recently published proteins associated with the rat colon mitochondria highlight the diverse functions of mitochondria in the colon (13). Studies investigating colon pathologies using proteomics approaches have revealed changes to mitochondrial proteins in response to oxidative stress (9), inflammation (14) and colon disease (15-16) but none have investigated the effect of obesity. The diet induced obesity rat model (DIO) provides a useful tool to investigate obesity in a polygenic environment (17-18). These rats have a propensity to increased obesity at a range of body weights much like humans. Increased energy intake by the diet induced obese sensitive (DIOS) rats leads to weight gain, and consequent alterations in biochemistry, metabolism, insulin and adipokine levels (8).

The application of organelle proteomics and parallel gene expression analysis is a potentially powerful method to elucidate the mechanisms whereby obesity increases risk of colon pathologies as observed epidemiologically. Previous studies reported extensive characterisation of mitochondrial-associated proteins extracted from rat colon (13) and a number of the identified mitochondrial proteins are regulated at gene transcription level (19). This study further investigates colon mitochondrial-associated protein profiles and parallel investigation of corresponding gene expression in colon in response to increased adiposity in a rat model of diet induced obesity (DIO).

Methods

Animals and Experimental Protocol

All procedures were licensed according to the Animals (Scientific Procedures) Act of 1986 and subject to approval by the Rowett Research Institute's Ethical Review Committee. Colons and plasma were procured from male out bred Sprague-Dawley rats (n=8) fed a high-energy diet (Research Diet D12266B, New Brunswick, NJ) as described in the previously reported study (8). Briefly, after two weeks of high-energy diet, rats were selected from the lowest (DIOR) and highest (DIOS) quartile of body weight for levels of obesity. All selected rats were maintained on the high-energy diet. After 10 weeks of feeding the high-energy diet and prior to sacrifice, fat mass was measured by Nuclear Magnetic Resonance using the EchoMRI 2004 Whole Body Composition Analyzer System (Echo Medical Systems, Houston, USA). After 23-24 weeks of feeding the high-energy diet, rats were euthanased with isoflurane to provide colons for mitochondrial proteomic analysis and RNA extraction for semi-quantitative real-time PCR. Colons were excised from the caecum at the ileo-caecal valve and rinsed with sucrose tris buffer to remove contents. Colon length measurements were recorded and a 2cm segment was dissected from the mid-point of the distal two thirds of the colon for mitochondrial extraction (13).

Circulating hormones and metabolites

Plasma leptin (Linco RL-83K), insulin (Linco RL-13K) and adiponectin (Linco MADP-60HK) were measured using commercially available rat/mouse-specific radioimmunoassay kits (Linco BioGenesis, Poole, UK) as described previously (8). Plasma glucose, triglycerides and non-esterified fatty acids were determined as previous (18) using the fully automated KONE analyser (20-21). Lipid peroxidation products were estimated in plasma as thiobarbituric acid reactive substances (TBARS) using HPLC and fluorimetric detection as previously described (22).

Proteomics of mitochondrial enriched fractions

Mitochondria-enriched extracts were prepared using a mitochondrial isolation kit (MITO-ISO1, Sigma, UK) according to the manufacturer's instructions as described and optimized for Sprague-Dawley rats for proteomic analysis previously (13). Tissues were minced immediately prior to homogenization then centrifuged twice for 5 minutes at 600 x g at 4C to remove debris and at 10000 x g to pellet mitochondria. Mitochondrial extracts were previously assessed for membrane integrity using transmission electron microscopy and JC-1 stain with flow cytometry (13).

150µg of mitochondrial-enriched protein extracts (as estimated by γ -globulin standard and a BioRad Bradford assay) were loaded onto BioRad IPG strips (17cm, pH 3-10) in 340µl of 7M urea, 2M thiourea, 4% Chaps, 2% biolyte (BioRad) and 3% DTT buffer to separate the proteins in the first dimension. A second dimension SDS-PAGE step was run on an 18x18cm linear SDS polyacrylamide gradient as described previously (9). The gels were then stained with colloidal Coomassie Blue staining as described by Anderson (23). Gels (n=16) were imaged on a BioRad GS710 flat bed imager followed by image analysis using BioRad PD Quest Version 7.1.1. The gel with highest spot number and quality was selected as the match set standard. Statistically significant altered spots were trypsin digested and identified by Nano LC/MS/MS as previously described (13). The presence of multiple protein expression forms (MPEFs) associated with the

identified proteins was assessed by reference to a previously published profile of rat colon mitochondrial-associated proteins (13).

RNA extraction

RNA was extracted from 0.5cm distal colon segments adjacent to that used for proteomic analysis using an RNeasy Midi Kit (Qiagen, Crawley, UK), incorporating DNase digestion. All RNA samples were subjected to analysis using a NanoDrop Spectrophotometer (NanoDrop Technologies, LabTech International, UK) for quantification and the Agilent Bioanalyser (Agilent Technologies, Bracknell, UK) to ensure quality was sufficient for semi-quantitative gene expression analysis.

Real-time PCR arrays

Total RNA (1ug) was reverse transcribed using an RT PCR Array First Strand kit (Tebu-Bio, UK) according to the manufacturer's instructions. A custom designed RT Profiler PCR Array (SuperArray Bioscience Corporation) was used to assess comparative gene expression of 44 target genes (Table 1) encoding the mitochondrial proteins showing altered expression patterns in response to increased adiposity. Three targets were excluded because primers were unavailable from SuperArray. Due to plate layout constraints, it was necessary to omit one further target hence calmodulin was also not analysed by PCR array. Two reference genes commonly used in real-time PCR studies were selected, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine guanine phosphoribosyl transferase (HPRT). GAPDH was selected since evidence from previous studies indicates absence of significant differences in GAPDH gene expression in rat colon tissue in response to disease (24). HPRT was also selected since this provides normalised expression data for cross-referencing results normalised to an alternative transcript, as advised by Ropenga *et al.*, (25). The design incorporated each target and reference genes in technical duplicates on each 96-well PCR array plate. Controls consisted of template RNA processed for PCR without the addition of reverse transcriptase to assess non-specific amplification and ensure that subsequent threshold cycles were assessed from specific amplification of gene targets and a synthetic RNA control to ensure efficient levels of reverse transcription are achieved. A melt curve programme was run immediately following the cycling programme to establish the absence of non-specific amplification products. Amplicons generated on the PCR array plates were also size checked by electrophoresis using 1% NuSieve GTG agarose (Cambrex, UK) gel and tri-acetate buffer with a Hyperladder V DNA ladder (Bioline, UK). A two-step thermocycling programme was performed using the ABI 7500Fast (Applied Biosystems) using SuperArray SYBR green master mix (Tebu-Bio, UK) formulated for use with the ABI platform. Cycling consisted of an initial step of 10 minutes at 95°C to activate the HotStart DNA polymerase, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The threshold cycle number (C_t) was measured using the ABI7500Fast and its associated software (Applied Biosystems). The PCR efficiency of each gene on the arrays was assessed and verified by the manufacturer (SuperArray, USA). The C_t values were obtained from linear amplified regions of the PCR profiles and exported to an Excel spreadsheet. Relative transcript levels were calculated for each reaction using the $\Delta\Delta C_t$ method with GAPDH and HPRT as the reference genes. Subsequent fold expression changes between the DIOR and DIOS groups relative to GAPDH and HPRT were calculated from the $\Delta\Delta C_t$ values.

Statistical Analysis

Student's t-test ($p < 0.05$) was carried out on all measured indices of obesity, logged proteomic data and ΔC_t real time PCR data. Principal component analysis was carried out on all resolved proteins; whilst partial least squares analysis was carried out on 69 significantly altered proteins together with the measured indices of obesity. A randomisation test on PLS analysis data revealed PLS plot was not a random result.

Results

Body weight and fat mass analysis

Body weights of the DIOR and DIOS rats (n=8) were significantly ($p < 0.05$) different between the groups throughout the experiment (Fig 1). Fat masses were also significantly different between the DIOR and DIOS groups (Table 2) and were significantly correlated with body weights $r^2 = 0.6534$, $p < 0.01$ level (2 tail test).

Circulating hormones and metabolites

Significant ($p < 0.05$) increases in circulating hormones (Table 2), leptin and insulin and triglycerides were measured in DIOS rats (n=8). There was no significant difference in adiponectin, glucose, NEFA or TBARS between DIOR and DIOS groups (data not shown).

Proteomics of colon mitochondrial enriched fraction

Seven hundred and fifty mitochondrial-associated proteins were resolved on 2D gels (n=8). Principal Component Analysis (PCA) is often used to reduce multidimensional data sets, such as those from proteomics and transcriptomics, to a simplified graph revealing the sometimes hidden simplified structure that underlie the data. Principal component analysis (Fig 2A) applied to all 750 resolved spots revealed increased adiposity was associated with altered profiles of colon mitochondrial-associated proteins with 16% of the variation most significantly correlated with body weight ($p < 0.01$). Student t-test of logged protein intensity data revealed 174 significantly altered spots ($p < 0.05$) that were subjected to visual inspection to ensure the spot was present on at least 4 replicate gels and to eliminate those of insufficient quantity for downstream analysis. This resulted in the final selection of a subset of 69 spots ranging in pI from 4.1 to 9.68 with a size range from 15.2 to 116.5 kDa that were excised and subject to LC/MS/MS analysis (Table 1). Partial Least Squares (PLS) analysis specifies the linear relationship between variables e.g. body mass and a particular protein. This is a useful tool identifying proteins for further investigation. PLS analysis (Fig 2B) of the 69 spots with the hormone, biochemical and body composition data (see above) identified potential relationships between the significantly altered mitochondrial-associated proteins and indices of obesity. Twenty-seven proteins were positively associated with body adiposity e.g. dnaK-type molecular chaperone hsc73 (Fig 2B, spot 3721), whilst 42 proteins (in the shaded area of Fig 2B) such as voltage-dependant anion selective channel protein 1 (Fig 2B, spot 8211) were inversely associated. However, measured indices of obesity were not associated with a particular functional group of modulated proteins.

LC/MS/MS of the 69 altered proteins identified proteins involved in redox regulation, calcium binding, energy metabolism, electron transport chain, protein synthesis and degradation, transport, structural proteins and protein folding (Table 1, Fig 3-7). Identified proteins (Table 1) were compared against proteins identified by Padidar *et al.*, (13) to establish the presence of MPEFs. This revealed that 48 out of the 69 significantly altered proteins were associated with one or more previously identified MPEFs in the rat mitochondrial-enriched extracts (Table 1). There was an overall down-regulation of MPEFs of proteins involved in energy metabolism (Fig 4A), electron transport chain (Fig 4B), protein folding (Fig 5A) and structural proteins (Fig 6A) with increased adiposity. The calcium binding protein calmodulin had altered expression profiles with a change in protein mass in colon from DIOS rats (Fig 7A-C), highlighting the importance of post-translational modifications to proteins.

Real-time PCR array

Real-time PCR array performed on 44 of the 48 genes encoding for the significantly altered mitochondrial-associated proteins by proteomics (listed in Table 1) were amplified successfully, yielding single amplified products of the correct size (data not shown). Fold expression changes of the selected nuclear-encoded mitochondrial target genes (listed in Table 1) from total RNA extracted from colon segments adjacent to that used for proteomic analysis of DIOR and DIOS rats were calculated relative to the reference genes GAPDH and HPRT. Despite alteration in the expression patterns of the proteins encoded by these gene targets in response to increased adiposity there was no evidence of significant altered regulation at the gene transcription level (data not shown).

Discussion

To our knowledge this is the first study to investigate the impact of increased adiposity on colon mitochondrial-associated proteins and their corresponding encoding genes. Employing the diet induced obesity rat model of obesity (17-18, 26), the DIOR and DIOS rats fed a high-energy diet have significant differences in body weight (Fig 1) and fat mass (Table 2) (8). This increased fat mass in DIOS rats was associated with significant elevation of obesity markers, leptin, insulin, and triglycerides (Table 2) (8), all linked to an increase risk of colon cancer (27-28). The elevated fat mass in DIOS rats did not result in significantly altered plasma adiponectin levels counter to reports of reduced plasma adiponectin with increased obesity (29).

Proteomics of mitochondrial-associated proteins

Sixty-nine mitochondrial-associated protein profiles were significantly altered as a result of increased adiposity in DIOS rats, compared with DIOR. Twenty-seven proteins were associated with increased adiposity and body weight (Fig 2B). Altered proteins were involved in calcium binding, protein folding, energy metabolism, electron transport chain, structural proteins, protein synthesis and degradation, redox regulation, and transport (Table 1, Figs 3-7), reflecting the broad functional groups of proteins previously found in colon mitochondria (13). Sixty one percent of the colon mitochondrial-associated proteins were down regulated in the DIOS rats. Principal Component Analysis (PCA) is often used to reduce multidimensional data sets, such as those from proteomics and transcriptomics, to a simplified graph revealing the sometimes hidden simplified structure that underlie the data. The PCA plot (Fig 2A) shows graphically there is a significant difference between the protein profiles of DIOR and DIOS rats, which was found correlating most significantly with body weight.

Energy Metabolism and Electron Transport Chain

Forty two percent of the colon mitochondrial-associated proteins modulated in DIOS rats were involved in the electron transport chain (ETC) and energy metabolism (Fig 4), indicative of a major function of mitochondria. Over half of these were down regulated in the DIOS rat (Fig 4A-B) suggesting a decrease in ATP production with increased adiposity, supporting observations of decreased muscle mitochondrial ATP production in rats fed a high-energy diet independent of diet composition (30). A multiple protein expression form (MPEF) of ATP synthase beta chain (Fig 4B, spot 1505) and electron transfer flavoprotein alpha (Fig 4A, spot 6111), down regulated in DIOS rats, have also previously been found to be down regulated in oxidative stress (9) and aged human colonic epithelium (31). Down-regulation of ATP synthase beta subunit in skeletal muscle of patients with type-2 diabetes is thought to be due to phosphorylation of this subunit, therefore altering the regulation of ATP synthesis and contributing to the pathogenesis of type-2 diabetes, a disease significantly associated with obesity (32) and colon cancer (33). Creatine kinase precursor was up regulated in the DIOS group (Fig 4A, spot 6414). Creatine kinases are normally found in cells with a high or fluctuating energy demands by shuttling 'high-energy' phosphates formed in mitochondria and transporting to the cytosol (34-35). Mitochondrial creatine kinase (EC 2.7.3.2) is located in the intermembrane compartment (36) and uses ATP to form phosphocreatine (37), which is transported to the cytosol using voltage-dependent anion channel (VDAC) (38-39) for the phosphorylation of ADP to ATP by cytosolic creatine kinase (34-35). Up regulation of mitochondrial creatine kinase precursor in the DIOS group may be a compensatory response to down regulation of ETC and VDAC1 proteins thus augmenting a decrease in ATP production in the DIOS group.

Protein Folding and Transport

Nine significantly modulated proteins were involved in transport and protein folding; seven of these proteins were down regulated in the DIOS rats (Fig 5). VDAC 1 (Fig 5B, spot 8211), forms a pore on the outer mitochondrial membrane, allowing the passage of small ionic and hydrophilic molecules e.g. ATP, into the membrane, in a calcium dependent manner. VDAC is a candidate component of the permeability transition pore, thought to participate in both apoptotic and necrotic cell deaths (40-41). VDAC1 has been shown to co-immunoprecipitate with the anti-apoptotic protein Bcl-2 and has been suggested to be involved in forming the pore that releases cytochrome c during apoptosis (42). Previous profiling of the colon mitochondrial-associated proteins of the rat colon has revealed three MPEFs of VDAC1 (13). This study found one of these MPEFs to be down regulated in the DIOS group. Since apoptosis requires the maintenance of >65% of normal ATP concentration in the cell and a severe or prolonged decrease in ATP concentration (<20%) leads to necrosis (43-44), it is possible that any decreased ATP level and down regulation of VDAC1 and ETC proteins, associated with increased adiposity, could lead to a delay in mitochondrial-coordinated apoptotic processes. Apoptosis is a key event in colon epithelial regulation where stem cells originating at the base of the crypt are thought to differentiate and migrate up the crypt length, followed by apoptosis and sloughing off into the luminal tract (reviewed in 45). Changes to apoptotic events may give rise to aberrant crypt foci, potentially an early stage marker of colon cancer histology (reviewed in 45).

Many proteins require an association with one or more types of molecular chaperones, during their synthesis, transport, folding and degradation within cells. Many expression forms of these proteins were found down regulated in DIOS rats. The heat shock proteins (Hsp) are a family of proteins that facilitate the folding of proteins (46). Hsp60 (Fig 5A, spot 1508), down regulated in the DIOS group, is an essential mitochondrial chaperone and promotes the folding of several proteins imported into the mitochondrial matrix (47-48). It may also prevent misfolding and promote the refolding and proper assembly of unfolded polypeptides generated under stress conditions in the mitochondrial matrix (49). Expression forms of HSC73 (Fig 5A, spot 3706, 3721) were found modulated in DIOS compared with DIOR rats, with a change in mass, similar to calmodulin (see below *Calcium Binding*). It is a member of the hsp70 protein family and is involved with the uncoating of clathrin-coated vesicles (50) and transport of cytoplasmic proteins to lysosomes for degradation (51). Alterations in these chaperone proteins may lead to misfolding and transport of proteins with downstream consequences.

Structural Proteins

Twenty three percent of the modulated colon mitochondrial-associated proteins were structural proteins (Fig 6A); with over half of these down regulated in DIOS rats. A down regulation of some MPEFs of actin beta (Fig 6A, spots 1803, 2211 and 2212) was found in DIOS rats, suggesting alterations in normal patterns of cell death (52). Modulation of structural proteins such as actin beta and keratins also highlight the close interaction of mitochondria with microtubules and actin filaments in their association, transport and positioning within cells (53-54). This implies that the proteins associated with mitochondrial-enriched fractions may be linked to normal functioning of mitochondria in the colon. For example, desmin (Fig 6A, spots 1304 and 1318) serves an important role in the maintenance of cellular integrity of muscle tissues (55-56). In desmin-null mice, mitochondrial abnormalities are the first visible defect, including loss of shape and

positioning, proliferation, aggregation, swelling, degeneration, diminished ADP-stimulated respiratory function and loss of creatine kinase coupling (reviewed in 54). This study has found changes to the desmin profile in the colons of DIOR and DIOS rats, thus supporting the hypothesis that obesity influences mitochondrial function in colonic tissue that may have implications for colon health.

Calcium Binding

Proteins involved in calcium binding (Fig 7C) such as calmodulin and calreticulin have been previously found modulated in colon cancer (57-58). The modulation of these proteins reflects the intimate relationship between mitochondria and other vesicular membranes such as the endoplasmic reticulum for calcium homeostasis (59). These proteins may also work closely with the structural proteins such as actins in exo- and endocytosis (60). The down regulation of 3 out of 4 significantly altered calcium binding proteins with increased adiposity suggests effects on calcium homeostasis known to regulate a number of cell processes including cell death. Calmodulin was found in a different location in DIOR and DIOS gels (Fig 7A-C, spots 10 and 107). An increase in molecular weight by 0.4kDa (15.64 and 16.04) and no change in pI (4.1) in the DIOS gels (Fig 7B), suggested a post-translational modification, such as phosphorylation as induced by insulin (61).

Redox regulation

There was a down regulation of some MPEFs of redox proteins such as superoxide dismutase and peroxiredoxin 2 (Fig 6C) in DIOS rats. MPEFs of these proteins were identified from the previous study profiling mitochondrial-associated proteins from rat colon (13). In the case of superoxide dismutase, three MPEFs were identified by Padidar *et al.*, (13) and one of these was significantly down regulated in the DIOS rats. Down regulation of redox proteins correlates with increased oxidative stress seen with chronic obesity (62), however, the DIOS rats did not have significantly increased plasma oxidative stress as measured by TBARS (data not shown). This may reflect a lack of sensitivity in the TBARS assay applied in this particular rat model as opposed to other rodent models studies investigating oxidative stress and application of the TBARS assay (9). Analysis of enzyme activity of superoxide dismutase using established assays (63) also did not reveal significant differences in activity associated with increased adiposity (unpublished observation). It may be that although one particular MPEF was down-regulated the overall level of total protein may not have significantly altered.

In summary, the changes to the colon mitochondrial-associated protein profiles associated with increased adiposity suggest a decrease in ATP production, apoptosis, protein folding and mitochondrial positioning. Colons taken from DIOR and DIOS rats do not show gross differences in morphology on histological analysis (8). The altered mitochondrial-associated protein profile changes observed in this study may provide clues to mitochondrial-regulated functions in the colons of obese subjects preceding colon pathologies (1-3).

Gene Expression Of Modulated Proteins

The MPEFs generated by post-translational modification, observed frequently as a consequence of proteomic analysis, are often the product of a single gene. A transcriptomic approach was employed here to further investigate regulation of the colon mitochondrial-associated proteins since it is known that many mitochondrial proteins are also regulated at the gene transcription level (for review see 34). Real-time PCR is costly and labour intensive and selecting targets from the numerous proteins identified from

proteomic analysis presents a dilemma. The RT PCR array approach adopted here presents a solution, allowing custom design and rapid screening of numerous targets simultaneously. Forty-four target genes corresponding to the majority (85%) of the identified mitochondrial proteins showing altered expression patterns were successfully assessed at the gene transcription level using a custom designed PCR array approach. There are many studies in the literature that have found a poor correlation between microarray data and proteomic data (64) suggesting various levels of regulation during protein synthesis e.g. translational, post-translational and post-transcriptional regulation. The lack of robust changes at the gene transcription level in this study is also perhaps indicative of the ability of the mitochondria to regulate homeostatic functions in response to increased adiposity at the post-transcription level. Notably, both reference genes gave similar results and increase confidence in the use of these two transcripts for further semi-quantitative gene expression studies in colon. Potentially the altered protein expressions observed may lead to altered regulation of downstream gene targets not present on the PCR array.

The mechanisms and genotype that cause the phenotypic differences between DIOR and DIOS rats have not yet been elucidated. Previous studies have found DIOS rats consume approximately 25% more energy (personal communication, Zoe Archer), changes to hypothalamic gene expression (reviewed in 65) and increased hepatic acetyl CoA carboxylase (66) This study found DIOS rats with a propensity to increased body weight to have an associated altered colon mitochondrial protein profile and biochemistry Since the Sprague Dawley strain used in this study is out bred it is considered unlikely that the altered mitochondrial profiles are due to a single genetic mutation. However, it this study does not rule out the possibility that there may be genotypic factors that contribute to the altered profiles observed.

Summary

Increased adiposity in DIOS rats was associated with altered protein expression of 69 mitochondrial-associated proteins involved in processes associated with calcium binding, protein folding, energy metabolism, electron transport chain, structural proteins, protein synthesis and degradation, redox regulation, and transport. Although this investigation did not find changes to mitochondrial protein profiles to correlate changes to gene expression, real-time PCR arrays were found to be a successful, high throughput and valid approach in investigating proteomic and gene expression in parallel.

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Table 1: Mitochondrial-associated proteins significantly altered with increased adiposity.

Spot No	Protein ID	Accession No	Gene	Mr/pI of spot	MPEF	Functional Group
10	Calmodulin	3CLN		15.64/4.1	2	CB
107	Calmodulin	3CLN		16.04/4.1	2	CB
802	Similar to protein kinase C substrate 80K-H	Q921X2	Prkcs_h_predicted	80.31/4.8	0	CB
604	calreticulin precursor	JH0819	Calr	51.11/4.88	3	CB & PF
2808	NADH-ubiquinone oxidoreductase 75 kDa subunit	Q8BM16	Ndufs1	77.89/6.00	2	EM
3404	NAD ⁺ -specific isocitrate dehydrogenase a-subunit	Q99NA5	Idh3a	39.03/6.32	4	EM
3405	NAD ⁺ -specific isocitrate dehydrogenase a-subunit	Q99NA5	Idh3a	38.69/6.48	4	EM
3611	Mitochondrial aldehyde dehydrogenase	Q8K3V8	Aldh2	51.26/6.44	2	EM
3617	Mitochondrial aldehyde dehydrogenase	Q8K3V8	Aldh2	52.46/6.59	2	EM
4702	glycerol-3-phosphate dehydrogenase (EC 1.1.99.5), mitochondrial precursor	A54051	Gpd2	67.44/6.73	1	EM
4721	Similar to methylmalonyl-coenzyme A mutase	Q8VED0	Mut	72.83/6.78	0	EM
5409	isovaleryl-CoA dehydrogenase (EC 1.3.99.10) precursor	C34252	Ivd	40.48/6.9	2	EM
6111	electron transfer flavoprotein alpha chain precursor	A31568	Etf_a	23.69/7.98	1	EM
6414	creatine kinase (EC 2.7.3.2) precursor, mitochondrial	S17189	Ckmt1	41.6/7.87	5	EM
6501	glutamate dehydrogenase [NAD(P)] (EC 1.4.1.3) precursor	S03707	Glud1	49.52/7.44	4	EM
6504	glutamate dehydrogenase [NAD(P)] (EC 1.4.1.3) precursor	S03707	Glud1	49.25/7.63	4	EM
1505	ATP synthase beta chain, mitochondrial precursor (EC 3.6.3.14)	ATPB_RAT	Atp5b	48.4/5.47	13	ETC
1510	ATP synthase beta chain, mitochondrial precursor (EC 3.6.3.14)	ATPB_RAT	Atp5b	48.98/5.7	13	ETC
1607	ATP synthase beta chain, mitochondrial precursor (EC 3.6.3.14)	ATPB_RAT	Atp5b	55.29/5.51	13	ETC
2003	cytochrome c oxidase subunit vb	BAA01743	Cox5b	15.2/6.04	1	ETC
2108	ATP synthase D chain, mitochondrial* (EC 3.6.3.14)	ATPQ_RAT	Atp5h	21.67/6.13	2	ETC
2213	F1-atpase beta chain	1MABB	Atp5b	27.71/5.81	13	ETC
2508	ubiquinol cytochrome c reductase	BAB27022	Uqcrc1	46.93/5.94	1	ETC
3108	ATP synthase D chain, mitochondrial* (EC 3.6.3.14)	ATPQ_RAT	Atp5h	22.72/6.16	2	ETC
3618	DLST dihydrolipoamide succinyltransferase	Q8CJG5	Dlst	53.13/6.6	4	ETC
3619	DLST dihydrolipoamide succinyltransferase	Q8CJG5	Dlst	52.89/6.45	4	ETC
3710	dihydrolipoamide S-acetyltransferase* (EC 2.3.1.12)	S21766	Dlat	63.97/6.36	4	ETC
4402	NADH dehydrogenase 1 alpha subcomplex 10-like protein	Q80WE0	Ndufa10	40.39/6.74	3	ETC
4406	NADH dehydrogenase 1 alpha subcomplex 10-like protein*	Q80WE0	Ndufa10	39.44/6.91	3	ETC
6108	ubiquinol-cytochrome-c reductase (EC 1.10.2.2) Rieske iron-sulfur protein precursor	A32296	Uqcrcf1	24.79/7.88	1	ETC
6202	cytochrome c1	BAB22380	Cyc1_predicted	29.43/7.44	1	ETC
6623	dihydrolipoamide dehydrogenase (e3 component)	AAH62069	Dld	52.57/7.44	4	ETC
8215	DLST dihydrolipoamide succinyltransferase	Q8CJG5	Dlst	27.76/9.56	4	ETC
319	Class II histocompatibility antigen RT1-D alpha(u) chain precursor	S06316	RT1-Da	30.16/4.93	0	Other
1320	SWIPROSIN 1	Q8C845	Efh2	31.07/5.52	0	Other
4304	Aflatoxin B1 aldehyde reductase member 2	AR72_RAT	Akr7a2	35.77/6.92	0	Other

4605	Hypothetical protein	Q8BH86		61.7/6.83	0	Other
1508	chaperonin groEL precursor	HHRT60	Hspd1	49.9/5.69	4	PF
1707	dnaK-type molecular chaperone precursor	HHRTGB	Hspa5	72.57/5.53	5	PF
1709	dnaK-type molecular chaperone precursor	HHRTGB	Hspa5	71.92/5.58	5	PF
3706	dnaK-type molecular chaperone hsc73	S07197	Hspa8	65.81/6.25	3	PF
3709	dnaK-type molecular chaperone grp75 precursor	I56581	Hspa9a_p redicted	67.45/6.32	5	PF
Spot No	Protein ID	Accession No	Gene	Mr/pI of spot	MPEF	Functional Group
3721	dnaK-type molecular chaperone hsc73	S07197	Hspa8	66.73/6.26	3	PF
2112	Phosphatidylethanolamine-binding protein	PEBP_RAT		22.55/5.99	0	PSD
3103	Mitochondrial Lon protease	Q924S5	Prss15	25.82/6.35	1	PSD
3109	Serine carboxypeptidase 1	AAK84661	Scpep1	18.07/6.22	0	PSD
3720	neurolysin (metallopeptidase m3 family)	CAA60630	Nln	72.79/6.61	1	PSD
5705	methycrotonoyl-coenzyme a carboxylase 1 alpha	AAH21382	Mccc1	74.69/7.15	1	PSD
7417	Branched chain aminotransferase 2	AAH61790	Bcat2	39.37/8.12	2	PSD
2110	thiol-specific antioxidant peroxiredoxin 2	A57716	Prdx2	21.43/5.84	1	Redox
7112	superoxide dismutase (EC 1.15.1.1) (Mn) precursor	DSRTN	Sod2	23.16/8.79	3	Redox
3104	RhoA precursor	BAC36896	Rhoa	25.32/6.53	0	Structural
5702	WD repeat domain 1	Q9DBM8		63.74/7.09	0	Structural
1304	Desmin	DESM_RAT	Des	34.81/5.28	6	Structural
1311	Keratin, type II cytoskeletal 8	K2C8_RAT	Krt2-8	36.29/5.58	12	Structural
1318	Desmin	DESM_RAT	Des	35.66/5.3	6	Structural
1419	keratin 21, type I, cytoskeletal	A40452	Krt20	39.55/5.38	2	Structural
1421	keratin 21, type I, cytoskeletal	A40452	Krt20	39.56/5.26	2	Structural
1507	Keratin, type II cytoskeletal 8	K2C8_RAT	Krt2-8	43.28/5.58	12	Structural
1803	Actin beta	ATRTC	Actb	78.86/5.44	9	Structural
1813	Filamin A	FLNA_MOUSE		78.7/5.52	0	Structural
2211	Actin beta	ATRTC	Actb	27.5/5.95	9	Structural
2212	Actin beta	ATRTC	Actb	25.72/5.69	9	Structural
2511	Actin gamma 1	ATHUG		43.46/5.94	0	Structural
2814	spectrin alpha	AAC33127	Spna2	116.5/5.81	1	Structural
3411	Lamin A	LAMA_RAT	Lmna	39.88/6.37	0	Structural
5605	Ezrin	Q8VHK3	Vil2	57.78/7.18	3	Structural
2116	GTP-binding protein Rab1	TVRTYP	Rab1	21.31/5.99	0	Transport
8211	Voltage-dependent anion-selective channel protein 1	POR1_RAT	Vdac1	30.05/9.68	3	Transport

CB = Calcium binding, EM = energy metabolism, ETC = electron transport chain, PF = protein folding, PSD = protein synthesis and degradation. Proteins in bold represent those significantly down regulated in DIOS rats ($p < 0.05$). Genes for the 44 targets investigated using real-time PCR array are indicated. Known MPEFs identified previously by Padidar *et al.*, (13)

Table 2 Significantly altered fat mass and plasma leptin, insulin and triglyceride levels in DIOR and DIOS rats.

	DIOR	DIOS
Fat mass (g)	61.2 (95% CI: 48.9-73.6)	121.2 (95% CI: 105.4-136.9)
Plasma leptin (ng/ml)	14.5 (95% CI: 11.2-17.8)	20.2 (95% CI: 17.1-23.4)
Plasma insulin (ng/ml)	1.45 (95% CI: 0.82-2.08)	1.96 (95% CI: 1.25-2.66)
Plasma triglycerides (mmol/l)	2.001 (95% CI: 1.08-2.80)	2.69 (95% CI: 1.88-3.79)

Body fat mass was significantly elevated ($p < 0.01$) in DIOS rats ($n=8$) Data for body fat mass prior to sacrifice is shown. Plasma leptin, insulin and triglycerides were significantly ($p < 0.05$) elevated in DIOS rats ($n=8$).







