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Label-free profiling of white adipose tissue of rats exhibiting high or low levels of intrinsic exercise capacity.

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Abstract

Divergent selection has created rat phenotypes of high- and low-capacity runners (HCR and LCR, respectively) that have differences in aerobic capacity and correlated traits such as adiposity. We analysed visceral adipose tissue of HCR and LCR using label-free HDMS^E profiling. The running capacity of HCR was 9-fold greater than LCR. Proteome profiling encompassed 448 proteins and detected 30 significant ($p < 0.05$; false discovery rate <10%, calculated using q-values) differences. Approximately half of the proteins analysed were of mitochondrial origin, but there were no significant differences in the abundance of proteins involved in aerobic metabolism. Instead, adipose tissue of LCR rats exhibited greater abundances of proteins associated with adipogenesis (e.g. cathepsin D), endoplasmic reticulum stress (e.g. 78 kDa glucose response protein) and inflammation (e.g. Ig gamma-2B chain C region). Whereas the abundance anti-oxidant enzymes such as superoxide dismutase [Cu-Zn] was greater in HCR tissue. Putative adipokines were also detected, in particular protein S100-B, was 431 % more abundant in LCR adipose tissue. These findings reveal low running capacity is associated with a pathological profile in visceral adipose tissue proteome despite no detectable differences in mitochondrial protein abundance.

Introduction

Worldwide the levels of obesity have almost doubled since 1980 and approximately 3.4 million adults die each year as a result of being overweight or obese. In particular, abdominal obesity is associated with a heightened risk of cardiovascular disease and type 2 diabetes mellitus (T2DM) and is a cardinal feature of the metabolic syndrome. Indeed, almost half of the worldwide diabetes burden and one-quarter of the ischaemic heart disease burden is attributable to overweight and obesity.

Diet-induced models of obesity are common as are targeted and spontaneous genetic models such as the Zucker fatty rat [1]. However, in addition to diet and energy intake, physical activity and energy expenditure also impact on whole body energy balance and composition. We have used divergent artificial selection on running capacity to create high-capacity runner (HCR) and low-capacity runner (LCR) rats that have profound differences in running capacity and exhibit numerous correlated traits relevant to human disease. While HCR animals are relatively lean and have a low disease-risk profile, LCR rats are overweight [2], hypertensive [3], insulin resistant [4] and have a significantly shorter life expectancy [5]. Moreover, in response to a high fat diet, LCR are more prone to exacerbation of this insulin resistant condition whereas HCR are protected despite their low levels of habitual activity and relatively greater calorific intake [2].

Biochemical assessment of carcass composition [6] reveals the percentage body fat of adult LCR (~36 %) is more than double that (~16 %) of HCR. More specifically, the greater adiposity of LCR is due to the increased masses of retroperitoneal, omental and periretroproductive fat pads [7], and enlargement of such visceral adipose depots, in particular, is intimately associated with cardiometabolic disease risk [8]. In striated muscle,

the weight of evidence from our HCR/LCR model points to mechanistic links between low respiratory capacity and mitochondrial dysfunction associated with defects in contractile function and substrate metabolism. In contrast, recent examination of visceral adipose tissue of LCR/HCR [9] reported no major differences in mitochondrial enzyme content despite overt differences in running capacity and adipose tissue mass. It seems unlikely artificial selection has left adipose tissue unaffected but as yet the nature of the effect in adipose tissue is unknown at the molecular level.

Herein, we use label-free proteomic profiling as a non-targeted approach to discover differences between HCR and LCR. This approach is designed to highlight candidate biomarkers or direct future research regarding the greater adiposity and enhanced rate of adipogenesis of LCR in response to environmental stresses such as a high-fat diet. We used a quadrupole time-of-flight (Q-TOF) mass spectrometer operated in data-independent acquisition (DIA) mode, which affords more detailed detection of peak profiles and improves measurement precision compared to classic data-dependent acquisitions [10]. Relative expression data generated by this instrument was analysed using the “Hi-3” method wherein label-free quantitation is performed on the summed ion counts from the 3 most intense peptides from each protein normalised to an exogenous protein spiked in to each sample [11]. This approach affords robust differential analysis based on the normalised abundance of proteins and was performed on biological replicates of HCR and LCR animals.

Method

2.1 Animal model and tissue collection

The inception of HCR-LCR strains from a founder population of genetically heterogeneous N:NIH rats has been described in detail [12]. Animals were housed (two per cage) in accordance with the University of Michigan Committee guidelines on the use and care of animals. Environmental conditions were 20-21 ºC, 40-50 % relative humidity with a 12 h light (0600-1800) and dark cycle, and food and water were available ad libitum.

Rats from generation 26 of selection were used in the current work. Consistent with our standard selection procedures [12], HCR and LCR animals were phenotyped by their maximum running capacity at 10 wk of age. Briefly, endurance trials were performed on an inclined (15 degrees) motorised treadmill (Columbus Instruments, OH) on 5 consecutive days. The treadmill velocity began at 10 m/min and was increased by 1 m/min every 2 min until exhaustion. Exhaustion was operationally defined as the third time a rat could no longer keep pace with the speed of the treadmill and remained on the shock grid for 2 s. At this point the total distance (m) run was recorded. The single best distance of the 5 trials was considered as the performance indicator most closely associated with the heritable component of endurance running capacity. Other than the aforementioned phenotyping trials, rats used in the current report were sedentary and did not perform exercise training. Therefore, the difference in running capacity between HCR and LCR is primarily an innate response to selection.

Two weeks after completing the exercise trials male HCR and LCR rats (n = 6, of each) were weighed and anesthetised using sodium pentobarbital (60 mg/kg body mass).

Retroperitoneal fat pads were surgically excised then freeze-clamped in liquid nitrogen and stored at -80 °C as part of our routine tissue archiving procedure.

2.2 Sample preparation

Visceral fat samples (~100 mg) were homogenised on ice in 10 volumes of 1 % (v/v) Triton x-100, 250 mM sucrose, 100 mM NaCl, 5 mM EDTA, 25 mM Tris, pH 7.4, at 4 ºC, containing Complete protease inhibitor (Roche Diagnostics). After centrifugation at 12,000 g, 4 ºC for 45 min supernatants were decanted and an aliquot containing 100 µg protein precipitated in 5 volumes of acetone for 1 h at -20 ºC. Pellets were resuspended in 1% (w/v) SDS and incubated at 90 ºC for 30 min before being washed twice through 5 kDa molecular weight cut-off spin columns (Vivaspin; Sigma-Aldrich, Gillingham, UK) using 0.1% (w/v) Rapigest SF (Waters; Milford, MA) in 50 mM ammonium bicarbonate (500 µL per wash). Protein suspensions were concentrated to 50 µL then incubated at 80 ºC for 15 min. DTT was added (final concentration 1 mM) and incubated at 60 ºC for 15 min followed by incubation whilst protected from light in the presence of 5 mM iodoacetamide at 4 ºC. Sequencing grade trypsin (Promega; Madison, WI) was added at a protein ratio of 1:50 and digestion allowed to proceed at 37 ºC overnight. Digestion was terminated by the addition of 2 µL concentrated TFA and peptide solutions were cleared by centrifugation at 13 000 g for 5 min. Samples were diluted 1:1 with a tryptic digest of yeast alcohol dehydrogenase (100 fmol/ µL) to enable the amount of each identified protein to be quantified, as described previously using a “Hi 3” methodology [11].

2.3 HDMS^{E}

Tryptic peptide mixtures were analysed by nanoscale ultra-performance liquid chromatography (nanoACQUITY, Waters, Milford, MA) and online nano electrospray

ionisation (ESI) ion-mobility mass spectrometry ($HDMS^E$; SYNAPT G2-S, Waters, Manchester, UK). Samples (200 ng tryptic peptides) were loaded in aqueous 0.1% (v/v) formic acid via a Symmetry C_{18} 5 µm, 5 mm x 300 µm pre-column (Waters, Milford, MA). Separation was conducted at 35 ºC through a HSS T3 C_{18} 3 µm, 15 cm x 75 µm analytical reverse phase column (Waters, Milford, MA). Peptides were eluted using a gradient rising to 40 % acetonitrile 0.1% (v/v) formic acid over 90 min at a flow rate of 300 nL/min. Additionally, a Lockmass reference (100 fmol/ µL Glu-1-fibrinopeptide B) was delivered to the NanoLockSpray source of the mass spectrometer at a flow rate of 500 nL/ min, and was sampled at 60 s intervals.

For all measurements, the mass spectrometer was operated in a data-independent positive ESI mode at a resolution of >20,000 FWHM. Prior to analysis, the time of flight analyser was calibrated with a NaCsI mixture from m/z 50 to 1990. $HDMS^E$ analyses were conducted within the Triwave ion guide. Accumulated ions were separated according to their drift time characteristics in the N_2 gas-filled mobility cell prior to collision induced dissociation (CID) alternating between low (4 eV) and elevated (14-40 eV) collision energies at a scan speed of 0.9 s per function over 50-2000 m/z.

Each biological sample was analysed in replicate. Analytical data were LockMass corrected post-acquisition using the doubly charged monoisotopic ion of the Glu-1-fibrinopeptide B. Charge reduction and deconvolution of potential parent-fragment correlation was achieved in the first instance by means of retention and drift time alignment, as described previously [13]. Briefly, protein identifications and quantification information were extracted using the dedicated algorithms employed in ProteinLynx GlobalSERVER (PLGS) v2.5 (Waters, Milford, MA). Peak lists were searched against the Uniprot database (date 18th Jan 2012) restricted to ‘Rattus’ (7500 entries). The enzyme specificity was trypsin allowing one missed cleavage, carbamidomethyl modification of

cysteine (fixed), oxidation of methionine (variable) and the parent and fragment ion ppm error automatically calculated from the data. Decoy databases were employed to allow the calculation of identification error rates and scoring of the database searches was refined by correlation of physicochemical properties of fragmented peptides from theoretical and experimental data. Data were uploaded and checked in PRIDE converter and inspector [14]. Functional enrichment testing was performed using the Database for Annotation, Visualisation and Integrated Discovery (DAVID v6.7; [15, 16]) and protein interactions were investigated using the search tool for interacting genes/proteins (STRING v9.1; [17]).

Results

3.1 Exercise capacity of HCR and LCR rats

Proteome profiling was performed on retroperitoneal fat from male HCR and LCR of generation 26. The average running capacity of HCR (1926 ± 160 m) was ~9-fold greater ($P < 0.0001$) than LCR (216 ± 34 m), whereas the average body weight of LCR (329 ± 23 g) was ~50 % greater ($P < 0.0001$) than HCR (242 ± 13 g). Fat pad masses were not recorded during the tissue archiving procedure, but as a point of reference, Table 1 in Demarco et al., [7] reports the masses of retroperitoneal, perireproductive and omental fat depots in male and female HCR and LCR rats from generation 27 at 30 wk of age. Consistent with a number of earlier studies (e.g. [2, 18-21]), Demarco et al., [7] reports retroperitoneal mass in male LCR (11.14 ± 1.2 g) was more than double ($P < 0.005$) that of HCR (4.2 ± 0.5 g).

3.2 HDMS^E analysis of visceral fat proteins

HDMS^E analysis of replicate adipose tissue homogenates from 6 HCR and 6 LCR rats produced a non-redundant list of 1,023 proteins at a false positive rate of <4 %. This number is close to the 1,493 proteins identified in Xie et al., [22] using LC-MS/MS analysis of 20 molecular weight fractions (i.e. GeLC-MS/MS) of adipocytes isolated from human subcutaneous adipose tissue. Adachi et al., [23] reports a more comprehensive catalogue (total 3,287 proteins) of adipocyte proteins, which was achieved by fractionating 3T3-L1 adipocytes to nuclear, mitochondrial, membrane and cytosolic components followed by GeLC-MS/MS to culminate in a total of 45 fractions per sample. MS data of technical replicates of each HCR and LCR sample is available in the PRoteomics IDEntification

(PRIDE) database (www.ebi.ac.uk/pride/). Supplementary table S1 reports the identity and relative abundance of a subset of 448 proteins that were replicated in at least 4 out of the 6 samples from each group and were used in differential statistical analysis of the HCR and LCR groups. Table S1 also includes data extracted from Adachi et al., [23] reporting homologues of proteins identified in the mouse adipocyte proteome.

Based on the estimated protein abundance on-column, the dynamic range of the current analysis spanned 3 orders of magnitude. Amongst the most abundant (amount on column) proteins identified were serum albumin (~75 pg), carbonic anhydrase (~28 pg) and fatty acid ethyl ester synthase (~26 pg), which is a major lipase in white adipose tissue. The least abundant (amount on column) proteins meeting the inclusion criteria for statistical analysis were galectin-5 (0.06 pg), dimethylarginine dimethylaminohydrolase 1 (0.063 pg) and mitochondrial fusion protein 1 (0.076 pg). Supplementary table S1 reports the protein scores, number of peptides, sequence coverage, estimated abundance, and differences in relative abundance between HCR and LCR for the 448 proteins included in the statistical analysis.

Gene ontology analysis performed using DAVID found the majority of proteins were of cytosolic (130 proteins) or mitochondrial (128 proteins) origin. Prominent Biological Processes included: alcohol catabolic process (30 proteins) and glucose catabolic process (27 proteins). While the main Molecular Functions related to GTP binding (50 proteins) and nucleotide binding (172 proteins). When clustered according to associations in the Kyoto Encyclopaedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/), the top ranking pathway was Glycolysis/gluconeogensis, which encompassed 27 proteins representing 6.2 % of the documented pathway. Other prominent pathways included Fatty acid metabolism (18 proteins) and Glutathione metabolism (19 proteins).

A total of 79 statistically significant (P<0.05) differences in protein abundance were detected between HCR and LCR adipose samples and after correction (q-value) for multiple testing, 30 significant differences exhibited a false discovery rate of less than 10 % (q<0.1). Table 1 reports the protein identity and differences in abundance between HCR and LCR for the top-ranked (P<0.05, FDR <10 %) 30 proteins. A number of the proteins that differed between HCR and LCR have previously been associated with adipogenesis or adipocyte differentiation in vitro. Proteins associated with antioxidant or cellular stress response, immune cell function and energy metabolism were other prominent features. Amongst the most abundant of the differentially expressed proteins were serotransferrin (~29 pg on column) and long-chain fatty acid CoA-ligase 1 (~15 pg on column) while the least abundant (>0.5 pg on column) differentially expressed proteins detected included sulfotransferase 1A1, IgG2B C-chain and mitogen-activated protein kinase 3.

The search tool for the retrieval of interacting genes/proteins (STRING) was used to construct a graphical network (Figure 1) based on evidence of co-expression, protein-protein interaction, co-occurrence in KEGG pathways and literature mining. The network constructed from 15 proteins that matched to the STRING DB and were significantly (P<0.05, FDR <10 %) more abundant in LCR included protein-protein interactions between Mapk3 and Hspa5, Aldh2, TF, Acsl1, Tpi1 and Gapdh. Protein-protein interactions were also found between Aldh2 and Tpi1, and for Acsl1 and Hspa5. Co-expression was reported between Tf and Hpx, Tpi1 and Gapdh, Acsl1 and Ctsd, Mapk3 and Ctsd, Igg-2a and 678757 (IgG-2B chain C region). In addition links were drawn based on co-occurrence in KEGG pathways and text mining.

Fewer interactions were found within the 13 proteins that were significantly (P<0.05, FDR <10 %) more abundant in HCR. The only notable evidence being co-expression of peroxiredoxin 6 and selenium-binding protein in 1 proteomic profiling experiment.

Discussion

Despite a 9-fold difference in innate running capacity there was no difference in the abundance of mitochondrial proteins between HCR and LCR visceral adipose tissue. Instead, our non-targeted analysis highlighted significant differences in putative adipokines and proteins involved in adipogenesis, oxidative stress and inflammation. Our label-free profiling encompassed 448 proteins and detected 30 statistically significant ($P<0.05$, FDR <10 %) differences in protein abundance. The majority of these proteins have previously been detected in proteomic analyses of adipocytes isolated from human [22] or mouse [23] adipose tissues. Notable exceptions include IgG and MHC proteins that were significantly more abundant in LCR. This may indicate greater infiltration of inflammatory cells in LCR visceral adipose tissue.

The role of macrophages and T lymphocytes in adipose tissue inflammation has long been recognised, whereas the role of B lymphocytes has more recently come to light. Herein we report the pro-inflammatory IgG-2B Chain C isotope, which is produced by B cells was 362 % more abundant in LCR visceral adipose tissue. The accumulation of B cells in adipose tissue is an early event in diet-induced obesity that precedes T cell infiltration, the onset of insulin resistance and macrophage accumulation [24]. During obesity, B cells within adipose tissue may instigate inflammation both by presenting antigen via MHC I to activate CD8+ (i.e. cytotoxic) T cells and through the production of pro-inflammatory IgG2c [25]. When studied in vtiro, B cells modulate T cell release of pro-inflammatory cytokines [26] and our data suggests low aerobic capacity may also be associated with similar pro-inflammatory interactions.

ER stress associated with adipocyte enlargement is another early mechanism associated with inflammatory responses such as the production of TNF-α and infiltration of macrophages to adipose tissue [27]. The 78 kDa glucose-regulated protein (GRP-78, also known as BiP) was more abundant in LCR and is a recognised marker of ER stress that is induced by the unfolded protein response associated with adipogenesis [28] and pharmacological agents such as tunicamycin [29]. ER stress can occur due to the accumulation of unfolded proteins caused by oxidative stress-related protein damage. Accordingly, anti-oxidant enzymes, including superoxide dismutase, peroxiredoxin 6 and glutathione S-transferase were significantly less abundant in LCR. This finding is consistent with the greater oxidative stress reported in the kidneys [30], liver [21], skeletal muscle [31] and heart [32] of LCR rats and further highlights oxidative stress as a common process associated with low aerobic capacity. Herein non-targeted proteomics also detected a significantly greater (43 %) abundance of mitogen-activated protein kinase 3 (ERK1), which is commonly activated in adipocytes exposed to pharmacologically induced ER stress [33] or inflammatory cytokines [34]. This finding adds weight to the hypothesis that low innate aerobic capacity *per se* is sufficient to predispose animals to obesity and adipose tissue inflammation.

Adipose tissue is recognised as an endocrine organ that secretes adipokines that affect tissues such as the hypothalamus, liver and skeletal muscle. In addition to well described adipokines, such as leptin, proteomic studies (e.g. [35-37]) point to a broad array of putative adipokines that may be released or secreted from adipocytes in vitro. In addition, comprehensive mining of the 3T3-L1 adipocyte proteome by Adachi et al [23] highlighted 554 adipocyte proteins with signal peptides for the ER-Gologi export pathway. Five of the proteins that differed in abundance between HCR and LCR (Table 1) either have a signal peptide or have been reported as secreted from adipocytes in vitro. Putative adipokines

that were more abundant in LCR visceral adipose tissue include S100-B, cathepsin D, serotransferrin and GRP-78/ BiP, whereas sulfotransferase 1A1 and galectin-1 were more abundant in HCR. The majority of the above proteins have been associated with obesity, metabolic syndrome or type 2 diabetes. For example mRNA expression of cathepsin D is up-regulated in adipose tissue of mice exposed to a high-fat diet and in obese humans, and studies in vitro suggest differentiation of pre-adipocytes is dependent upon cathepsin D expression [38]. Sulfotransferase 1A1 does not have a signal peptide for the golgi excretory pathway but has previously been highlighted as a gene associated with T2DM by genome-wide scans. Indeed, sulfotransferase 1A1 mRNA expression is down regulated in response to HFD alongside other candidates close to genetic loci for obesity [39]. Galectin-1 also lacks a signal peptide but is reported to be more abundant in plasma of T2DM patients, which contrasts with our findings. However, galectin-1 is highly expressed in skeletal muscle [40] and exposure of skeletal muscle to glucose increases galectin-1 [41]. Thus, the elevations in plasma galectin-1 in T2DM patients may primarily arise from skeletal muscle rather than adipose tissue.

S100-B does not have a canonical signal peptide but is relatively abundant in adipose tissue (HCR 0.37 ± 0.28; LCR 1.7 ± 0.57 pg on coulmn) and is widely documented as a secreted protein. Circulating levels of S100-B correlate positively with BMI [42] and visceral obesity [43] but this is not evident in all studies (e.g [44]) and because S100-B is also released from brain astrocytes it is also regarded as a biomarker of blood-brain barrier permeability. Nonetheless, the current finding that an estimated 100 % difference in adipose tissue mass is associated with a >400 % increase in S100-B abundance supports the role of this protein as a sensitive biomarker of adiposity. Furthermore, S100-B has recently been implicated mechanistically in the interaction between adipocytes and macrophages in vitro. Fujiya et al., [45] report S100-B stimulates the release of TNF-α

from RAW macrophages and primary monocytes, and up-regulates markers of M1 macrophages. Correspondingly, TNF-α augments S100-B secretion from 3T3-L1 adipocytes creating a reciprocal interaction that may amplify the inflammatory response of adipose tissues in obese subjects.

When fed a high-fat diet (HFD), LCR gain significantly more weight than HCR despite the fact that HCR consume a greater amount of calories relative to body mass [2] In the current work, many of the proteins that were more abundant in LCR visceral adipose tissue are also recognised markers of adipocyte differentiation. For example the greater abundances of glycerol-3-phosphate dehydrogenase and long chain fatty acid CoA ligase may contribute to the relatively greater propensity for obesity in LCR rats. Our non-targeted analysis was able to detect important adipose tissue proteins highlighted through hypothesis-led research, including lipoprotein lipase, fatty acid translocase, adipose fatty acid binding protein, adipose triglyceride lipase (ATGL), hormone sensitive lipase, fatty acid synthase, perilipin 1 and retinol binding protein 4. However, the abundances of these proteins were not significantly different between HCR and LCR visceral adipose tissue. Interestingly, LCR did exhibit lesser abundance ($P<0.05$, FDR >10 %) of comparative gene identification-58, which enhances the lipase activity of ATGL [46] and has previously been reported to be less abundant in LCR adipose tissue [9].

Summary

Whilst adiposity is a correlated trait of selection on running capacity, it is not associated with overt differences in the profile of enzymes of aerobic metabolism in adipose tissue. Instead, our non-targeted proteomic analysis has revealed LCR visceral adipose tissue has relatively poor defences against oxidative stress and exhibits markers of adipocyte differentiation, ER stress and inflammation. Moreover, several putative adipokines differed in abundance between HCR and LCR. In particular S100-B protein, which has recently been mechanistically linked in adipose tissue inflammation was 431 % more abundant in LCR and may represent candidate biomarker of clinical relevance.

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Figure Legend

Figure 1- Association network drawn from proteins that differed in abundance in HCR and LCR visceral adipose tissue.

Proteins that exhibited statistically significant (P<0.05, FDR <10 %) differences in abundance (Table 1) were imported to the statistical tool for the retrieval of interacting genes/proteins (STRING). Nodes represent individual proteins annotated with common gene names: **678757**, Ig kappa chain C region, B allele; **Acsl1**, long-chain fatty acid Co-A ligase 1; **Aldh2**, aldehyde dehydrogenase 2; **Ctsd**, cathepsin D; **Gapdh**, glyceraldehyde-3-phosphate dehydrogenase; **Gpd1**, glycerol-3-phosphate dehydrogenase [NAD+] cytosolic; **Hpx**, Hemopexin; **Hspa5**, 78-kDa glucose-regulated protein; **Hspb1**, heat shock protein beta-1; **IgG-2a**, Ig gamma-2B Chain C region; **Kng1l1**, T-kininogen 2; **Mapk3**, mitogen-activate protein kinase 3; **Pfn1**, profilin-1; **Pgk1**, phosphoglycerate kinase; **Prdx6**, peroxiredoxin-6; **Selenbp1**, selenium binding protein-1; **Slc25a4**, ADP/ATP translocase 1; **Sod1**, superoxide dismutase [Cu-Zn]; **Tf**, serotransferrin; **Tpi1**, triosephosphate isomerase.

Vectors between nodes are colour coded to represent different levels of information, including: evidence of co-expression (black), protein-protein interaction (pink), concurrence in databases such as the Kyoto encyclopaedia of genes and genomes (KEGG; blue) or PubMed abstracts (green). A medium confidence cut-off was used and no additional interactions were added.