Proteomic Analysis of Cardiac Adaptation to Exercise by High Resolution Mass Spectrometry

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Regular exercise has many health benefits, among which is a significant reduction of cardiovascular risk. Although many beneficial effects of exercise are well described, the exact mechanisms by which exercise confers cardiovascular benefits are yet to be fully understood. In the current study, we have used high resolution mass spectrometry to determine the proteomic responses of the heart to exercise training in mice. The impact of exercise-induced oxidative stress on modifications of cardiomyocyte proteins with lipid peroxidation biomarker 4-hydroxynonenal (4-HNE) was examined as well. Fourteen male mice were randomized into the control (sedentary) group and the exercise group that was subjected to a swim exercise training program for 5 days a week for 5 months. Proteins were isolated from the left ventricular tissue, fractionated and digested for shotgun proteomics. Peptides were separated by nanoliquid chromatography and analyzed on an Orbitrap Fusion mass spectrometer using high-energy collision–induced dissociation and electron transfer dissociation fragmentation. We identified distinct ventricular protein signatures established in response to exercise training. Comparative proteomics identified 23 proteins that were upregulated and 37 proteins that were downregulated with exercise, in addition to 65 proteins that were identified only in ventricular tissue samples of exercised mice. Most of the proteins specific to exercised mice are involved in respiratory electron transport and/or implicated in glutathione conjugation. Additionally, 10 proteins were found to be modified with 4-HNE. This study provides new data on the effects of exercise on the cardiac proteome and contributes to our understanding of the molecular mechanisms underlying the beneficial effects of exercise on the heart.

Keywords: exercise, left ventricle, proteomics, oxidative stress, 4-hydroxynonenal

INTRODUCTION

In the early 1950s, Morris and others showed the association between physical activity and reduced deaths from coronary heart disease, which led to increased scientific interest in the potential of physical activity to combat diseases (Morris et al., 1953). Current knowledge shows a clear link between an active lifestyle and overall health. Physical exercise is found to benefit the heart as it increases aerobic fitness (VO\textsubscript{2max}), enhances contraction, and accelerates relaxation, as well as

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decreasing the risk of developing cardiomyopathies (Roof et al., 2013). It is found that different types, intensities, durations, and frequencies of exercise interventions are crucial for cardiovascular health (Gevaert et al., 2020). Endurance exercise can trigger several adaptational mechanisms resulting in exercise-induced cardioprotection changes. Those changes include an enhanced antioxidative defense system with increased expression of glutathione peroxidase-1 and manganese superoxide dismutase. Moreover, cardiac function is improved due to changes in the expression of enzymes involved in energy metabolism (Burniston and Hoffman, 2011).

Many studies suggest the beneficial effects of exercise-induced oxidative stress. It is suggested that the released reactive oxygen species (ROS) post-physical exercise can lead to cardiac adaptation, including protection against infarction (Frasier et al., 2011). At a molecular level, excess oxidants can damage macromolecules. Lipids are one of the target sites of ROS-induced damage, where it was found that maximal and supra-maximal exercise can significantly increase lipid peroxidation (Mohamed et al., 2016).

Lipid peroxidation can result in the formation of many reactive species as secondary products, known as reactive carbonyl compounds (RCCs), due to the highly reactive carbonyl group. Those secondary products have high stability with an average half-life of minutes or even hours, compared to the short half-life of ROS, which can only last for nanoseconds or milliseconds (Jaganjac et al., 2016). As a result, RCCs can diffuse through cellular membranes and attack biomolecules that are far away from the site of origin (Pamplona, 2011; Jaganjac et al., 2013). Among all secondary products studied, of particular biochemical and biomedical relevance is 4-hydroxynonenal (4-HNE) (Živković et al., 2005).

The physiological levels of 4-HNE vary between different cell types or tissues. Physiological concentrations of 4-HNE are in the submicromolar range, and for the heart samples, a 4-HNE concentration of around 0.25 nmol/mg of tissue (Nakagawa et al., 2014) or 6 nmol/mg of protein (Benes et al., 2013) was reported. 4-HNE can bind to the nucleophilic amino acid side chain of proteins via the Michael addition or the Schiff base formation modifying protein function (Zarkovic et al., 2013). It is crucial for cells to control 4-HNE concentrations, as low concentrations are needed for normal physiological processes, while high concentrations can have detrimental effects (Jaganjac et al., 2020a).

In this study, conducted in experimental animals (mice), we aimed to determine the effect of intense exercise training on the left ventricle proteome and on the formation of 4-HNE protein adducts by using in-depth proteomics.

MATERIALS AND METHODS

Animals

Experiments were performed on 14 male C57BL/6J mice that were 8–10 weeks old, with water and food given ad libitum. All experiments were performed in accordance with the European Commission Directive 2010/63 (European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes) and the United Kingdom Home Office (Scientific Procedures) Act (1986) with project approval by the Institutional Animal Welfare and Ethical Review Committee of the University of Manchester (PBA6A87CA).

Training Protocol and Sample Collection

Animals were randomized into two groups: a sedentary group and an exercised group that was trained by swimming for 5 months, for 60 min per day for 5 days per week. After 5 months, the animals were terminated by cervical dislocation, following which the heart was rapidly excised. A 2-mm biopsy of the left ventricle free wall was sampled, approximately midway between the base and the apex, and stored at −80°C until further analysis. At the time of sample collection, the mice were 7–7.5 months old.

Protein Isolation and Digestion

Left ventricular tissue was homogenized in a glass homogenizer in ice-cold lysis buffer (20 mM HEPES, 20 mM NaCl, 5 mM EDTA, 1% w/v CHAPS, and protease inhibitors). 7 ml of buffer was added per 1 g of tissue. Lysates were then sonicated for 10 min, followed by centrifugation at 14,000 × g for 10 min at +4°C. Supernatants were collected into new Eppendorf tubes, and protein concentration was determined using a BCA Protein Assay Kit (Pierce).

Protein samples were prepared for proteomics analysis in a similar manner to that described before (Al-Thani et al., 2018). Briefly, normalized protein samples were electrophoretically separated on an SDS-PAGE using non-reducing sample buffer and whole lanes were excised and divided into 8 equal parts that were then reduced with 10 mM dithiothreitol and alkylated with 100 mM iodoacetamide, followed by overnight digestion at 37°C with 20 ng/μl Trypsin Gold MS grade (Promega). The mixture of 45% water, 50% acetonitrile, and 5% formic acid was used to extract peptides.

Shotgun Proteomics

Samples were analyzed using an Orbitrap Fusion Tr ibrid mass spectrometer (Thermo Scientific, Waltham, United States) coupled with an Easy n-LC II (Thermo Scientific, Waltham, United States) for nano-LC gradient separation. Thermo Xcalibur (version 3.0) software was used to control instrument setup. The nanoelectrospray ionization (NSI) mode was used, which is the preferred ionization mode for peptides and proteins. Peptide mixtures were separated on a reverse-phase C18 column (25 cm, 75 μm, Thermo Scientific, Waltham, United States) attached to a pre-trapping C18 column (2 cm, 75 μm, Waters, Waltham, United States), and elution was carried out at a constant flow rate of 300 nl/min over a 108-min step gradient. Solvents used for separation were solvent A (HPLC-grade water with 0.1% (v/v) formic acid) and solvent B (HPLC-grade acetonitrile with 0.1% (v/v) formic acid). The separation gradient was set to 5% B for 5 min, 5–37% B for 90 min, 37–80% B for 4 min, 80% B for 2 min, 80–5% B for 2 min, and 5% B for 5 min.
The Orbitrap Fusion Tribrid analysis was set to data-dependent acquisition (DDA), using an Orbitrap mass analyzer to acquire the full MS spectra and an IonTrap to acquire the MS/MS fragment ion spectra. First full-scan mode acquisition was performed with a resolution of 120,000 orbitrap and a scan range of 400–1,600 m/z. Automatic gain control (AGC) was set at 200,000 with an injection time of 100 ms. This scan mode was carried out by enabling a monoisotopic precursor selection (MIPS) filter and dynamic exclusion with a duration of 30 s and a mass tolerance of 10 ppm. This is followed by two different second scan modes based on decisions of two scan event types. Scan event type 1 uses higher-energy collision–induced dissociation (HCD) fragmentation at the quadrupole isolation mode, which generates b and y ions, with an isolation width of 2 m/z and a collision energy of 30% and at 10,000 AGC and a maximum injection time of 70 ms. The neutral loss ion is triggered with masses of 52 or 78 m/z from the 156-Da 4-HNE-modified peptide by the Michael addition or masses of 46 or 69 m/z from the 138-Da 4-HNE—modified peptide by the Schiff bases (Carini et al., 2004). Selected peptides are further fragmented by electron transfer dissociation (ETD), which generates c and z ions and cleaves the amide group, leaving the side chain intact, using the quadrupole isolation mode with an isolation width of 2 m/z and a collision energy of 30% and at 10,000 AGC and a maximum injection time of 70 ms. Meanwhile, scan event type 2 is HCD fragmentation at the quadrupole isolation mode with an isolation width of 1.6 m/z and a collision energy of 30% and at 10,000 AGC and a maximum injection time of 70 ms.

Data Processing and Analysis

The raw data generated by the Orbitrap Fusion were processed using Proteome Discoverer 2.2 (Thermo Scientific, San Jose, California, United States). The spectrum selector filter was assigned with 350 Da as the minimum precursor mass and 5,000 Da as the maximum precursor mass. The MS/MS spectra search was carried out using SEQUEST HT search algorithms against the Uniprot Mus musculus (Mouse) protein database, and the FASTA file was retrieved on the 9th of October, 2017. Two scan event filters were used: one with the HCD activation type, followed by Sequest HT search with the following parameters: full trypsin digestion, a maximum of 2 missed cleavages sites, a minimum peptide length of 6 residues, a tolerance of 20 ppm for precursor mass and 0.6 Da for fragment mass, dynamic modification of oxidation of methionine (+15.995 Da), and static modification of carboxymethyl of cysteine (+57.021 Da). The second scan event filter included the ETD activation type with 1,000 set as the maximum collision energy, followed by another Sequest HT search using a comprehensive workflow that included additional dynamic modifications: 4-HNE adduction to cysteine, histidine, lysine, leucine, methionine, and arginine (+156.115 Da); 4-HNE + H₂ for the Michael adducts in cysteine, histidine, lysine, leucine, methionine, and arginine (+158.131 Da); and 4-HNE-H₂O for the Schiff bases in cysteine, histidine, lysine, leucine, methionine, and arginine (+138.104 Da). The target decoy PSM validator node was used for peptide validation with an FDR of 0.01. For precursor ion quantification, label-free quantification using the Minora Feature Detection was used, followed by statistical analysis using t-test (p < 0.05) to measure proteins that were significantly upregulated in the exercised group compared to the control group. On the other hand, the consensus workflow parameters used were as follows: a peptide validator with an automatic validation mode of 0.01 FDR, peptides and proteins were searched using a high-confidence peptide filter, and the precursor ion quantifier calculated abundance based on the intensity. For high confidence, XCorr values were set to at least 1.8 for singly, 2.4 for doubly, 2.5 for triply, and 2.6 for quadruply or more charged peptides. The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD026558.

Only cardiac proteome changes detected in six or more samples per group were considered for further data visualization and interpretation. Differential expression analysis was performed using Perseus 1.6.15.0. (Tyanova et al., 2016) according to the protocol of Tyanova & Cox (Tyanova et al., 2018), and the FDR value was set to 0.05. The results are reported with the corrected p-value (q-value).

Data analysis of proteins identified in both groups was performed using InfernoRDN v1.1.7234 software (Polpitiya et al., 2008). Data were log-transformed and normalized by central tendency adjustment, followed by partial least square (PLS) analysis. Moreover, variables upregulated or downregulated with exercise with p-values ≤ 0.05 were visualized on the heat map, while all proteins that met the criteria and were identified in the database are plotted in a volcano plot.

Protein ANalysis THrough Evolutionary Relationships (PANTHER) classification system software (version 14.0) was used to analyze changes in different classes of proteins based on identified proteins and to analyze gene ontology annotations available in the UniProt database (Mi et al., 2019). The Reactome 3.7 database of reactions, pathways, and biological processes with database release 73 was used for pathway analysis of left ventricular proteins upregulated or only identified in the exercised group (Haw and Stein, 2012).

RESULTS

High-resolution mass spectrometry was used to study changes in the left ventricular tissue proteome after long-term intensive swimming exercise. The total number of identified proteins in the control and the exercise group was 1,839 and 1,915, respectively, while the total number of identified peptides was 10,092 and 10,129, respectively. Further analyses were based on the criteria that the protein/peptide must be common for tissue samples of the same group of animals. Based on that criteria, a total of 804 proteins were identified in the control group with a total number of 2,913 peptides, while 816 proteins were found in the exercised group with a total of 3,254 peptides.

To evaluate the impact of exercise on the cardiac proteome, we have used the comparative proteomics and label-free proteomics
| UniProtKB accession | Gene       | Description                                              | # AAs | MW [kDa]     | Number of positive samples |
|---------------------|------------|----------------------------------------------------------|-------|--------------|---------------------------|
| G3UYJ7              | Gm20441    | Predicted gene 20441 (fragment)                          | 252   | 28.6         | 7                         |
| Q9WLK2              | Eif4h      | Eukaryotic translation initiation factor 4H              | 248   | 27.3         | 7                         |
| Q99JP6              | Nmnat3     | Nicotinamide/nicotinic acid mononucleotide adenylytransferase 3 | 245   | 27.7         | 7                         |
| Q9D7J4              | Cox20      | Cytochrome c oxidase protein 20 homolog                  | 117   | 13.2         | 7                         |
| Q9D819              | Ppa1       | Inorganic pyrophosphatas                                  | 289   | 32.6         | 7                         |
| Q9RIP3              | Psmb2      | Proteasome subunit beta type-                            | 201   | 22.9         | 7                         |
| Q8BSB7              | Comt       | Catechol O-methyltransferase                             | 265   | 29.5         | 7                         |
| E9QMD2              | Ube2g1     | Ubiquitin-conjugating enzyme E2Q-like protein 1           | 304   | 32.6         | 7                         |
| Q6ZWQ0              | Synet2     | Nesprin-2                                                | 6,874 | 782.2        | 7                         |
| Q99J6               | Rap1b      | Ras-related protein Rap-1b                               | 184   | 20.8         | 7                         |
| P68899              | Tub1a      | Tubulin alpha-1A chain                                   | 451   | 50.1         | 7                         |
| Q8KOC6              | Srt5       | NAD-dependent protein deacetylase sirtuin-5, mitochondrial | 310   | 34.1         | 7                         |
| Q99131              | Gsto1      | Glutathione S-transferase omega-1                         | 240   | 27.5         | 7                         |
| P10649              | Gstm1      | Glutathione S-transferase Mu 1                            | 218   | 26           | 7                         |
| Q7TMG8              | Nipsnap2   | Glioblastoma amplified sequence                           | 281   | 32.9         | 7                         |
| Q9CYR6              | Pgm3       | Phosphoacetylglucosamine mutase                          | 542   | 59.4         | 6                         |
| Q8BS520             | Shoc2      | Leucine-rich repeat protein SHOC-2                        | 582   | 64.9         | 6                         |
| P62748              | Hpcal1     | Hippocalcin-like protein 1                                | 193   | 22.3         | 6                         |
| Q32ME0              | Kcnh6      | Potassium voltage-gated channel, subfamily H (Eag-related), member 6 | 950   | 105.6        | 6                         |
| Q8RSGS              | Actr1b     | Beta-catenin                                              | 376   | 42.3         | 6                         |
| D2YYS6              | Mgl1       | Monoglyceride lipase                                      | 351   | 36.6         | 6                         |
| D3YWD3              | Tmem245    | Transmembrane protein 245                                 | 880   | 97.7         | 6                         |
| Q9CPUW4              | Arpc5      | Actin-related protein 2/3 complex subunit 5               | 151   | 16.3         | 6                         |
| Q9DAW9              | Cnn3       | Calponin-3                                               | 330   | 36.4         | 6                         |
| A2A5Y6              | Mapt       | Microtubule-associated protein                            | 749   | 78           | 6                         |
| P62827              | Ran        | GTP-binding nuclear protein Ran                           | 216   | 24.4         | 6                         |
| Q9CR21              | Ndufa1     | Acyl carrier protein, mitochondrial                       | 156   | 17.4         | 6                         |
| Q9RS26              | Pcd111     | Polycystic kidney disease protein 1-ike 1                 | 2,615 | 290.7        | 6                         |
| B7ZCF1              | Psmc3      | 26S proteasome regulatory subunit 6A                      | 451   | 50.4         | 6                         |
| E9QCL4              | Iti207     | Interferon-activated gene 207                             | 978   | 104.4        | 6                         |
| Q7BYS3              | Pdc26      | Phosducin-like protein 2                                  | 240   | 27.8         | 6                         |
| Q9EQY4              | Zfp94      | Zinc finger protein 94                                    | 486   | 55.5         | 6                         |
| P51163              | Uros       | Uroporphyrinogen-III synthase                             | 265   | 28.5         | 6                         |
| P113S2              | Gpx1       | Glutathione peroxidase 1                                  | 201   | 22.3         | 6                         |
| A2A4S2              | Cul4b      | Cullin-4B                                                | 970   | 110.6        | 6                         |
| Q923D2              | Blrb       | Flavin reductase (NADPH)                                  | 206   | 22.2         | 6                         |
| P51855              | Gss        | Glutathione synthetase                                    | 474   | 52.2         | 6                         |
| Q95505              | Fah        | Fumarylacetoacetase                                       | 419   | 46.1         | 6                         |
| Q90892              | Itpa       | Inosine triphosphate pyrophosphatase                      | 198   | 21.9         | 6                         |
| Q4VAE3              | Tmemr65    | Transmembrane protein 65                                  | 234   | 24.9         | 6                         |
| H5UBL2              | Atpaf1     | ATP synthase mitochondrial F1 complex assembly factor 1   | 348   | 38.8         | 6                         |
| Q6NZN1              | Pprc1      | Peroxisome proliferator-activated receptor gamma coactivator-mediated protein 1 | 1,644 | 175         | 6                         |
| Q9JII6              | Akr1a1     | Alcohol dehydrogenase [NADP (+)]                          | 325   | 36.6         | 6                         |
| Q9D8S4              | Rexo2      | Oligoribonuclease, mitochondrial                          | 237   | 26.7         | 6                         |
| Q8BHE8              | Mapi1      | m-AAA protease-interacting protein 1, mitochondrial       | 291   | 33           | 6                         |
| Q9VC2C              | Ces1       | Liver carboxylesterase                                   | 565   | 62.6         | 6                         |
| E9PWM3              | Armcx4     | Armadillo repeat-containing, X-linked 4                  | 2,356 | 242.8        | 6                         |
| E9QT5J              | Acyp1      | Acylphosphatase                                           | 157   | 17.3         | 6                         |
| Q9JKL5              | Tesc       | Cacinein B homologal protein 3                           | 214   | 24.6         | 6                         |
| Q2NL51              | Gsk3a      | Glycogen synthase kinase-3 alpha                         | 490   | 51.6         | 6                         |
| Q8BXX9              | Clic5      | Chloride intracellular channel protein 5                 | 251   | 28.3         | 6                         |
| Q9EPS6              | Apoc3      | Apolipoprotein C-III                                     | 137   | 15.2         | 6                         |
| Q9D1X0              | Nol3       | Nucleolar protein 3                                       | 220   | 24.6         | 6                         |
| Q9WUM4              | Coro1c     | Coronin-1C                                               | 474   | 53.1         | 6                         |
| Q9LJH8              | Tmod4      | Tropomodulin-4                                            | 345   | 39.2         | 6                         |
| E9PVA8              | Gcn1       | elf-2-2-alpha kinase activator GCN1                      | 2,671 | 292.8        | 6                         |
| Q8VE22              | Mrps23     | 28S ribosomal protein S23, mitochondrial                 | 177   | 20.3         | 6                         |
| Q6RIPO              | Sr2        | SMCS-SMC6 complex localization factor protein 2          | 1,278 | 143.9        | 6                         |
| P04938              | Mup11      | Major urinary protein 11                                  | 181   | 20.7         | 6                         |
| P28883              | Rkbp1a     | Peptidyl-prolyl cis-trans isomerase FKBP1A                | 108   | 11.9         | 6                         |

(Continued on following page)
Exercise is marked, among others, with elevated ROS production that can lead to peroxidation of lipids, yielding biologically active reactive aldehyde 4-HNE (Al-Menhali et al., 2020). Previously, it was shown that 4-HNE regulates metabolism in the skeletal muscle cells either directly or by adducting to proteins (Al-Menhali et al., 2020). In the present work, modification of proteins by 4-HNE was determined to gain insight into the effects of long-term intensive swimming exercise on the left ventricle and on the potential signaling role of 4-HNE (Table 3). Thirteen different proteins were found to be prone to exercise-induced 4-HNE modifications, compared to eight proteins in the control group. Among the identified 4-HNE–modified proteins, only three proteins were found to be commonly modified for both groups. Those proteins are myoglobin, sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (SERCA2), and laminin subunit alpha-1.

**DISCUSSION**

In the past decade, significant advances have been made to understand the effects of exercise on the heart. This study aimed to identify alterations in the left ventricle proteome after long-term intensive swimming exercise training. Proteomics depends mainly on mass spectrometry, and by the use of high-resolution instruments and appropriate search engines, changes in the proteome can be detected (Al-Thani et al., 2018). Our earlier study on the erythrocyte proteome demonstrated that besides information on the comparative and quantitative proteome changes, high resolution mass spectrometry is also a vital tool for redox proteomics (Al-Thani et al., 2018). In addition, we have demonstrated that redox modifications of proteins can alter their function (Ludtmann et al., 2018). 4-HNE modification of proteins is one of the common types of oxidative modifications, and the preferred mass spectrometry methods for the detection and identification of proteins modified by 4-HNE use neutral loss-triggered electron capture dissociation tandem mass spectrometry (Rauniyar et al., 2009), collision-induced dissociation (CID), and electron transfer dissociation (ETD) MS/MS (Fritz et al., 2012). We therefore employed a high-resolution tridium mass spectrometer using HCD and ETD fragmentation methods to investigate changes in the proteome of the left ventricle of the heart, including the identification of 4-HNE–modified proteins, of control and exercised mice. According to the study published several months ago, it is possible that lowering the value for the mass resolution of the

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**TABLE 1 (Continued)** Left ventricular tissue proteins identified only in samples of exercised mice. Proteins identified in ≥6 left ventricular tissue samples isolated from exercised animals but in none of the samples of the control group are listed. The table shows the UniProt name of the protein, main description, gene coding of the protein, number of amino acids (AAs), and molecular weight (MW).

| UniProtKB accession | Gene | Description | # AAs | MW [kDa] | Number of positive samples |
|---------------------|------|-------------|-------|----------|---------------------------|
| P00687              | Amy1 | Alpha-amyrase 1 | 511   | 57.8     | 6                          |
| P48542              | Kcnj6| G protein-activated inward rectifier potassium channel 2 | 425   | 48.6     | 6                          |
| Q9CO65              | Mtap | S-methyl-5’-thiodenosine phosphorylase | 283   | 31        | 6                          |
| S4R1W1              | Gm3839| Glyceraldehyde-3-phosphate dehydrogenase | 333   | 35.8     | 6                          |

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approach. A total of 125 proteins displayed statistically significant changes in the ventricular tissue of the swimming exercise group compared to the control group (Tables 1, 2). Table 1 lists 65 proteins that were identified in ≥6 ventricular tissue samples of exercised mice, while the same were not identified in the control mice. Proteins that were identified in 6/7 samples for each group were selected for PCA analysis, to determine the multivariate exercise signature of proteins (Figure 1A). The semiquantitatively assessment of proteins upregulated/downregulated with exercise identified 23 proteins that were upregulated and 37 that were downregulated with exercise (Figure 1B), and details of each are included in Table 2. The changes in the protein abundances are shown using a volcano plot in Figure 1C. The largest increase following exercise training was observed for mitochondrial cytochrome c oxidase subunit 7A2, cathepsin B, and ubiquitin carboxyl-terminal hydrolase isozyme L3. In addition, the most significant exercise-induced upregulation was observed for mitochondrial cytochrome c oxidase subunit 7A2, delta-sarcoglycan, and mitochondrial cysteine desulfurase. PLS analysis demonstrated good separation between the two groups with distinct protein signatures in the ventricular samples of exercised animals.

Furthermore, all proteins upregulated or identified only in samples of exercised mice were classified into several major classes of proteins (Figures 1D–F). Only those classes represented with at least 3 different proteins are shown in Figure 1D. Exercise increases the metabolic rate in order to meet the energy demand. Thus, as predicted, more than 40% of proteins belong to the metabolite interconversion enzyme class. Also, close to 16% of proteins upregulated with swimming exercise belong to the cytoskeletal protein class (Figure 1D).

To understand the effect of exercise on the transcription of proteins, the identified proteins specified above in the ventricular samples of exercised mice were searched for their known molecular function (Figure 1E) and biological processes (Figure 1F), using the PANTHER classification system. The molecular function of these proteins was mostly involved in catalytic activity (51.0%) and binding (32.7%) (Figure 1E). The cellular processes were primarily classified as various cellular processes (29.3%) and metabolic processes (22.2%). Additionally, more than 10% of proteins have a role in biological regulation and cellular component organization or biogenesis (Figure 1F).

The pathway analysis of the ventricular proteins altered with swimming exercise using Reactome (Figure 1G) suggested that significantly affected pathways were tRNA aminoacylation, pyrophosphate hydrolysis, glutathione conjugation, and respiratory electron transport (Entities FDR <0.03 for all).
TABLE 2 | Left ventricular tissue proteins ubiquitously identified in both groups but altered with exercise. Only proteins upregulated in ≥6 samples in each group are listed.

| UniProt accessions | Description | Gene | Exercised % of control ± SE | q-value |
|---------------------|-------------|------|----------------------------|---------|
| A3KGL9              | Spectrin alpha chain, non-erythrocystic 1 | Sptan1 | 24.5 ± 2.4 | 0.0160 |
| P48771              | Cytochrome c oxidase subunit 7A2, mitochondrial | Cox7a2 | 718.8 ± 49.8 | 0.0100 |
| P82347              | Delta-sarcoglycan | Sgcd | 471.4 ± 41.7 | 0.0133 |
| P17182              | Alpha-enolase | Eno1 | 19.4 ± 1.1 | 0.0150 |
| Q9ZUJ3              | Cysteine desulfurase, mitochondrial | Nfs1 | 231.3 ± 12.7 | 0.0120 |
| E9Q7L0              | Oxoglutarate dehydrogenase-like | Gdhh | 19.2 ± 2.6 | 0.0120 |
| Q62261              | Spectrin beta chain, non-erythrocystic 1 | Spibt1 | 19.9 ± 2.5 | 0.0223 |
| Q61234              | Alpha-1-syntrophin | Snta1 | 342.6 ± 26.1 | 0.0230 |
| Q8BTM8              | Filamin-A | Flna | 13.8 ± 1.7 | 0.0213 |
| Q3UJ28              | Myosin light chain kinase 3 | Mylk3 | 6.8 ± 1.1 | 0.0196 |
| Q8BUJ6              | Isoleucine--tRNA ligase, mitochondrial | Irs2 | 27.0 ± 3.8 | 0.0193 |
| Q70622              | Reticulon-2 | Rtn2 | 46.4 ± 5.2 | 0.0183 |
| H7BX01              | Dynamin-like 120 kDa protein, mitochondrial | Dmp1 | 46.1 ± 5.0 | 0.0175 |
| Q9WUR2              | Enoyl-CoA delta isomerase 2, mitochondrial | Eci2 | 302.7 ± 22.1 | 0.0171 |
| G3X9J1              | Sodium/calcium exchanger 1 | Slc8a1 | 32.9 ± 5.2 | 0.0168 |
| A0A0R4J0P1          | Acyl-coenzyme A dehydrogenase family, member 8 | Acad8 | 43.2 ± 2.9 | 0.0165 |
| P45952              | Medium-chain acyl-CoA dehydrogenase, mitochondrial | Acadm | 160.0 ± 5.1 | 0.0170 |
| Q9242X              | Carminine O-palmitoyltransferase 1, muscle isoform | Cpt1b | 46.2 ± 3.9 | 0.0189 |
| P17710              | Hexokinase-1 | Hk1 | 33.7 ± 5.5 | 0.0198 |
| P51881              | ADP/ATP translocase 2 | Stc2a5a | 585.8 ± 63.6 | 0.0200 |
| Q8BH80              | Vesicle-associated membrane protein, associated protein B and C | Vapb | 367.4 ± 41.3 | 0.0198 |
| Q9D6J6              | NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial | Ndufl2 | 355.7 ± 39.3 | 0.0195 |
| P28474              | Alcohol dehydrogenase class-3 | Adh3 | 282.7 ± 24.5 | 0.0191 |
| G3X9J1              | Inter-alpha trypsin inhibitor, heavy chain 2 | Itpi2 | 4.8 ± 3.6 | 0.0200 |
| Q9DC93              | NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10 | Nduflb10 | 372.6 ± 37.2 | 0.0200 |
| O55143              | Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 | Atp2a2 | 61.1 ± 3.9 | 0.0192 |
| Q7TX57              | 26S proteasome non-ATPase regulatory subunit 1 | Psmd1 | 33.7 ± 4.4 | 0.0197 |
| P29758              | Ornithine aminotransferase, mitochondrial | Oat | 217.3 ± 16.4 | 0.0196 |
| P00905              | Integrin beta-1 | Itgb1 | 13.2 ± 2.1 | 0.0207 |
| Q9DCT2              | NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial | Ndufs3 | 596.9 ± 70.5 | 0.0200 |
| Q02035              | Ubiquitin-like modifier-activating enzyme | Ubam1 | 143.3 ± 1.8 | 0.0194 |
| Q9R069              | Basal cell adhesion molecule | Bcam | 37.8 ± 3.6 | 0.0190 |
| Q792Z1              | MCG1440784 | Try10 | 610.5 ± 70.3 | 0.0186 |
| Q9JK81              | Ubiquitin carboxyl-terminal hydrolase isozyme L3 | Uch3 | 1,573.5 ± 202.3 | 0.0187 |
| Q9KB83              | Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial | Sdhb | 49.5 ± 2.3 | 0.0183 |
| P10493              | Nidogen-1 | Nid1 | 46.2 ± 4.5 | 0.0181 |
| Q9VDMD              | 26S proteasome non-ATPase regulatory subunit 2 | Psmd2 | 44.4 ± 1.4 | 0.0182 |
| Q35639              | Annexin A3 | Anxa3 | 541.7 ± 73.5 | 0.0178 |
| Q60597              | 2-oxoglutarate dehydrogenase, mitochondrial | Gdh | 22.6 ± 2.5 | 0.0200 |
| Q64727              | Vinculin | Vcl | 47.4 ± 7.3 | 0.0196 |
| P69566              | Leucine-rich PPR motif-containing protein, mitochondrial | Lrpprc | 8.5 ± 1.4 | 0.0198 |
| P28665              | Murinoglobulin-1 | Mug1 | 19.5 ± 2.7 | 0.0196 |
| P62908              | 40S ribosomal protein S3 | Rps3 | 242.7 ± 16.5 | 0.0193 |
| Q99LX0              | Protein/nucleic acid deoxyglycase DJ-1 | Park7 | 483.9 ± 74.9 | 0.0197 |
| Q99L5C              | Electron transfer flavoprotein subunit, mitochondrial | Efta | 223.7 ± 16.5 | 0.0196 |
| Q91V9M              | Inorganic pyrophosphatase 2, mitochondrial | Ppa2 | 394.2 ± 43.7 | 0.0192 |
| Q61941              | NAD(P) transhydrogenase, mitochondrial | Npt | 48.3 ± 5.6 | 0.0188 |
| P70670              | Nascent polypeptide-associated complex subunit alpha, muscle-specific form | Napa | 51.8 ± 4.2 | 0.0187 |
| P18242              | Cathepsin D | Ctsd | 346.1 ± 39.6 | 0.0196 |
| Q8K1M3              | Protein kinase, cAMP dependent regulatory, type II alpha | Prkar2a | 39.0 ± 2.5 | 0.0197 |
| P16005              | Cathepsin B | Ctsb | 1895.7 ± 357.0 | 0.0196 |
| P10027              | Complement C3 | C3 | 30.8 ± 7.2 | 0.0192 |
| 961922              | 4-aminobutyrate aminotransferase, mitochondrial | Abat | 22.1 ± 2.0 | 0.0193 |
| Q93880              | Mitochondrial import inner membrane translocase subunit TIM50 | Tim50 | 28.7 ± 3.0 | 0.0190 |
| Q922B2              | Aspartate--tRNA ligase, cytoplasmic | Pbrf6 | 23.8 ± 3.4 | 0.0190 |
| O52324              | Proteasome subunit beta type-5 | Psmb5 | 568.5 ± 69.7 | 0.0191 |
| Q90880              | Elongation factor 1-gamma | Eef1g | 52.8 ± 3.5 | 0.0189 |

...upregulated with exercise in addition to 65 proteins identified only in exercised mice samples and also demonstrated a distinct proteome signature of the hearts of exercised and sedentary animals. Indeed, long-term endurance exercise triggers...
significant physiological and autonomic adaptations to maintain the cardiac function (Rivera-Brown and Frontera, 2012; Gourine and Ackland, 2019). Several studies have demonstrated the effect of endurance exercise-induced transcriptional regulations with regard to increasing mitochondrial biogenesis. The training protocol used in this study increases mitochondrial biogenesis.
mitochondrial volume density and number, and both basal and insulin-stimulated glucose uptake in the left ventricle (Vettor et al., 2014). In line with our previous work using this training protocol model (D’Souza et al., 2017; D’Souza et al., 2014; Bidaud et al., 2020), the training efficiency in this study was also evidenced by the development of phenotypic characteristics of the ‘athletes heart,’ including ventricular hypertrophy determined by echocardiography and sinus bradycardia determined by ECG. The cardiac response to swim training observed in this study was similar to that produced by treadmill running in rats where the training status was assessed by measuring the maximal oxygen uptake (VO2max) (D’Souza et al., 2017). This study confirmed that more than 10% of proteins identified only after exercise or upregulated proteins in the ventricular tissue of exercised animals have a role in biological regulation and cellular component organization or biogenesis. Moreover, the transcriptional coactivator peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), which is a master regulator of mitochondrial biogenesis, is found to be activated in the hearts of mice after endurance swimming exercise (Boström et al., 2010). In addition, we also found peroxisome proliferator-activated receptor gamma coactivator-related protein one in the exercised group but not in the control group. During exercise, the continuous energy demand is regulated with adequate oxygen supply to support oxidative phosphorylation (Rivera-Brown and Frontera, 2012). This study identified a number of proteins significantly specific to the left ventricle of the exercised group that are directly involved in the mitochondrial electron transport, including acyl carrier protein, ATP synthase mitochondrial F1 complex assembly factor 1, NADH dehydrogenase [ubiquinone] flavoprotein 2, NADH dehydrogenase [ubiquinone] one beta subcomplex subunit 10, NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, cytochrome c oxidase subunit 7A2, cytochrome c oxidase protein 20 homolog, and electron transfer flavoprotein subunit alpha. This is in agreement with earlier studies that reported exercise-induced remodeling of the cardiac mitochondrial proteome (Sun et al., 2008; Kavazis et al., 2009), along with exercise-induced reprogramming of the cardiac mitochondrial phosphoproteome (Ferreira et al., 2014), suggesting that these changes contribute to the cardioprotective phenotype. Mitochondria are one of the main sites of intracellular ROS production and, thus, the main contributors to exercise-induced oxidative stress. Exercise-induced oxidative stress could lead to elevated 4-HNE modulating various cellular processes. Acute 4-HNE exposure can lead to transient adaptation (Al-Menhali et al., 2020), while 4-HNE accumulation, reaching larger concentrations, can have negative effects on cells (Elrayess et al., 2013). Indeed, our recent work has demonstrated an important function of 4-HNE on the regulation of mitochondrial metabolism and cellular energy production as well as in inducing adaptation when present at physiological levels (23). In addition, calcium-dependent upregulation of the mitochondrial metabolism involves lipid peroxidation and, in particular, 4-HNE, which might explain 4-HNE modification of calcium-dependent protein binding protein transport protein Sec31A. Exercise-induced 4-HNE modification of desmoplakin and lysine-specific demethylase 2B could suggest 4-HNE involvement in cardiac development (Garcia-Gras et al., 2006). 4-HNE modification of coronin-1C, also observed in this study, was reported to have a role in phagocytosis (Chacko et al., 2016), which is increased by exercise. Vascular endothelial growth factor receptor 2 (VEGFR2) is another protein modified by 4-HNE in the left ventricles of trained mice. Low levels of 4-HNE (0.5–5 μM) upregulate VEGF in retinal endothelial cells and were suggested to promote angiogenesis (Vatsyayan et al., 2012); however, at high levels, 4-HNE (75 μM) was shown to downregulate the expression of VEGFR2, decreasing angiogenesis (Roy and Palaniyandi, 2020). Still, the underlying mechanisms of the role of 4-HNE in the
CONCLUSION

In summary, this study highlights the significant effect of exercise training on mitochondrial biogenesis and the antioxidant defense system. Among the biochemical pathways regulated by proteins upregulated after long-term intensive exercise are respiratory electron transport and glutathione conjugation, while identification of proteins modified by 4-HNE suggest its role in adaptation. These data provide further evidence for the adaptive and beneficial role of physical exercise in maintaining cardiovascular health.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/pride/archive/, PXD026558.

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ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Welfare and Ethical Review Committee of the University of Manchester (PBA6A87CA).

AUTHOR CONTRIBUTIONS

MJ, AG, AA, and AD’S designed the study. CA and AD’S performed the animal experiments and collected the samples. ASA-M and MJ performed the proteomics experiments and analyzed the data. AA and MJ supervised the progress of the proteomics work. ASA-M wrote the initial draft of the article with the support of MJ. ASA-M, AG, AD, and MJ prepared the final version of the manuscript. All authors approved the final version of the article.

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TABLE 3 | 4-HNE-modified proteins in the left ventricular samples of the control and exercised groups. The table shows the UniProt accession number, main description, gene coding of the protein, number of amino acids (AAs), molecular weight (MW), and number of samples in the control or exercised group having the same protein.

| UniProt accession | Description | Gene | # AAs | MW [kDa] | Number of positive samples |
|------------------|-------------|------|-------|---------|---------------------------|
| P04247 | Myoglobin | Mb | 154 | 17.1 | 6 3 |
| O55143 | Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 | Atp2a2 | 1,044 | 114.8 | 6 5 |
| P19137 | Laminin subunit alpha-1 | Lama1 | 3,084 | 338 | 5 5 |
| P58854 | Gamma-tubulin complex component 3 | Tubgcp3 | 905 | 103.4 | 7 n.d |
| A7L528 | Calcium-transporting ATPase type 2C member 2 | Atp2c2 | 944 | 102.5 | 5 n.d |
| E9O2A2 | Zinc finger protein 644 | Zfp644 | 1,323 | 148.3 | 6 n.d |
| Q60739 | BAG family molecular chaperone regulator 1 | Bag1 | 355 | 39.7 | 5 n.d |
| Q80Y72 | Cystatin-like 1 | Cstl1 | 140 | 16.2 | 5 n.d |
| D3YWD3 | Transmembrane protein 245 | Tmem245 | 880 | 97.7 | n.d 6 |
| K7NB5 | Predicted gene 8,011 (fragment) | Gm8011 | 207 | 24.8 | n.d 5 |
| Q9DB54 | Oligoribonuclease, mitochondrial | Rlxo2 | 237 | 26.7 | n.d 5 |
| E9QB10 | Complement factor H | Cfh | 1,252 | 141.2 | n.d 5 |
| Q34PL0 | Protein transport protein Sec31A | Sec31a | 1,230 | 133.5 | n.d 5 |
| P35918 | Vascular endothelial growth factor receptor 2 | Kdr | 1,367 | 152.4 | n.d 5 |
| Q5CFN8 | Serine/threonine-protein kinase pim-1 | Pim1 | 313 | 35.4 | n.d 5 |
| Q9WUM4 | Coronin-1C | Coro1c | 474 | 53.1 | n.d 5 |
| E9KS77 | Desmoplakin | Dsp | 2,883 | 332.7 | n.d 5 |
| Q6IPG2 | Lysine-specific demethylase 2B | Kdml2b | 1,309 | 149.6 | n.d 5 |

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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