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Effect of a 12-Week Endurance Training Program on Circulating Extracellular Vesicle Proteome in Sedentary Adults With Obesity

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ABSTRACT

Systemic inflammatory state found in obesity increases the risk of developing numerous diseases. While endurance training seems effective to reduce this inflammation, the underlying mechanisms are not fully understood. Among those, extracellular vesicles (EVs) have been proposed to be actors in the anti-inflammatory intercellular crosstalk induced by exercise training. This study aimed to investigate how endurance training modulates the EV proteome in the context of an inflammatory state in adults with obesity. Thirteen lean sedentary adults and 10 sedentary adults with obesity participated in a 12-week endurance training programme. Skeletal muscle, abdominal subcutaneous adipose tissue and venous blood samples were taken prior to and after the training period. The systemic and adipose tissue inflammatory states were assessed, and plasma EVs were isolated by size exclusion chromatography. EV content was analysed by mass spectrometry. EVs isolated from the medium of myotubes stimulated by electrical pulse stimulation in vitro were quantified, and their content was analysed by western blot. After the endurance training, C-reactive protein (CRP) levels decreased in participants with obesity. In abdominal subcutaneous adipose tissue, the phosphorylation state of nuclear factor-kappa B (NF-κB) was not affected by training, but interleukin (IL)-6 and IL-1β protein levels were reduced after the 12 weeks in both groups. Conversely, interferon gamma (IFNγ) level reduction was exclusively found in the obesity group. Despite no changes in EV abundance, EV proteome was modified by training. Among the modified proteins in participants with obesity, the antioxidant enzyme peroxiredoxin (PRDX) 1 abundance was increased after training. Additionally, the PRDX1 content of EVs isolated from stimulated myotubes was increased compared to control conditions. In conclusion, our results suggest that the anti-inflammatory effects of exercise training are not directly mediated by EV anti-inflammatory proteome changes. However, exercise training increases circulating EV antioxidant content, possibly through contractile activity of skeletal muscle during repeated exercise.

1 | Introduction

The World Health Organization defines obesity as 'a chronic complex disease defined by excessive fat deposits that can impair health' (WHO 2025). Obesity prevalence has dramatically increased in the last 50 years worldwide. With the current trend, a global prevalence could reach 18% in men and 21% in women by 2025 (WHO 2016). This represents a real epidemiologic problem, as obesity is a major risk factor for several noncommunicable diseases (WHO 2025). Indeed, excessive fat accumulation triggers local white adipose tissue inflammation and ectopic lipid deposition in non-adipose metabolically important tissues, ultimately leading to systemic low-grade inflammation (Longo et al. 2019; Reilly and Saltiel 2017). This systemic inflammatory state then increases the risk of developing cardiovascular diseases, diabetes, musculoskeletal disorders, and some cancers (WHO 2025; Pahwa et al. 2023; Unamuno et al. 2018). In order to reduce these local and systemic inflammatory signatures, non-pharmacological interventions such as caloric restriction and exercise are usually advised by practitioners (You et al. 2013). Endurance exercise alone or combined with resistance training appears to be an effective method to reduce low-grade systemic inflammation (You et al. 2013; Gonzalo-Encabo et al. 2021). Although the underlying mechanisms are not fully understood, exercise training could reduce local/systemic inflammation through (1) the acute release of interleukin (IL)-6 by skeletal muscle; (2) the reduction of hypoxia within the adipose tissue; (3) the reduction of adhesion molecules produced by endothelial cells; (4) the modulation of immune cell function characterized by a decrease in pro-inflammatory monocytes, an increased M2/M1 macrophage polarization ratio and an increase in circulating regulatory T immune cells (You et al. 2013; Metsios et al. 2020).

Beyond those mechanisms, extracellular vesicles (EVs) have been recently proposed to be actors in the anti-inflammatory intercellular crosstalk induced by exercise training (Catitti et al. 2022). While EV cargo seems to be altered with metabolic impairments such as insulin resistance and type 2 diabetes, exercise could restore a healthy and functional cargo (Mastrototaro and Roden 2024). By transporting exerkines during exercise, EVs could participate in the inflammation reduction observed after a training period. For instance, Sullivan et al. (2022) have shown that a single week of concurrent exercise training was sufficient to modulate muscle-derived EV cargo in individuals with obesity (Sullivan et al. 2022). In response to training, miRNA EV content related to IL-10, IL-6, toll-like receptor and nuclear factor-kappa B (NF-κB) signalling pathways was modified (Sullivan et al. 2022). In obese mice, an 8-week treadmill endurance training resulted in a significant modulation of EV miRNA content as well (i.e., reductions in miR-22-3p, -122 and -192 levels) (de Mendonca et al. 2020). This downregulation might, in part, explain the positive outcomes measured at the adipose level, as those miRNAs are associated with impaired adipogenesis, increased insulin and increased fat mass. Indeed, after training, an increased number of small adipocytes and a reduced number of larger adipocytes, increased expression of adipogenesis markers, and increased activity of lipogenesis enzymes were observed (de Mendonca et al. 2020). Those adaptations might in turn contribute to the reduction of adipose tissue inflammation (Longo et al. 2019; Reilly and Saltiel 2017). In humans with obesity exhibiting insulin resistance, it was also shown that a 12-week high-intensity interval training (HIIT) could modulate EV proteome (Apostolopoulou et al. 2021). Additionally, an upregulation of the 20S core proteasome complex was observed, which could reduce inflammation via degradation of NF-κB. While systemic inflammation was not addressed in that study, skeletal muscle NF-κB expression was decreased after the training period (Apostolopoulou et al. 2021). This led the authors to hypothesize that EV content may target specific myocellular signalling pathways and thus reduce inflammation. Although growing evidence suggests the involvement of EVs in the anti-inflammatory effect of exercise (Catitti et al. 2022; Sullivan et al. 2022; Apostolopoulou et al. 2021), our understanding remains limited. Here, we investigated the modulation of circulating EV proteome by endurance training in the context of an inflammatory state in sedentary adults with obesity.

Our research revealed several key findings. First, we observed that low-volume endurance training (90 min·week^{-1}) effectively reduced systemic inflammation, independent of any weight or fat mass loss. Second, after the 12-week training programme, EV proteome abundance and diversity were significantly modulated. Third, endurance training was found to regulate the antioxidant EV content rather than pro- or anti-inflammatory proteins. Lastly, we found that contractile activity increased the antioxidant content of EVs released from human myotubes.

2 | Materials and Methods

2.1 | Participants

Twenty-three sedentary male adults (30–55 years old) were recruited based on their body mass index (BMI) (between 20 and 28 kg·m^{-2} or above 30 kg·m^{-2}). Only male participants were included in this study to reduce hormonal variability and isolate the effects of endurance training on EV proteome modulation. All participants gave their written consent to voluntarily participate in the experiment, which was approved by the ethical committee of the UCLouvain (2021/22MAR/134) and conducted in accordance with the Declaration of Helsinki. A medical check-up was organized, and exclusion criteria for participation were set as regular physical activity (90 min/week), diagnosed cancer, diabetes, untreated thyroid condition, liver and renal diseases, long-term use of anti-inflammatory medication (e.g., chronic use of anti-rheumatics or analgesics), steroids and any health risk that could compromise participant's safety during maximal oxygen uptake ($\dot{V}O_2$ peak) testing. After a DEXA scan analysis (HOLOGIC, Discovery W [S/N 84165]), participants were separated into two groups, lean or with obesity (Table S1), based on their fat percentage, as BMI is often misleading (De Lorenzo et al. 2019; Okorodudu et al. 2010). Indeed, individuals with high adiposity but low muscle mass can be misclassified as normal with BMI, as this indicator does not take body composition into account. Individuals above 25% of fat percentage were therefore classified as individuals with obesity (Macek et al. 2020). The participants were asked to maintain their usual dietary habits through the study period and to avoid unusual activities the day before sample collection or testing visits. Of the 23 participants involved, three could not complete the entire study protocol due to COVID, COVID quarantine, and personal reasons, respectively.

2.2 | Experimental Protocol

The study was conducted between September 2021 and June 2022 in two experimental periods. During each period, 3 phases were organized: 3 visits of pre-testing, 12 weeks of training, and 2 visits of post-testing (Figure S1). During the first visit, participants came fasted to the lab to undergo a medical check-up with the physician to ensure their eligibility for the study. A first blood sample was collected to assess their metabolic and inflammatory status. The second visit consisted of a body composition analysis followed by an incremental exercise test performed on a cycle ergometer (Cyclus II; RBM Electronics, Leipzig, Germany) to assess aerobic fitness, as determined by $\dot{V}O_2$ peak and peak power output (PPO). The starting load was set at 70 W and was incremented by 30 W every 2 min until exhaustion. Heart rate (HR) (Polar Team System 2; Polar Electro, Kempele, Finland) and respiratory exchanges (Ergocard Clinical, Medisoft, Sorinnes, Belgium) were continuously monitored through the test. Finally, for the last visit of the pre-test period, blood, skeletal muscle, and abdominal subcutaneous adipose tissue samples were collected at rest in a fasted state. After those three test visits, the participants followed a supervised training on a cycle ergometer 3 times a week for 12 weeks. Each session lasted 30 min at an intensity of 60% of $\dot{V}O_2$ peak. The short duration of these sessions helped to limit time constraints, and the moderate intensity enabled obese people to tolerate exercise well (Baillot et al. 2021; Dalle Grave et al. 2011), while still being intense enough to induce adaptations (Gonzalo-Encabo et al. 2021). After 4 and 8 weeks, the intensity was increased to 70% and 80% of initial $\dot{V}O_2$ peak, respectively. At the end of the intervention period, body composition and aerobic fitness were re-evaluated, and 96 h after, blood, skeletal muscle and subcutaneous adipose tissue samples were collected at rest in a fasted state at the same time as performed in pre-testing.

2.3 | Blood, Skeletal Muscle and Adipose Tissue Sample Collection

Blood drawing from an antecubital vein of the forearm was performed in EDTA and heparin lithium tubes. Blood was then centrifuged for 15 min at 2000 × *g* at 4°C to obtain plasma. An extra 15 min centrifugation at 2000 × *g* was performed for EDTA tubes to remove remaining platelets. Platelet-free plasma and heparinized plasma were frozen at −80°C until EV isolation and metabolic/inflammatory analysis, respectively.

Skeletal muscle biopsies were taken in the vastus lateralis according to the modified Bergström technique with suction (Tarnopolsky et al. 2011). After local anaesthesia (xylocaine 1% without epinephrine, Aspen, Dublin, Ireland), a 5 mm incision was made with a scalpel, and pressure was applied for 10 min to reduce bleeding. The Bergström needle was then inserted into the incision, at least 1 cm beyond the fascia, to reach the muscle, and three successive cuts were performed to obtain ~100 mg of sample. The samples were immediately frozen in liquid nitrogen and stored at −80°C. Due to high thigh adiposity, skeletal muscle biopsies could not be taken in 3 participants with obesity ($n = 7$). Skeletal muscle biopsy could not be taken in one lean participant as well ($n = 12$).

Subcutaneous adipose tissue biopsies were collected from the periumbilical abdominal subcutaneous adipose tissue depot using a Menghini liver biopsy set (Hepafix, Braun, Germany). The tissue samples were immediately placed in saline solution to remove blood. The tissue was then frozen in liquid nitrogen and stored at −80°C. Due to the procedure duration, subcutaneous adipose tissue biopsies were taken in 8 lean participants and 7 participants with obesity.

2.4 | Metabolic and Inflammatory Status

The plasma levels of blood glucose, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and C-reactive protein (CRP) were assessed using an automatic analyser (PENTRA C200, HORIBA Medical, Japan). The plasma levels of insulin were determined by ELISA with an ultra-sensitive insulin kit from Mercodia (Winston Salem, NC). Both analyses were performed on heparinized plasma.

Insulin sensitivity was estimated by the homeostatic model assessment (HOMA) and quantitative insulin sensitivity check index (QUICKI). Those indexes were calculated from fasting blood samples. HOMA of insulin resistance (HOMA-IR) and β-cell function (HOMA-β) were calculated with the following formula: HOMA-IR = [fasting insulin ($mUI \cdot L^{-1}$) × fasting glucose ($mg \cdot dL^{-1}$)/405] and HOMA-β = [360 × fasting insulin ($mUI \cdot L^{-1}$)/fasting glucose ($mg \cdot dL^{-1}$) − 63] (Matthews et al. 1985). QUICKI was calculated with the following formula: 1/[log(fasting insulin) + log(fasting glucose)] (Katz et al. 2000). Plasma and subcutaneous adipose tissue levels of pro- and anti-inflammatory cytokines were analysed by electrochemiluminescence immunoassay (ECLIA) with the U-PLEX Proinflam Combo 1 kit from Meso Scale Discovery (MSD).

2.5 | EV Isolation and Concentration

The EV isolation and characterization were performed in accordance with the International Society for Extracellular Vesicles (ISEV) guidelines available at the time of the study (Théry et al. 2018). The procedure was performed on platelet-free plasma obtained from blood collected in EDTA tubes. Apoptotic bodies and large vesicles were removed from platelet-free plasma by differential centrifugation at 2000 × *g* and at 20,000 × *g*, respectively, for 1 h. The supernatant was then filtered through 0.22 µm syringe filters, and EV isolation was performed by size exclusion chromatography (SEC) using qEVoriginal SEC columns (Izon Science) (Figure 1A). One millilitre of platelet-free plasma was loaded on the column, and 500-µL fractions were collected according to the manufacturer's recommendations. Fractions 7 to 9 were considered EV-rich and protein- and lipoprotein-poor (Figure 1B, D), as described in a previous work of our group (Warnier et al. 2021). For each sample, two separate isolations by SEC were performed (2 × 1 mL of plasma). The duplicated (2 × 500 µL) fractions 7, 8, and 9 obtained after SEC were pooled and ultracentrifuged at 4°C for 18 h at 100,000 × *g* (TLA55 rotor, k-factor 66, Beckman). The supernatant was removed to concentrate the samples 10×, and the three pellet fractions (7, 8, and 9) were pooled to obtain one sample per participant for further western blot and mass spectrometry analyses (see below).

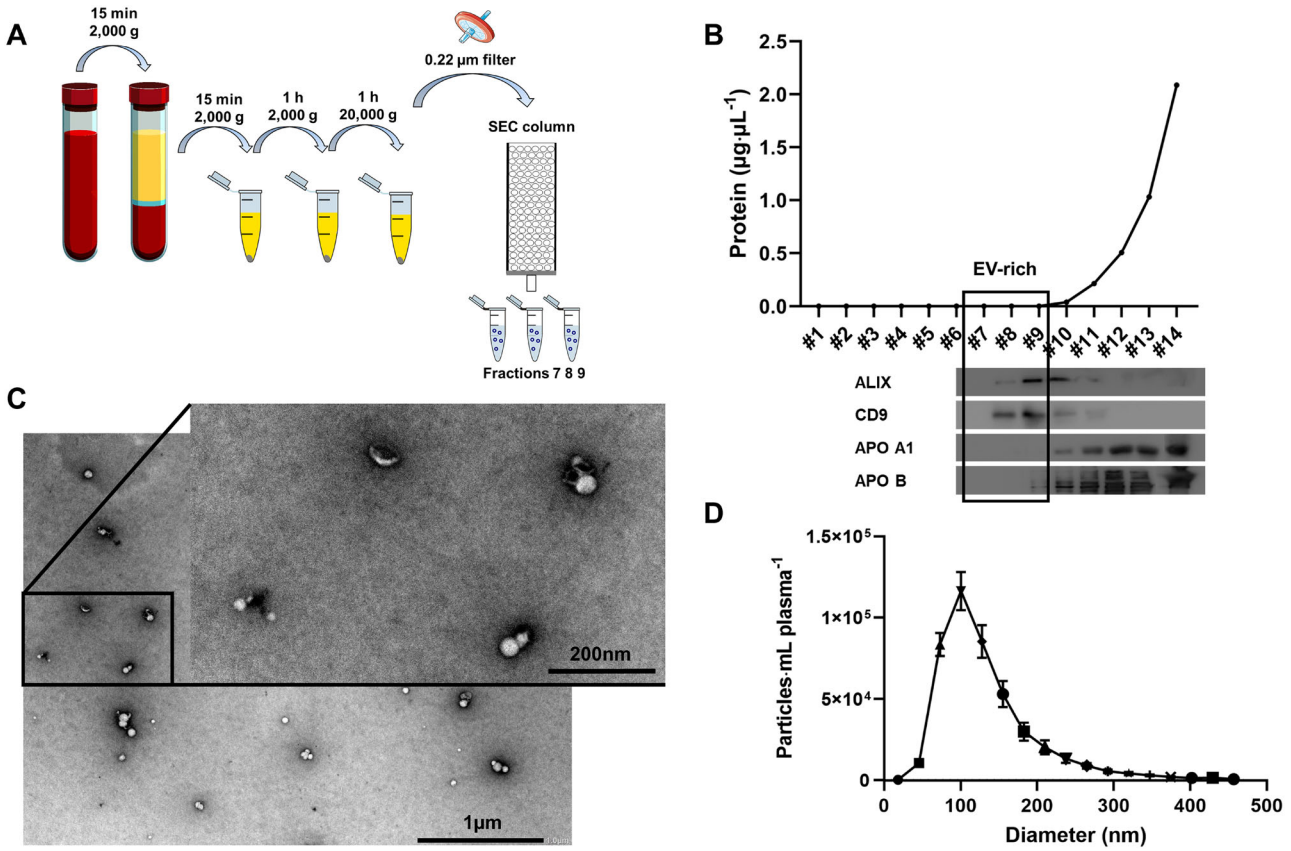

FIGURE 1 | Extracellular vesicle isolation method from plasma samples. (A) Differential centrifugations followed by filtration and size exclusion chromatography (SEC). (B) Protein concentration (line) on the 14 SEC fractions, combined with western blot analysis using EV-markers (ALIX and CD9) and Apolipoprotein A1 (APO A1) and B (APO B) as non-EV markers on fractions 7 to 14. Fractions 7 to 9 are considered EV-rich and protein- and lipoprotein-poor and were pooled. (C) Transmission electron microscopy image of the pooled fractions (negative staining), scale bar refers to 1 µm and 200 nm. (D) Particles size distribution in pooled fractions analysed by NTA.

2.6 | Nanoparticle Tracking Analysis (NTA)

Before ultracentrifugation, a small volume of samples (20 µL) from fractions 7, 8 and 9 were pooled for NTA. EVs were counted by the Zetaview (Particle Metrix, GmbH), which captures Brownian motion through a laser scattering microscope combined with a video camera to obtain size distribution (50–1000 nm) and concentrations. Samples were diluted in UltraPure DNase/RNase-free distilled water at 1:20–1:4000 to reach 50–200 particles/frame, corresponding to ~2.10^7–1.10^8 particles·mL^{-1}. Sensitivity was set to 80 and camera shutter to 100 to detect less than 5–10 particles/frame as background when UltraPure water alone was injected. Measurements were performed with medium resolution for 2 cycles at 11 different positions of the cell chamber containing the sample. The Zetaview software was used to generate results in text files.

2.7 | Transmission Electron Microscopy (TEM)

Before ultracentrifugation, EV images were taken by negative staining in TEM. Maze Ni-grids (formvar- and carbon-coated [thickness of carbon layer: 3 nm]) were used and were given glow discharge at 15 mA for 40 s. Aliquots (5 µL) of the sample were blotted on the grids held with self-locking tweezers. The samples were adsorbed on the grid for 1 min by leaving the drop on the coated side of the grid facing up and dabbed dry with filter paper. The grids were washed five times in droplets of Milli-Q water placed on a parafilm sheet. Excess liquid was dabbed dry with filter paper and stained with 25% uranyl acetate replacement stain (UAR-EMS, a non-radioactive substitute for uranyl acetate with comparable results) for 1 s and in a second drop of UAR for 1 min. Excess stain was removed with filter paper. Grids were air-dried for at least 4 h before imaging on the transmission electron microscope. Samples were viewed with a JEM 1400plus TEM (JEOL, Tokyo, Japan) operating at 80 kV.

2.8 | Mass Spectrometry Analysis

Each EV sample was transferred to 0.5 mL polypropylene Protein LoBind Eppendorf tubes and precipitated with 5 volumes of ice-cold acetone for 1 h at −20°C. Pellet was solubilized in denaturing lysis buffer containing 6 M urea and 2 M thiourea in 100 mM triethylammonium bicarbonate at pH 8.5 by 1 h vortexing at room temperature and was sonicated 2 × 5 min in an H-40 ultrasonic bath (EMAG Emmi) at 20°C. Proteins were reduced by adding 5 mM dithiothreitol and alkylated by adding 15 mM iodoacetamide. Sample was diluted 5 times in 100 mM triethylammonium bicarbonate at pH 8.5. Proteolysis was performed by adding 2 µg of trypsin overnight at 37°C. Each sample was dried under vacuum with the Savant Speed Vac Con-

centrator. Peptide separation was performed on 200 ng of digested proteins using nanoUPLC (Waters). For online liquid chromatography mass spectrometry (LC-MS) analysis, the nanoUPLC was coupled to the mass spectrometer through a nano-electrospray ionization (nanoESI) source emitter. Ion mobility separation—high-definition enhanced mobility separation (IMS-HDMS^E) analysis was performed on a SYNAPT G2-Si high-definition mass spectrometer (Waters) equipped with a NanoLockSpray dual electrospray ion source (Waters). HDMS^E data were processed with Progenesis QI (Nonlinear DYNAMICS, Waters) software using a species-specific database (UNIPROT). Venn diagrams were generated with the free web tool DeepVenn (Hulsen 2022). Signalling pathways were identified using PantherDB 18.0.

2.9 | Protein Isolation and Western Blotting

The samples (~20 mg of skeletal muscle and ~50 mg of abdominal subcutaneous adipose tissue) were homogenized with a Polytron mixer in ice-cold lysis buffer (20 mM Tris–HCl pH 7.0, 270 mM sucrose, 5 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM B-glycerophosphate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM dithiothreitol, 1% Triton-X 100 and a complete protease inhibitor tablet). Skeletal muscle samples were centrifuged at 10,000 × *g* for 10 min at 4°C, and the supernatant was aliquoted to be stored at −80°C. Subcutaneous adipose tissue samples were centrifuged at 20,000 × *g* for 10 min, and the infranatant (the transparent layer between the upper fat layer and the lower cell debris) was collected and stored at −(the transDC protein assay (Bio-Rad, Hercules, CA, USA) kit was used to determine the protein concentration of each sample using bovine serum albumin as a standard.

Twenty to forty micrograms of protein for skeletal muscle and subcutaneous adipose tissue samples and equal volumes of vesicle isolate were loaded for all samples. The proteins were separated by SDS-PAGE (8%–15% gels) and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon Transfer Membrane; Millipore). Membranes were blocked in tris buffered saline (TBS)-Tween containing 5% skimmed milk for 1 h at room temperature. Membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The following antibodies were applied: Apolipoprotein (APO) B (1:1000, Santa Cruz, sc-376818), APO A1 (1:1000, Santa Cruz, sc-393636), ALG-2-interacting protein X (ALIX) (1:500, Cell Signaling Technology (CST), E6P9B), cluster of differentiation (CD) 9 (1:1000, CST, D3H4P), CD63 (1:1000, Santa Cruz, sc-5275), CD81 (1:1000, CST, D3N2D), tumour susceptibility gene 101 (TSG101) (1:1000, Abcam, Ab30871), calnexin (1:1000, CST, 2433), LC3b (1:1000, Sigma, SAB4200361), TOM20 (1:1000, CST, 42406), peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1α) (1:1000, Santa Cruz, sc517380), oxidative phosphorylation (OXPHOS) (1:1000, Abcam, Ab110413), vascular endothelial growth factor A (VEGF-A) (1:500, Santa Cruz, sc-7269), citrate synthase (CS) (1:1000, CST, D7V8B), succinate dehydrogenase A (SDHA) (1:1000, Santa Cruz, sc-390381), extracellular signal-regulated kinase (ERK) 1/2 (1:1000, CST, L34F12), p-ERK 1/2 (1:1000, CST, D13.14.4E), NFκB (1:1000, CST, D14E12), p-NFκB (1:1000, CST, 93H1), hypoxia inducible factor 1 alpha (HIF-1α) (1:500, CST, D2U3T), peroxiredoxin (PRDX) 1 (1:1000, CST, D5G12), actin (1:5000, BD Biosciences, 612656) and eukaryotic translation elongation factor 2 (eEF2) (1:1000, CST, 2332). Membranes were thereafter scanned, and proteins were quantified with GeneSnap software and tools (Syngene, Cambridge, United Kingdom). Skeletal muscle proteins were normalized to eEF2, while subcutaneous adipose tissue proteins were normalized to actin. For the protein phosphorylation state, the phosphorylated form was reported to the total form if the latter was not impacted by training. If the total form changed in those conditions, only the phosphorylated form normalized to eEF2 was presented. For PRDX1 EV enrichment, PRDX1 abundance was normalized to the geometric mean of EV markers used for validation and quantification (ALIX, TSG101 and CD9).

2.10 | Oxidative Stress Assessment

The OxyBlot Protein Oxidation Detection Kit (Millipore) was used to detect the presence of carbonyl groups in protein side chains. Briefly, 10 µg of protein lysates were derivatized to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by reacting with a 2,4-dinitrophenylhydrazine solution for 15 min. Then, the solution was neutralized. The derivatized samples were separated by SDS-PAGE and transferred to a PVDF membrane, and a rabbit anti-DNP primary antibody was used to detect total protein carbonylation.

2.11 | RNA Isolation and Real Time-qPCR

About 15 mg of skeletal muscle sample and 50 mg of subcutaneous adipose tissue sample were homogenized in 1 mL Trizol reagent (Invitrogen, Merelbeke, Belgium) using a Polytron mixer. RNA isolation was achieved according to the manufacturer's instructions. RNA quality and quantity were assessed by Nanodrop spectrophotometry. Reverse transcription was performed from 1 µg RNA using the iScript cDNA Synthesis Kit from Bio-Rad Laboratories (Nazareth, Belgium), following the manufacturer's instructions. The primers used are presented in Table S2. RT-qPCR was run using the following conditions: 3 min at 95°C, followed by 40 cycles of 15 s at 95°C and 30 s at 60°C. All samples were run in duplicate, and each reaction was processed in a 10-µL volume containing 4.8 µL SsoAdvanced Universal SYBR Green SuperMix (Bio-Rad Laboratories), 0.1 µL of each primer (100 nM final), and 5 µL cDNA at the appropriate dilution. Melting curves were systematically performed for quality control. To compensate for variations in input RNA amounts and efficiency of reverse transcription, beta-2-microglobulin (B2M) for skeletal muscle and peptidylprolyl isomerase A (PPIA) for subcutaneous adipose tissue were quantified. The results of each gene of interest were normalized to the normalization genes (the expression of each was not affected by the experimental conditions).

2.12 | Cell Culture and Electrical Pulse Stimulation

Human satellite cells were isolated as routinely performed in the lab from the muscle vastus lateralis of healthy male adult donors (36 ± 12 years, 25 ± 2 kg·m^{-2}) and grown until myoblasts before freezing (Balan et al. 2020). Human myoblasts (passage

5) were seeded in 35 mm dishes in six-well plates and incubated at 37°C in a humidified air atmosphere with 5% CO_2. Cells were grown in DMEM (Life Technologies) supplemented with 20% FBS, 1% penicillin/streptomycin, and 0.5% Ultroser G. When cells were 90% confluent, the proliferation medium was replaced by a differentiation medium (DMEM) containing 2% FBS and 1% penicillin/streptomycin. The differentiation medium was replaced every 2 days. On differentiation day 5, the medium was replaced by an EV-depleted medium in all plates. Fully mature myotubes were stimulated with electrical pulse stimulation (EPS, 1 Hz, 10 V, 2 ms for 24 h) using the C-Pace EP Cell culture stimulator, with C-dish and carbon electrodes (IonOptix, Milton, MA, United States), or kept unstimulated in the control conditions. Thereafter, the culture medium was collected for EV isolation and analysis. The above experiment was conducted five times ($n = 5$). A video of contracting myotubes is available in online Supporting Information.

2.13 | EV Isolation From Culture Media and Analysis

For all five experiments, the medium from the six wells in each EPS plate and from the six wells in each control plate was pooled to become a single sample to get enough material. Thereafter, EVs were isolated from the culture medium by differential and ultracentrifugation (Théry et al. 2006). Cell debris, apoptotic bodies and large vesicles were removed by differential centrifugation at 300 × *g* for 10 min, at 2000 × *g* for 20 min and at 10,000 × *g* for 30 min, respectively. Ultimately, the supernatant was ultracentrifuged two times at 100,000 × *g* for 105 min (TLA55 rotor, k-factor 66, Beckman). The final pellet containing EVs was resuspended in 100 µL of phosphate-buffered saline (PBS).

The presence of EVs was confirmed by western blot, and sample purity was verified with markers of intracellular components (endoplasmic, autophagosome and mitochondrial). EV release was quantified by calculating the delta of EV markers between EPS and control conditions, normalized to the initial cell protein concentration. EV antioxidant enrichment was assessed based on the delta of PRDX1 abundance between EPS and control conditions, normalized to the geometric mean of EV markers used for quantification (ALIX, TSG101 and CD9).

2.14 | Statistics

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS v.25.0; IBM, Armonk, NY, USA). The normality of distribution was assessed via the Shapiro–Wilk test. For the participant characteristics, a comparison between groups before training was performed using the independent *t*-test or Mann–Whitney test based on normality. For training intervention, a linear mixed model was applied using time and group as fixed factors and the participants as random factor. Training and obesity effects were analysed for skeletal muscle, subcutaneous adipose tissue and plasma EVs. Statistical significance was set at $p \leq 0.05$, and post-hoc pairwise comparisons using the Sidak test were only applied when the interaction (time*group) was significant. For the cell culture experiments, data are presented as the delta between EPS and control conditions. A nonparametric one-sample *t*-test comparing mean delta with 0 was used to assess the EPS effect. The interquartile range (IQR) method was used to detect outliers. A value greater than 3rd quartile + 1.5IQR or less than 1st quartile − 1.5IQR was considered statistically abnormal and removed from the analysis. Graphs were created using GraphPad Prism 8.4.0. The data are reported as means (and individual points for graphs) ± SD. Specific statistical analyses were performed for proteomics using the R (version R-4.3.0) software. Protein abundances were $\log_2$-transformed and then normalized by the median. Differential abundance analyses were performed with the R package limma (Ritchie et al. 2015) to compare the training and the health conditions' effects based on contrasts and moderated *t*-statistics. The biological replicate was included as a blocking effect in the limma regression to adjust for differences between them. The obtained *p* values were adjusted with the false discovery rate. All tests were two-tailed. The resulting adjusted *p* values and $\log_2$ fold changes are represented in the volcano plots.

3 | Results

3.1 | Body Composition and Fitness Assessment

After 12 weeks of training, neither body weight nor fat percentage changed in either group. Regarding fitness level, although $\dot{V}O_2$ peak and PPO were found to be superior in the lean compared to the obesity group ($p < 0.001$; $p = 0.014$), the intervention resulted in a similar improvement of $\dot{V}O_2$ peak ($p = 0.016$) and PPO ($p < 0.001$) in both groups. Finally, the respiratory exchange ratio (RER) at different submaximal intensities, reflecting substrate utilization, significantly decreased after 12 weeks of training but only in participants with obesity ($p < 0.05$). All data can be found in Table S3.

3.2 | Metabolic and Systemic Inflammatory Status

Endurance training induced several adaptations regarding metabolism and systemic inflammation, mainly in individuals with obesity (Table 1). Fasting plasma insulin levels, higher in participants with obesity compared to lean participants ($p < 0.001$), were not modified by endurance training. In contrast, while glucose concentrations were not different between the groups, endurance training induced different responses in the lean and obesity groups (interaction, $p = 0.019$). Post-hoc analysis showed a difference between groups pre-training ($p = 0.005$) and increased concentrations after endurance training in lean participants only (+3%, $p = 0.048$). HOMA-IR and HOMA-β indexes, higher in participants with obesity ($p < 0.001$), were not affected by training. Moreover, QUICKI values were lower in participants with obesity compared to lean participants ($p < 0.001$) but were not modified by endurance training either. Finally, no differences were found for HDL and LDL following the training intervention.

Main effects of time (training: $p = 0.045$), group ($p < 0.001$) and interaction ($p = 0.030$) were found for plasma CRP levels, reflecting changes in the systemic inflammatory status over time and between groups (Luan and Yao 2018). Post-hoc analysis revealed that a difference between the two groups was present

TABLE 1 | Metabolic and inflammatory status.

Systemic metabolic and inflammatory status							
	Lean		**Obesity**		***p* value**		
	Pre-training (*n* = 13)	**Post-training (*n* = 11)**	**Pre-training (*n* = 10)**	**Post-training (*n* = 8)**	**Time**	**Group**	**TxG**
Insulin ($mU \cdot L^{-1}$)	4.6 ± 1.9	4.9 ± 1.8	11.5 ± 5.5	7.7 ± 3.5	0.087	< 0.001	0.053
Glucose ($mg \cdot dL^{-1}$)	105.1 ± 6.9	111.7 ± 6.5 *	115.5 ± 7.9 ##	110.0 ± 8.9	0.831	0.083	0.019
HOMA-IR	1.3 ± 0.6	1.3 ± 0.5	3.4 ± 1.8	2.2 ± 1.2	0.098	< 0.001	0.057
HOMA-β (%)	33.6 ± 9.4	36.2 ± 13.3	76.7 ± 29.6	56.5 ± 20.5	0.146	< 0.001	0.062
QUICKI	0.38 ± 0.04	0.37 ± 0.02	0.33 ± 0.03	0.35 ± 0.03	0.521	< 0.001	0.193
HDL ($mmol \cdot L^{-1}$)	1.2 ± 0.3	1.3 ± 0.3	1.1 ± 0.2	1.1 ± 0.1	0.527	0.055	0.831
LDL ($mmol \cdot L^{-1}$)	2.9 ± 0.5	3.3 ± 0.7	3.5 ± 0.9	3.4 ± 0.7	0.685	0.128	0.325
CRP ($mg \cdot L^{-1}$)	0.5 ± 0.4	0.6 ± 0.5	3.5 ± 2.8 ##	1.5 ± 1.0 # **	0.045	< 0.001	0.030
Subcutaneous adipose tissue inflammation							
	Lean		**Obesity**		***p* value**		
	Pre-training (*n* = 6)	**Post-training (*n* = 6)**	**Pre-training (*n* = 7)**	**Post-training (*n* = 7)**	**Time**	**Group**	**TxG**
IL-6 ($pg \cdot mL^{-1}$)	5.9 ± 4.8	2.4 ± 2.2	12.1 ± 11.1	2.7 ± 1.8	0.025	0.221	0.262
IL-2 ($pg \cdot mL^{-1}$)	8.0 ± 11.2	2.5 ± 1.8	21.5 ± 16.6	3.1 ± 3.0	0.012	0.111	0.139
IL-1β ($pg \cdot mL^{-1}$)	6.3 ± 4.1	5.2 ± 2.9	13.6 ± 8.3	6.2 ± 0.9	0.049	0.055	0.140
IFNγ ($pg \cdot mL^{-1}$)	76.4 ± 41.4	64.0 ± 37.4	183.5 ± 116.6	55.8 ± 15.0 **	0.020	0.083	0.047
IL-8 ($pg \cdot mL^{-1}$)	5.7 ± 1.2	7.1 ± 4.7	15.4 ± 9.3	8.2 ± 3.3	0.258	0.041	0.096
IL-12p70 ($pg \cdot mL^{-1}$)	4.6 ± 3.0	8.9 ± 6.8	6.9 ± 3.9	9.9 ± 4.6	0.065	0.377	0.723
IL-4 ($pg \cdot mL^{-1}$)	1.7 ± 1.1	3.2 ± 2.5	2.6 ± 1.3	3.4 ± 1.7	0.095	0.431	0.581
IL-10 ($pg \cdot mL^{-1}$)	5.2 ± 3.0	4.3 ± 2.8	10.6 ± 6.9	4.7 ± 0.9	0.058	0.096	0.147
IL-13 ($pg \cdot mL^{-1}$)	4.9 ± 6.9	2.0 ± 4.5	28.8 ± 27.2	6.6 ± 17.4	0.111	0.074	0.212
TNF-α ($pg \cdot mL^{-1}$)	3.2 ± 2.4	5.8 ± 4.7	5.8 ± 3.3	8.0 ± 5.9	0.199	0.210	0.906

Note: Values are means ± SD.

Abbreviations: CRP, C-reactive protein; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-β, homeostasis model assessment of insulin resistance beta cells function; IFNγ, interferon gamma; IL, interleukin; LDL, low-density lipoprotein; QUICKI, quantitative insulin sensitivity check index; TNF-α, tumour necrosis factor alpha.

Sidak post-hoc are indicated in the table as follow: *$p \leq 0.05$; **$p \leq 0.01$ vs. pre-training; #$p \leq 0.05$; ##$p \leq 0.01$ vs. lean at same timing.

both pre- ($p = 0.002$) and post-training ($p = 0.027$). Training reduced CRP levels only in the obesity group (−57%, $p = 0.006$).

3.3 | Subcutaneous Adipose Tissue Inflammatory State

Inflammation within the subcutaneous adipose tissue was assessed via the measurement of pro- and anti-inflammatory cytokine levels (Table 1). In response to endurance training, IL-6, IL-2, IL-1β and IFNγ levels decreased ($p < 0.05$). More particularly, IFNγ levels were modified by training ($p = 0.020$), being reduced only in the obesity group (−70%, $p = 0.003$). No training effect was found for IL-8, but there was a significant difference between the groups ($p = 0.041$), with higher values measured in participants with obesity. Finally, abdominal subcutaneous adipose tissue levels of IL-12p70, IL-4, IL-10, IL-13 and TNF-α were neither modified by training nor by the obesity status.

3.4 | Skeletal Muscle Adaptations

The protein expression of PGC-1α was not changed by endurance training (Figure S2A). The expression of OXPHOS complexes III and V was modulated in a distinct manner between the two groups (interaction, CIII: $p = 0.003$; CV: $p = 0.007$). Post-hoc analysis revealed an increased expression of OXPHOS CIII in participants with obesity only (+52%, $p = 0.008$; Figure S2B). Conversely, OXPHOS CV expression decreased in the lean group only (−26%, $p = 0.014$; Figure S2C). The expression of the other complexes remained unchanged after training (Figure S2D–F). The expression of CS (Figure S2G), SDHA (Figure S2H), the phosphorylated form of ERK1/2 (Figure S2I) and the expression of VEGF-A (Figure S2J) were not modified after training. Finally, the inflammation pathway, assessed by the phosphorylation state of NF-κB (Lawrence 2009), was not modified after 12 weeks of training (Figure S2K). Macrophage infiltration, indirectly measured by the mRNA levels of CD68, was unaffected by training (Figure S2L). Finally, we did not observe any effects of obesity or training on protein carbonylation (Figure S2M).

3.5 | Subcutaneous Adipose Tissue Adaptations

After 12 weeks of training, PGC-1α mRNA levels were not modified by training (Figure 3A). OXPHOS CII was not modified by training, but a significant interaction effect was found ($p = 0.032$). The post-hoc analysis revealed higher values in lean participants pre-training only ($p = 0.036$; Figure S3B). In a similar way, lower expression of OXPHOS CIII was found for participants with obesity compared to lean participants ($p = 0.018$; Figure S3C). The protein expression of OXPHOS complexes I, IV and V was not modified by training (Figure S3D–F). The protein expression of CS was not modified in response to training either but was lower in participants with obesity compared to lean participants ($p = 0.045$; Figure S3G). The protein expression of SDHA, VEGF-A, and HIF-1α, as well as the mRNA levels of CD68 and protein carbonylation, were similar between the two groups and remained unchanged after training (Figure S3H–L). The phosphorylation state of NF-κB did change after training but was higher in lean participants compared to those with obesity ($p = 0.046$; Figure S3M).

3.6 | EV Biogenesis Pathway

In skeletal muscle, the expression of the different proteins involved in EV biogenesis was not modified by training (Figure 2A, D). However, CD9 expression was superior in the lean compared to the obesity group (training: $p = 0.035$; Figure 2A). TSG101 mRNA levels decreased after 12 weeks of training ($p < 0.001$; Figure 2E). CD9, CD81 and ALIX mRNA levels remained unchanged after training and were not modified by obesity (Figure 2F, H). In subcutaneous adipose tissue, none of the different proteins involved in EV biogenesis were modified in response to training (Figure 2I, L), nor were the mRNA levels of CD81, ALIX, TSG101 and CD9 (Figure 2M, P).

3.7 | Circulating EV Regulation

Endurance training did not change the number of circulating particles (pre-training vs. post-training ± SD; lean: $2.1 \pm 4.3 \times 10^{13}$ vs. $6.1 \pm 8.4 \times 10^{13}$; obesity: $1.7 \pm 2.4 \times 10^{13}$ vs. $6.5 \pm 1.2 \times 10^{13}$). No differences between the groups could be detected either. The median diameter of particle size did not change after training, but the values were lower in the obesity group compared to the lean one ($p = 0.026$; Figure 3A). ALIX, CD9, CD81 and TSG101 protein abundance did not change in response to training. No differences between the groups could be found either (Figure 3B, E).

In addition to the modulation of circulating vesicle number, the context of obesity and inflammation led us to consider the EV content with particular attention. A total of 580 different proteins were identified using mass spectrometry. When analysing circulating EV proteome pre-training, participants with obesity and lean participants exhibited distinct profiles in both variety and abundance. We identified 285 proteins in lean participants and 198 proteins in participants with obesity (Figure 4A). Among the 166 proteins found in the two groups, the abundance of 4 was upregulated and the abundance of 12 was downregulated in the obesity group compared to the lean group (Figure 4B). The biological processes of those 16 proteins were related to biological regulation (GO:0065007), cellular process (GO:0009987), homeostatic process (GO:0042592), immune system process (GO:0002376), metabolic process (GO:0008152) and response to stimulus (GO:0050896). The molecular functions were associated with binding (GO:0005488), catalytic activity (GO:0003824), and molecular function regulator activity (GO:0098772). More specifically, participants with obesity had a higher abundance of syntenin-1 and a lower abundance of HSP70, two proteins involved in EV formation. Additionally, the abundance of proteins related to glycolysis, antioxidant process and immunity, alpha-enolase, peroxidoxin-6, immunoglobulins and alpha-2-macroglobulin-like protein 1 was downregulated in participants with obesity compared to the lean participants. All proteins regulated pre-training can be found in Table 2.

After 12 weeks training, we observed changes in variety and abundance of the EV proteome in our two groups. In lean participants, we identified 79 proteins that were exclusively found

TABLE 2 | List of modified EV protein abundance in participants with obesity versus lean participants pre-training.

Gene	Protein	Uniprot accession	Obesity vs. lean adjusted *p* value	Obesity vs. lean $\log_2$ fold change
IGKV3-7	Probable non-functional immunoglobulin kappa variable 3-7	A0A075B6H7; A0A0C4DH55; A0A0C4DH90	0.00051	−2.47
HSPA1A	Heat shock 70 kDa protein 1A	P0DMV8; P0DMV9; P17066	0.00275	−1.18
PTPRC	Receptor-type tyrosine-protein phosphatase C	P08575	0.00275	−1.45
PRDX6	Peroxiredoxin-6	P30041	0.00275	−1.55
SDCBP	Syntenin-1	O00560	0.00317	1.09
APOC3	Apolipoprotein C-III	P02656	0.00642	1.33
C16orf54	Transmembrane protein C16orf54	Q6UWD8	0.00642	−1.85
A2ML1	Alpha-2-macroglobulin-like protein 1	A8K2U0	0.00642	−1.55
ENO1	Alpha-enolase	P06733	0.00642	−1.22
COCH	Cochlin	O43405	0.00925	1.68
IGKV3D-11	Immunoglobulin kappa variable 3D-11	A0A0A0MRZ8; P04433	0.0096	−1.4
IGHV5-51	Immunoglobulin heavy variable 5-51	A0A0C4DH38; A0A0J9YXX1	0.01082	−1
H2AC19	Histone H2A type 2-A	Q6FI13; P04908; P0C0S8; P20671; Q16777; Q7L7L0; Q93077; Q96KK5; Q99878; Q9BTM1; P16104; Q96QV6	0.01082	1.3
IGKV1-39	Immunoglobulin kappa variable 1-39	P01597; P04432; A0A0C4DH72; P01601	0.01225	−1.18
TGM3	Protein-glutamine gamma-glutamyltransferase	Q08188	0.02584	−1.44
CALML5	Calmodulin-like protein 5	Q9NZT1	0.03759	−2.05

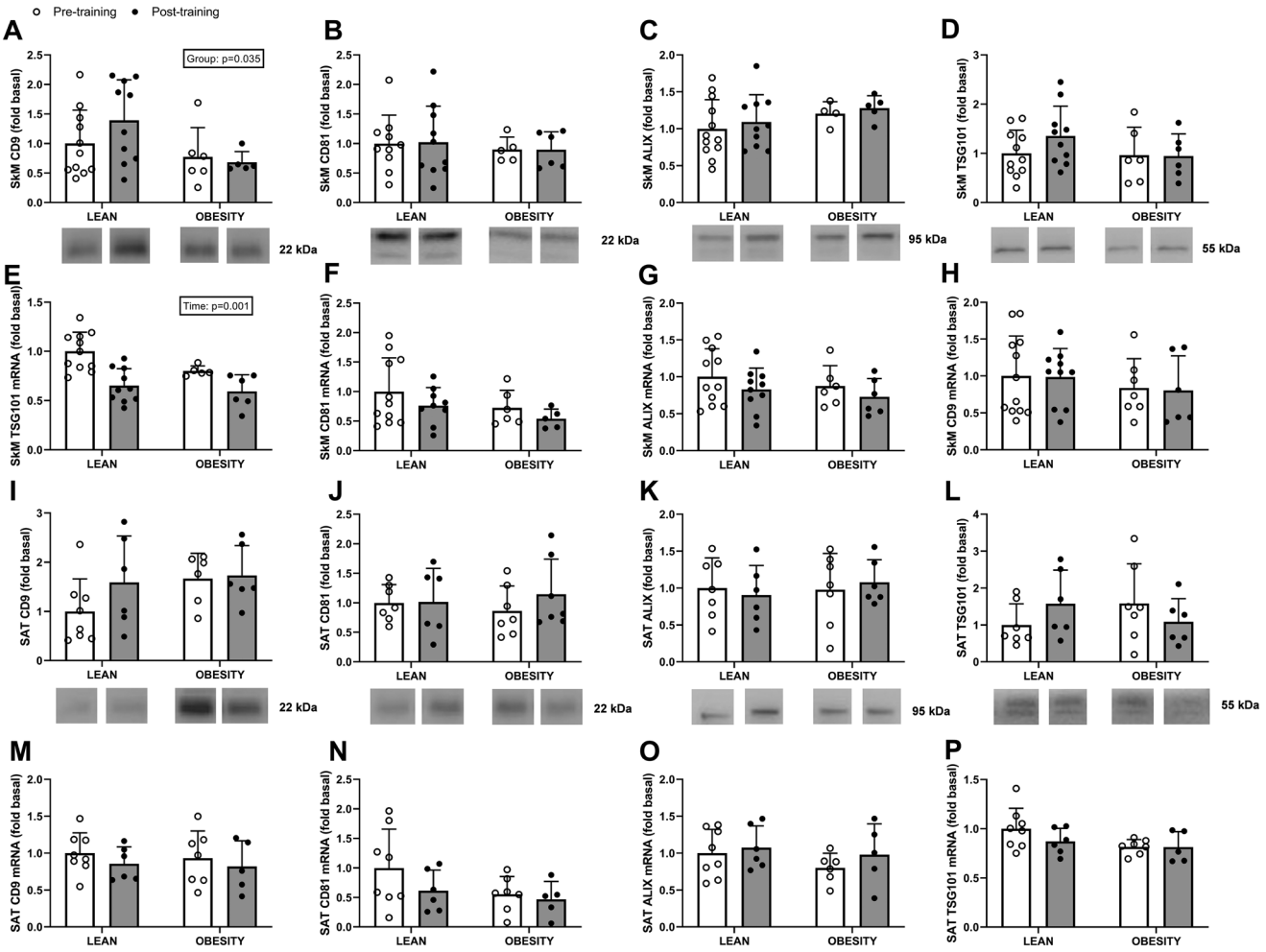

FIGURE 2 | Effects of obesity and training on the extracellular vesicle biogenesis pathways. Protein and mRNA expression pre- and post-training in skeletal muscle (SkM) and subcutaneous adipose tissue (SAT). Values are presented as individual values and means ± SD. The boxes contain the main statistical group and time (training) effects. 'Fold basal' means fold changes compared to the pre-training value from the lean group.

after training (Figure 4C). Among the proteins shared between pre- and post-training conditions, the abundance of 6 proteins was regulated (Figure 4D). The abundance of mitogen-activated protein kinase (MAPK) 15 and cochlin was upregulated whereas the abundance of alpha-enolase, desmoglein-1, immunoglobulin kappa variable 1–39 and putative lipocalin 1-like protein 1 was downregulated. In the obesity group, of the 246 proteins identified after the training period, 81 were exclusively found in that condition (Figure 4E). Additionally, the abundance of two proteins was regulated with an upregulation of PRDX-1 and a downregulation of probable non-functional immunoglobulin kappa variable 3–7 (Figure 4F). We further validated the abundance of PRDX1 in circulating EVs using western blot (Figure 4G).

Finally, similarly to pre-training, participants with obesity and lean participants exhibited distinct EV proteome abundance after training. Among the 170 proteins found in the two groups, the abundance of 6 was upregulated, and the abundance of 9 was downregulated in the obesity group compared to the lean one (Figure 4H, I). All proteins regulated post-training can be found in Table 3.

3.8 | Effect of Electrical Pulse Stimulation on EV Antioxidant Content Released by Human Myotubes

The presence of EVs in the cell culture medium was confirmed by the presence of the positive markers ALIX, TSG101, CD9, CD63 and CD81 (Figure 5A). Additionally, the samples were considered pure, as markers of the intracellular components calnexin, LC3B and TOM20 were undetectable (Figure 5A). EPS did not induce any increase in EV release from myotubes (Figure 5B). However, EV PRDX1 content was increased with EPS (+78%, $p = 0.046$; Figure 5C) and tended to be increased (+21%, $p = 0.08$; Figure 5C) when normalized to the geometric mean of EV markers.

4 | Discussion

The aim of this study was to assess the modulation of EV proteome by endurance training in the context of an inflammatory state in sedentary adults with obesity. The main results included: (1) low-volume (90 min·week^{-1}) endurance training is efficient to reduce inflammation independently of weight/fat mass loss; (2)

TABLE 3 | List of modified EV protein abundance in participants with obesity versus lean participants post-training.

Gene	Protein	Uniprot accession	Obesity vs. lean adjusted *p* value	Obesity vs. lean $\log_2$ fold change
IGKV3-7	Probable non-functional immunoglobulin kappa variable 3-7	A0A075B6H7; A0A0C4DH55; A0A0C4DH90	< 0.0001	−3.75
PRDX1	Peroxiredoxin-1	Q06830	< 0.0001	2.44
PTPRC	Receptor-type tyrosine-protein phosphatase C	P08575	< 0.0001	−2.21
NFIC	Nuclear factor 1 C-type	P08651	0.00016	−1.4
CTSD	Cathepsin D	P07339	0.00294	1.14
HRG	Histidine-rich glycoprotein	P04196	0.00305	−1.32
KCTD12	BTB/POZ domain-containing protein KCTD12	Q96CX2	0.00406	1.12
PRDX6	Peroxiredoxin-6	P30041	0.00275	−1.55
AMY1B	Alpha-amylase 1B	P0DTE7; P0DTE8; P0DUB6; P19961; P04746	0.00984	−1.96
HBA2	Haemoglobin subunit alpha	P69905	0.01788	1.01
EEF1A1	Elongation factor 1-alpha 1	P68104; Q5VTE0; Q05639	0.01788	−2.85
TGFBI	Transforming growth factor-beta-induced protein ig-h3	Q15582	0.02002	1.26
IGHV3-7	Immunoglobulin heavy variable 3-7	P01780	0.02537	1.24
H4C16	Histone H4	P62805	0.04246	−2.15
C4B_2	Complement C4-B	P0C0L5	0.04639	−1.23

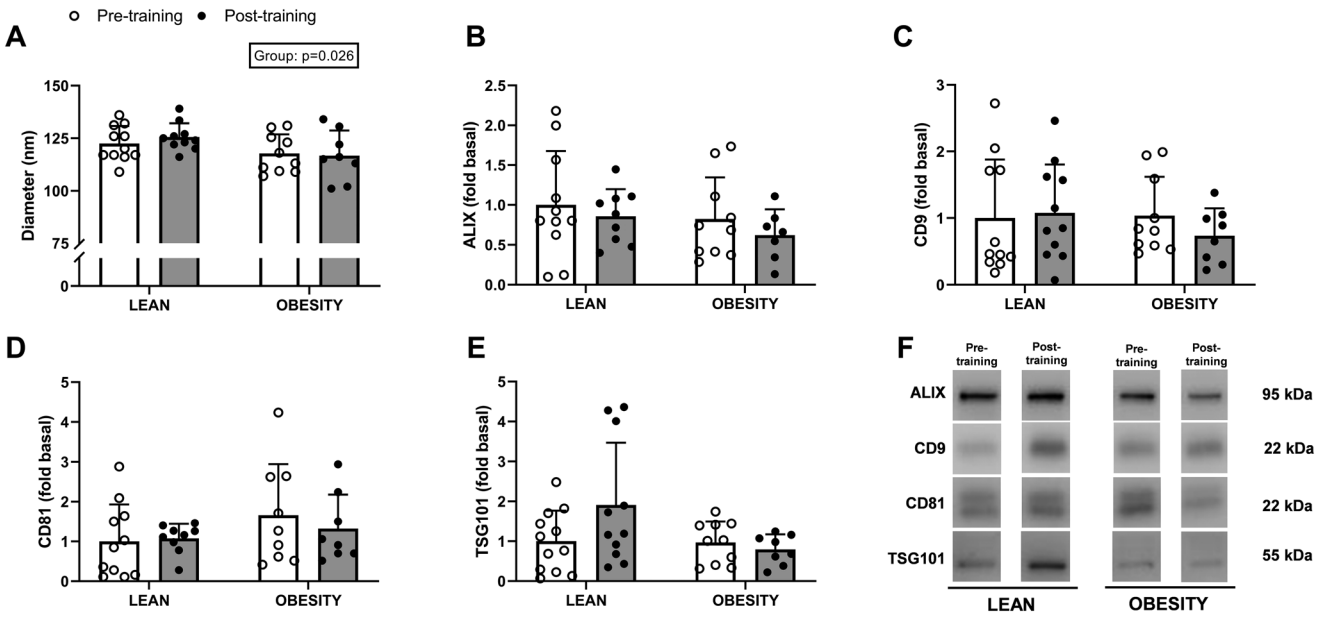

FIGURE 3 | Effects of obesity and training on circulating extracellular vesicles. (A) Particle median diameter pre- and post-training. (B–E) EV marker protein abundance pre- and post-training. Values are presented as individual values and means ± SD. The boxes contain the main statistical group effects. (F) Representative western blot analysis of circulating EVs (pooled fractions 7–9) using EV markers. "Fold basal" means fold changes compared to the pre-training value from the lean group.

12-week training modulated circulating EV proteome abundance and diversity; (3) endurance training seems to regulate antioxidant EV content rather than pro- and anti-inflammatory proteins in individuals with obesity; (4) contractile activity increases the antioxidant content of EVs released from human myotubes.

At the end of the 12-week endurance training, neither body weight nor fat percentage changed. Due to the relatively low volume (90 min·week^{-1}) and the diet maintenance, our study did not intend to reduce fat mass. Although fat mass loss may be an effective strategy for reducing inflammation, the aim of the present study was to investigate the training effects without specifically looking for a reduction in fat mass. Regarding fitness level, similarly to the literature (van Baak et al. 2021), we observed an increase in $\dot{V}O_2$ peak (~4 mL·min^{-1}·kg^{-1}) in both groups. Beyond $\dot{V}O_2$ peak, we also found submaximal adaptations with decreased RER in participants with obesity, indicating an improved lipid metabolism during exercise. Such adaptation is commonly found in individuals with obesity after endurance training (Bruce et al. 2006; Jabbour and Iancu 2017; Venables and Jeukendrup 2008). Despite trends to interaction effects for insulin, HOMA-IR and HOMA-β with decreased values in participants with obesity, no major metabolic adaptations occurred after training. For a more marked improvement in insulin sensitivity, longer duration, higher volume, higher intensity or a combination of these may be required (Houmard et al. 2004; Kang et al. 1996).

At a systemic level, low-grade inflammation, assessed by CRP levels, decreased after training in participants with obesity (−57%). This result, although expected, goes in line with the meta-analysis of Gonzalo-Encabo and colleagues showing decreased levels of CRP (from −9% to −53%) in response to exercise training of different durations (8 weeks to 2 years) (Gonzalo-Encabo et al. 2021). The NF-κB pathway is known to be involved in the development of adipose tissue inflammation in obesity (Longo et al. 2019; Reilly and Saltiel 2017). However, when looking at the NF-κB phosphorylation state in adipose tissue, participants with obesity exhibited lower levels when compared to lean participants. Additionally, the training intervention did not induce significant reduction of NF-κB phosphorylation, indicating that local inflammation reduction might be related to the immune environment rather than adipocyte inflammation per se. Of note, although NF-κB is a key piece in the inflammation response, other components, including but not limited to MAPK and signal transducer and activator of transcription (STAT) pathways or the inflammasome, are involved (Vandanmagsar et al. 2011; Chen et al. 2018). Together, our results highlight that low-volume, moderate-intensity endurance training is efficient to reduce systemic inflammation in individuals with obesity. Moreover, as no significant fat loss was observed, this reduced inflammation might be related to other processes, possibly through EVs.

Contrary to acute exercise, for which well-designed studies are available (Warnier et al. 2021; Brahmer et al. 2019; Frühbeis et al. 2015), the understanding of how long-term exercise regulates basal circulating EV levels is far from clear. Contrasting results from rodent studies indicated that chronic interventions (2 to 8 weeks) could either result in an increase (Bei et al. 2017; Bertoldi et al. 2018; Chaturvedi et al. 2015) or no changes (Hou et al. 2019; Ma et al. 2018) in basal circulating vesicles. However, among those studies, very heterogenous methods were applied, the collection period was not always stated and the ISEV guidelines were rarely entirely followed (Nederveen et al. 2020). In the present study, particle diameter was lower (−8 nm) in participants with obesity in comparison with their lean counterparts. While it was previously shown that obesity leads to the release of specific EV subsets characterized by typical EV marker enrichment and specific content (Camino et al. 2022; Camino et al. 2020), the

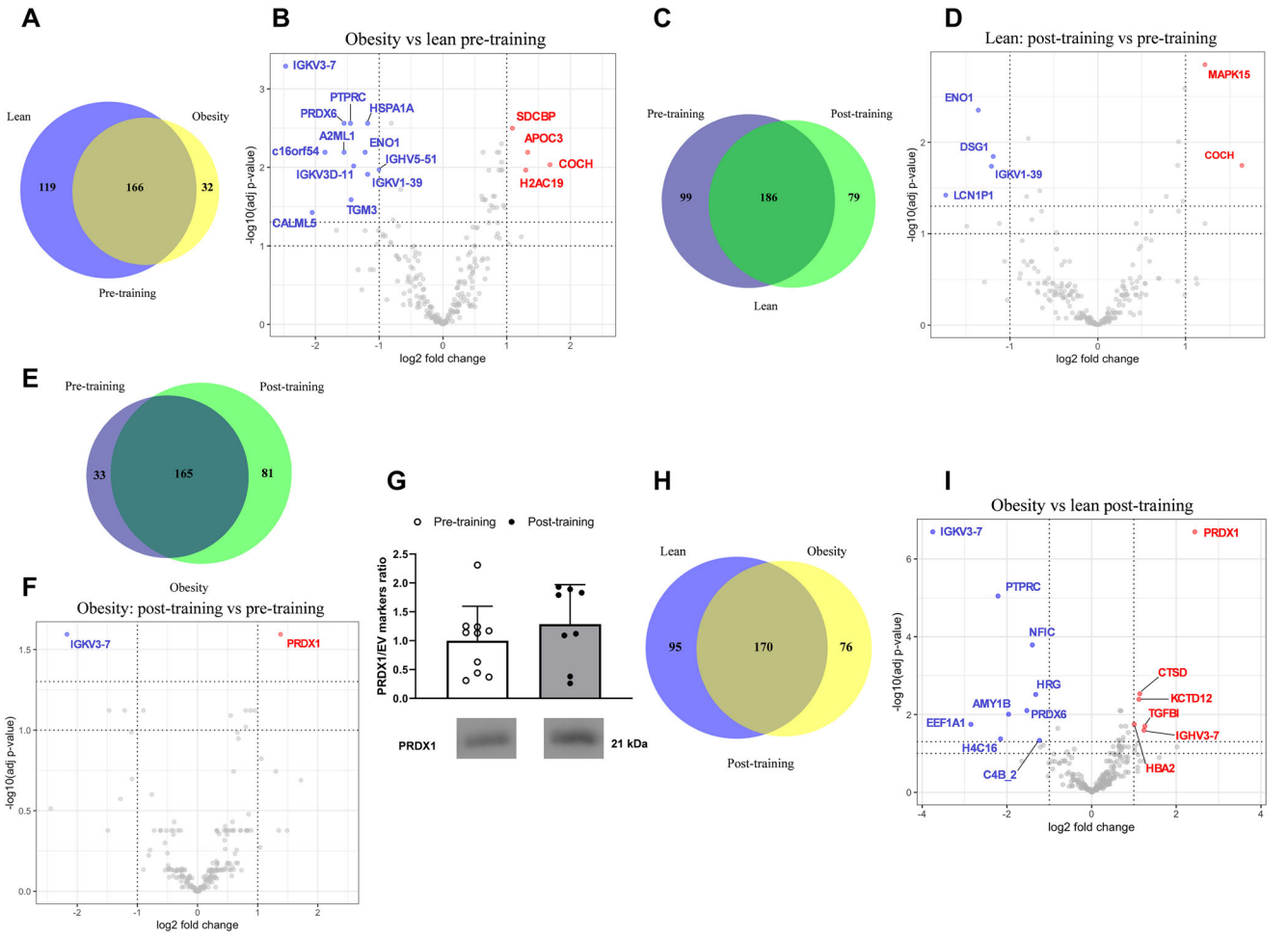

FIGURE 4 | Effects of obesity and training on circulating extracellular vesicle proteome. (A) Venn diagram of EV proteome overlap between lean participants and participants with obesity pre-training. (B) Volcano plot of up- and downregulated proteins in participants with obesity versus lean participants pre-training. Red dots represent proteins that are more abundant (≥ 1-fold) and blue dots represent proteins that are less abundant (≤ −1-fold). (C) Venn diagram of EV proteome overlap between pre- and post-training in the lean group. (D) Volcano plot of up- and downregulated proteins after training in the lean group. Red dots represent proteins that are more abundant (≥ 1-fold) and blue dots represent proteins that are less abundant (≤ −1-fold). (E) Venn diagram of EV proteome overlap between pre- and post-training in the obesity group; (F) Volcano plot of up- and downregulated proteins after training in the obesity group. Red dots represent proteins that are more abundant (≥ 1-fold) and blue dots represent proteins that are less abundant (≤ −1-fold). (G) Extracellular vesicle enrichment in peroxiredoxin (PRDX) 1 pre- and post-training expressed as PRDX1/EV markers ratio. (H) Venn diagram of EV proteome overlap between lean participants and participants with obesity post-training. (I) Volcano plot of up- and downregulated proteins in participants with obesity versus lean participants post-training. Red dots represent proteins that are more abundant (≥ 1-fold) and blue dots represent proteins that are less abundant (≤ −1-fold).

slight difference observed in our study should be interpreted with caution given the large variability of EV size. Endurance training did not induce any changes in basal circulating EVs, considering EV marker abundance or particle count. This lack of change observed in response to training goes in line with a previous study from our group where trained cyclists and triathletes were recruited to participate in a 6-week sprint interval training (SIT) protocol at different simulated altitudes. After the training intervention, no clear changes in EV abundance could be noticed regardless of the altitude used (Warnier et al. 2023). Two other groups investigated the effects of chronic endurance exercise on basal circulating EV amount in humans (Apostolopoulou et al. 2021; Hou et al. 2019). On the one hand, the longitudinal study of Hou et al. (2019) showed no effect of regular training on vesicle quantity (Hou et al. 2019). On the other hand, Apostolopoulou et al. (2021) observed an increase, but exclusively in metabolic responders displaying improved insulin resistance after training (Apostolopoulou et al. 2021). In both cases, EVs were solely quantified by particle analysis, which limits the interpretation of the results (Nederveen et al. 2020). While the aforementioned results relate to endurance exercise (Apostolopoulou et al. 2021; Bei et al. 2017; Bertoldi et al. 2018; Chaturvedi et al. 2015; Hou et al. 2019; Ma et al. 2018; Warnier et al. 2023), two recent studies have been conducted with a resistance modality (Estebanez et al. 2021; Xhuti et al. 2023). In the first one, untrained older adults (males and females, 70–85 years old) participated in resistance training twice a week for 8 weeks (Estebanez et al. 2021). After the training period, the amount of circulating EVs remained unchanged as assessed by the abundance of the EV markers CD9, CD63, CD81 or Flot-1 (Estebanez et al. 2021). In the second study, Xhuti et al. (2023) proposed a 12-week home-based resistance exercise training for older men (~75 years old) (Xhuti et al. 2023).

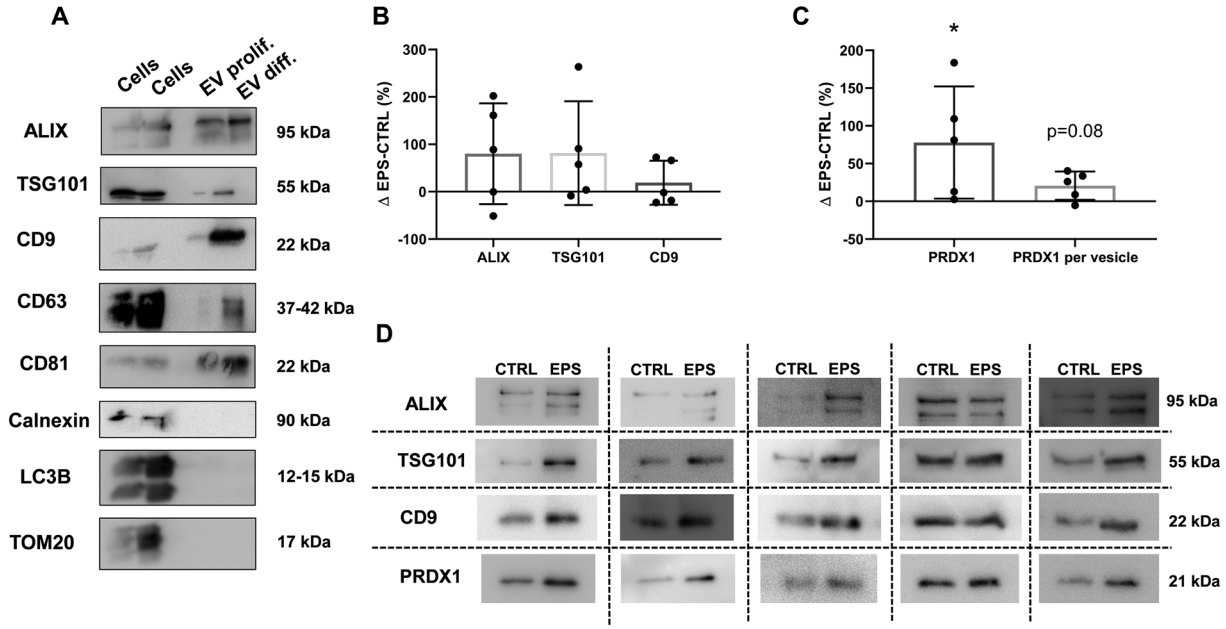

FIGURE 5 | Effect of electrical pulse stimulation on extracellular vesicle release and antioxidant content. (A) Western blot analysis of extracellular vesicles (EV) markers (ALIX, TSG101, CD9, CD63 and CD81) and non-EV intracellular markers (calnexin, LC3B and TOM20) in EV released from cells in proliferating (myoblasts) or differentiating cells (myotubes). (B) Delta between electrical pulse stimulation and control conditions for EV marker abundance (ALIX, TSG101 and CD9). (C) Delta between electrical pulse stimulation and control conditions for EV PRDX1 and EV PRDX1 normalized to the geometric mean of EV markers. (D) Western blot analysis of EV markers and PRDX1 content in all five cell culture experiments.

In response to this training intervention, the abundance of the EV marker TSG101 was increased (~40%), suggesting an increased amount of circulating EVs. While the load prescription is difficult to compare, the total volume (16 sessions of 24 sets vs. 36 sessions of 36 sets) might, in part, explain the differences observed on EV response, as training volume was almost 4 times greater in Xhuti's study (2023). Taken together, chronic endurance or resistance exercise might lead to a higher level of basal circulating EVs or at least of certain EV subpopulations/content. Whereas young and trained individuals might be less responsive, older adults displaying lower circulating vesicles or deconditioned subjects might experience an increase after several weeks of exercise training. In addition, a minimal threshold of training volume and intensity might be required to induce significant changes. In our case, 90 min per week at moderate intensity might have been an insufficient training dose to induce an increase in basal EV amount.

In line with the absence of changes in circulating EV amount, no major adaptations occurred in skeletal muscle or subcutaneous adipose tissue regarding EV biogenesis pathway markers. Where few studies reported skeletal muscle EV pathway modulation in response to acute exercise (Warnier et al. 2021; Garner et al. 2020; Garner et al. 2022), our data are the first on long-term training effects. To our knowledge, this is also the first investigation on the regulation of the human adipose tissue EV pathway. Further studies will be required to explore the regulation of EV biogenesis pathways by both exercise and training and obesity in various tissues, including but not limited to skeletal muscle and adipose tissue. Of note, caution should be taken when analysing complex tissues such as skeletal muscle and adipose tissue constituted of several other cell types (e.g., immune cells, endothelial cells, stem cells, fibroblasts, and mesenchymal progenitors).

Although an increase in the amount of circulating vesicles alone can result in beneficial adaptations (Bei et al. 2017), their content is no less important (Hou et al. 2019). Circulating vesicle cargo, such as miRNAs or proteins, can regulate gene expression and metabolism of the recipient cell/tissue (Thomou et al. 2017; Ying et al. 2017; Abdelsaid et al. 2022). In the exercise and inflammation context, it is therefore important to understand how EV cargo is modulated, as EVs could participate in positive adaptations by transporting specific molecules such as exerkines. When analysing blood samples taken pre-training, we found significant differences in EV proteome between the lean and obesity groups. While the two populations shared 166 EV-related proteins, 119 and 32 proteins were exclusively found in lean participants and participants with obesity, respectively. Moreover, the abundance of 16 proteins was altered in participants with obesity compared to the lean ones. Among those proteins, we found the EV markers HSP70 and syntenin-1, reinforcing once again the idea of specific EV subpopulation/content release between individuals with different metabolic statuses (Camino et al. 2022; Camino et al. 2020). The abundance of proteins involved in oxidative stress control, glucose metabolism and immunity was also altered in participants with obesity in comparison with lean participants. Those results confirm previous findings indicating that obesity and metabolic impairment lead to a modulation of circulating and tissue-derived EV content (Sullivan et al. 2022; Camino et al. 2022; Camino et al. 2020; Wang et al. 2023; Doncheva et al. 2022). For instance, Sullivan et al. (2022) showed that obesity alters the abundance of skeletal EV miRNAs targeting the insulin-like growth factor 1 (IGF-1), phosphoinositide 3-kinase (PI3K)/Akt, Wnt/β-catenin, and SERPINF-1 pathways (Sullivan et al. 2022). Obesity seems also to modulate adipose tissue-derived EV lipid composition. Known to be increased in the circulation during obesity, adipose tissue EVs could transport various lipids, includ-

ing triacylglycerol (Matilainen et al. 2024). Specifically looking at the training effects, our low-volume moderate-intensity training was sufficient to modulate the circulating EV proteome, despite the lack of changes in circulating EV abundance. Interestingly, the EV proteome was not only modulated in terms of abundance but also in terms of diversity. Seventy-nine and 81 proteins were exclusively found after endurance training in lean participants and participants with obesity, respectively. Training regulated the abundance of six proteins in lean participants, whereas only two were regulated in participants with obesity, suggesting that the training effects are impacted by the obesity status. Our results corroborate those from Apostolopoulou et al. (2021), who found that the proteins up- or downregulated after 12 weeks of HIIT were different based on the metabolic status. Specific patterns of modulation were found in patients with type 2 diabetes, insulin resistance and insulin sensitivity (Apostolopoulou et al. 2021). Together, our results confirm previous findings showing that training can induce significant changes in EV protein cargo loading (Apostolopoulou et al. 2021; Estébanez et al. 2023), as does a single bout of exercise (Abdelsaid et al. 2022; Chong et al. 2024; Kobayashi et al. 2021; Lisi et al. 2023). In addition to modulating EV proteome at rest, metabolic impairment seems to impact the EV response to endurance training in terms of cargo selection and transport.

The involvement of EVs in training-related inflammation reduction has been recently suggested in the literature (Catitti et al. 2022). However, the potential mechanisms are poorly described, and the role of the EV proteome remains hypothetical. When looking at the proteins regulated by training in our obesity group, we found none specifically involved in the inflammation process. On the one hand, we found that the abundance of probable non-functional immunoglobulin kappa variable 3–7, involved in immune response, was decreased after training. This finding confirms recent data from Chong et al. (2024) showing that EV transported proteins related to the immune system and that acute exercise modulated their EV abundance (Chong et al. 2024). Here, we found that such changes might last with regular exercise. On the other hand, after 12 weeks, the abundance of PRDX1 was increased. This result goes in line with two recent studies showing an upregulation of proteins involved in the antioxidant pathway by endurance training (Apostolopoulou et al. 2021; Abdelsaid et al. 2022). Abdelsaid et al. (2022) observed higher levels of EV superoxide dismutase (SOD) 3 (~100%) in exercised mice (2 weeks) compared to their sedentary counterparts (Abdelsaid et al. 2022). This effect of endurance training was also observed in the study conducted in humans by Apostolopoulou et al. (2021). The authors found that a 12-week HIIT training upregulated the abundance of EV PRDX-1 and 2 as well as SOD2 (Apostolopoulou et al. 2021). Taken together, the above results highlight a consistent antioxidant response within EVs with endurance training, whereas changes in anti-inflammatory proteins were not observed. Despite the current study not intending to assess the functional effect of exercise-derived EVs on recipient cells, we conducted an in vitro experiment to investigate the link between skeletal muscle contractions and the antioxidant response. In our experiment, PRDX1 EV content tended to increase by 21% after 24 h of EPS. This suggests that the systemic antioxidant response we found in EVs in vivo might be, in part, related to contractile activity. However, previous work has shown that skeletal muscle-derived EVs (SkM-EVs) account for only 1 to 5% of the total circulating pool (Estrada et al. 2022; Guescini et al. 2015). In this view, one may wonder whether muscle, through repeated contractions over time, is really a major contributor to the change observed in antioxidant content of EVs at a systemic level. From a mechanistic perspective, the elevation of reactive oxygen species in skeletal muscle cells during exercise might trigger specific cargo selection in EVs. Indeed, several studies in various cell types have shown that EV release and content are modulated under oxidative stress (Chiaradia et al. 2021). More specifically, some studies highlight that EVs released under oxidative stress carry antioxidant cargo (Mas-Bargues et al. 2023; Patel et al. 2017; Saeed-Zidane et al. 2017) and have antioxidant properties on recipient cells (Patel et al. 2017; Saeed-Zidane et al. 2017; Eldh et al. 2010; Shi et al. 2018). Despite the literature suggesting that oxidative stress could play an important role in EV content regulation in skeletal muscle, other processes related to muscle contraction could be involved as well. Ultimately, while a small proportion of those antioxidant-rich SkM-EVs might end up in the circulation, a vast majority would rather act in auto/paracrine manner within the muscle. Further studies are required to better understand the link between contractile activity, oxidative stress and vesicular content regulation.

Some limitations must be considered with regard to the presented results. The first limitation of our experiment resides in the relatively low number of participants. However, this number was sufficient to detect some differences in training-induced changes in the EV proteome, with distinct patterns between obese individuals and their lean counterparts. Second, only male participants were included. Knowing that both sex and menstrual phase seem to influence microvesicle release (Durrer et al. 2015; Lansford et al. 2016; Toth et al. 2007), it would be interesting to investigate whether it is also the case for smaller vesicles. Although some studies suggest differences in EV release during exercise between men and women (Conkright et al. 2022; Rigamonti et al. 2020), robust studies following international guidelines are still required. Finally, while the vesicle proteome changes and the systemic and local inflammation reduction were concomitant, causality could not be established. Future studies combining human and cellular experiments are needed to test the hypothesis that inflammation reduction is driven by EVs. Based on our results, underlying mechanisms might involve antioxidant enzymes.

In conclusion, low-volume endurance training seems to be an efficient strategy to reduce the general inflammatory status in sedentary adults with obesity, irrespective of fat mass loss. After 12 weeks of endurance training, EV proteome abundance and diversity were significantly modulated. Among those proteins, none were related to the inflammatory process. In contrast, the abundance of the antioxidant enzyme PRDX1 was increased in circulating EVs in participants with obesity in response to endurance training. Ultimately, in vitro experiments showed that human myotubes release EV with increased antioxidant content in response to EPS, suggesting that the changes observed at the systemic level might be partly related to repeated muscle activity.

Author Contributions

Geoffrey Warnier: methodology, investigation, validation, formal analysis, writing – original draft, writing – review and editing, data curation, project administration. **Sophie van Doorslaer de Ten Ryen**: data curation, investigation, writing – review and editing. **Camille Lannoy**: investigation, data curation, writing – review and editing. **Théo Mahy**: investigation, writing – review and editing, data curation. **Nancy Antoine**: data curation, investigation. **Emilien Boyer**: investigation, data curation. **Pascal Kienlen-campard**: validation, supervision, writing – review and editing. **Kenneth Verboven**: conceptualization, methodology, writing – review and editing, resources. **Sylvie Copine**: data curation, methodology, investigation, writing – review and editing, resources. **Marc Francaux**: methodology, writing – review and editing, resources, validation, formal analysis. **Louise Deldicque**: resources, formal analysis, project administration, supervision, methodology, conceptualization, investigation, funding acquisition, writing – original draft, writing – review and editing, validation.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting Materials: jex270087-sup-0001-VideoS1.mp4 Figure S1. Experimental protocol. Pre-test period consisted of three visits during which the eligibility to the study was assessed, body composition and fitness level were evaluated, and tissue collection (blood, skeletal muscle, and subcutaneous adipose) was performed. The 12-week endurance training was composed of three sessions of 30 min at 60% of $\dot{V}O_2$ peak from week 1 to 4, at 70% of $\dot{V}O_2$ peak from week 5 to 8 and at 80% of $\dot{V}O_2$ peak from week 9 to 12. At the end of the protocol, body composition and aerobic fitness were re-evaluated, and 96 h after, blood, skeletal muscle and subcutaneous adipose tissue samples were collected. Figure S2. Skeletal muscle adaptations in response to endurance training. Protein expression pre- and post-training in skeletal muscle. Values are presented as individual values and means ± SD. The boxes contain the main statistical group and interaction (group*time) effects. *$p < 0.05$, **$p < 0.01$ vs. pre-training; \$$p < 0.05$, \$\$$p < 0.01$ vs. lean group post-training. 'Fold basal' means fold changes compared to the pre-training value from the lean group. Panel N: representative western blots showing levels of protein carbonylation. Panel O: representative western blots. Figure S3. Subcutaneous adipose tissue adaptations in response to endurance training. Protein expression pre- and post-training in subcutaneous adipose tissue. Values are presented as individual values and means ± SD. The boxes contain the main statistical group and interaction (group*time) effects. *$p < 0.05$ vs. pre-training; \$$p < 0.05$ vs. lean group post-training. 'Fold basal' means fold changes compared to the pre-training value from the lean group. Panel N: representative western blots showing levels of protein carbonylation. Panel O: representative western blots. Supplementary table 1. Participant characteristics Supplementary Table 2. RNA sequence primers. Supplementary table 3. Body composition and fitness.