Free Radical Biology and Medicine 209 (2023) 282–291

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Antioxidant enzymes and Nrf2/Keap1 in human skeletal muscle: Influence of age, sex, adiposity and aerobic fitness

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ARTICLE INFO

Keywords:
Obesity
ROS
Free radicals
Aging
Nrf2
SOD
Catalase
Physical activity

ABSTRACT

Ageing, a sedentary lifestyle, and obesity are associated with increased oxidative stress, while regular exercise is associated with an increased antioxidant capacity in trained skeletal muscles. Whether a higher aerobic fitness is associated with increased expression of antioxidant enzymes and their regulatory factors in skeletal muscle remains unknown. Although oestrogens could promote a higher antioxidant capacity in females, it remains unknown whether a sex dimorphism exists in humans regarding the antioxidant capacity of skeletal muscle. Thus, the aim was to determine the protein expression levels of the antioxidant enzymes SOD1, SOD2, catalase and glutathione reductase (GR) and their regulatory factors Nrf2 and Keap1 in 189 volunteers (120 males and 69 females) to establish whether sex differences exist and how age, VO_2max and adiposity influence these. For this purpose, *vastus lateralis* muscle biopsies were obtained in all participants under resting and unstressed conditions. No significant sex differences in Nrf2, Keap1, SOD1, SOD2, catalase and GR protein expression levels were observed after accounting for VO_2max, age and adiposity differences. Multiple regression analysis indicates that the VO_2max in mL.kg LLM^{-1}.min^{-1} can be predicted from the levels of SOD2, Total Nrf2 and Keap1 ($R = 0.58$, $P < 0.001$), with SOD2 being the main predictor explaining 28 % of variance in VO_2max, while Nrf2 and Keap1 explained each around 3 % of the variance. SOD1 protein expression increased with ageing in the whole group after accounting for differences in VO_2max and body fat percentage. Overweight and obesity were associated with increased $pSer^{40}$-Nrf2, $pSer^{40}$-Nrf2/Total Nrf2 ratio and SOD1 protein expression levels after accounting for differences in age and VO_2max. Overall, at the population level, higher aerobic fitness is associated with increased basal expression of muscle antioxidant enzymes, which may explain some of the benefits of regular exercise.

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https://doi.org/10.1016/j.freeradbiomed.2023.10.393
Received 12 August 2023; Received in revised form 27 September 2023; Accepted 13 October 2023
Available online 17 October 2023

1. Introduction

Ageing, a sedentary lifestyle and obesity are associated with increased oxidative stress, a condition characterized by the oxidation of cellular structures due to an imbalance between oxidant and antioxidant mechanisms [1]. Regular exercise, via hormetic processes elicited by reactive oxygen and nitrogen species (RONS) bursts generated during muscle contractions, may cause an elevation of some antioxidant enzymes [2–4]. However, whether a greater enzymatic antioxidant capacity is necessary to reach a higher VO_2max remains unknown.

Abbreviations

AREs	antioxidant response elements
BMI	body mass index
CAT	catalase
GPx	glutathione peroxidase
GR	glutathione reductase
HRmax	maximal heart rate
Keap1	kelch-like ECH-associated protein 1
LLM	lower extremities lean mass
MHC I	myosin heavy chain type I
MHC IIa	myosin heavy chain type IIa
MHC IIx	myosin heavy chain type IIx
Nrf2	nuclear factor (erythroid-derived 2)-like 2
PKC	protein kinase C
RONS	reactive oxygen and nitrogen species
ROS	reactive oxygen species
SODs	superoxide dismutase
VO_2	oxygen uptake
VO_2max	maximal oxygen uptake
W	watts
Wmax	peak power output during the incremental exercise test to exhaustion

The antioxidant system comprises both enzymatic and nonenzymatic antioxidants. The most abundant enzymatic antioxidants in skeletal muscle are superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase [5]. Muscle fibres express the cytosolic (SOD1, 65–85 % of the total SOD activity present in skeletal muscle) and the mitochondrial (SOD2, 15–35 % of the total SOD activity present in skeletal muscle) isoforms of SOD [5]. The SODs catalyse the dismutation of O_2^- to produce H_2O_2, an oxidant converted into H_2O by the action of GPx and catalase [5], while glutathione reductase (GR) plays a critical role in free radical scavenging by catalysing the reduction of oxidized glutathione (GSSG) back to its active and reduced form (GSH).

These antioxidant enzymes are regulated by the nuclear factor erythroid-derived 2-like 2 (Nrf2) [6–8]. Nrf2 is a transcription factor that, under unstressed conditions, binds to Kelch-like ECH-associated protein 1 (Keap1), an adaptor protein for a ubiquitin E3 ligase complex that ubiquitinates Nrf2 for proteasomal degradation [9]. Thus, in unstressed situations, most Nrf2 is tagged for degradation. Under oxidative or electrophilic stress, Keap1 undergoes a conformational change that stabilizes the Keap1-Nrf2 complex, preventing the degradation of Nrf2 [10]. When the amount of free Keap1 is low, the newly synthetized Nrf2 remains free, and it moves into the nucleus, where it interacts with specific DNA sequences called antioxidant response elements (AREs), triggering the transcription of antioxidant genes [9,10]. Nrf2 can be phosphorylated at Ser^{40} by several ROS-sensitive kinases facilitating Nrf2 release from the Nrf2-Keap1 complex and its subsequent translocation to the nucleus for interaction with AREs [11].

By upregulating the antioxidant capacity of muscles, regular exercise may prevent oxidative stress [12]. Oxidative stress may be caused by increased production of RONS, reduced availability of antioxidants, or a combination of the two [5]. Oxidative stress is facilitated by sedentarism and has been reported in association with ageing, obesity, endothelial dysfunction, hypertension, insulin resistance and cancer [13–17]. However, subjects with better mitochondrial function and higher aerobic fitness are less prone to suffer the detrimental effects of oxidative stress [5]. Thus, part of the benefits of exercise in reducing the incidence of chronic diseases and facilitating healthy ageing may be related to its effects on the antioxidant enzymes and their regulatory factors. Aerobic fitness can be assessed by measuring maximal oxygen uptake (VO_2max), which provides an integrated assessment of the cardiorespiratory system and the capacity of the muscle to extract and utilize O_2. A high VO_2max is a distinctive characteristic of elite endurance athletes, whilst a low VO_2max is associated with shortened life expectancy [18] and increased mortality in clinical populations [19]. A low VO_2max may be due to a reduced O_2 delivery, as observed in patients with cardiorespiratory diseases or diminished O_2 utilization capacity due to a reduced muscle mass, capillarization or mitochondrial respiratory capacity, or a combination of them [20,21]. Despite the lower blood haemoglobin concentration in females, VO_2max values are similar in males and females when normalized to the lean mass of the lower extremities [22]. This is likely explained by females' higher O_2 extraction capacity, attributed to their superior mitochondrial respiratory capacity [23]. Some mitochondrial respiratory complexes and dehydrogenases generate O_2^-, which may damage mitochondrial structures and enzymes and hamper mitochondrial respiration and ATP generation [24]. The latter is efficiently prevented by mitochondrial and sarcoplasmic superoxide dismutases [5]. Research to date has not yet determined if a high level of mitochondrial antioxidant enzymes may be required for a high VO_2max.

Table 1
Physical characteristics, performance, and myosin heavy chain myosin composition (mean ± SD).

	Males (n = 120)	range	Females (n = 69)	range	*P*
Age (years)	31.4 ± 10.5	65.2–18.6	33.0 ± 10.5	54.9–18.2	0.311
Height (cm)	177.6 ± 7.4	198.0–161.0	163.6 ± 6.4	180.0–150.0	**0.000**
Weight (kg)	90.9 ± 18.0	136.9–55.9	81.5 ± 17.3	126.1–41.3	**0.001**
Body fat (%)	28.9 ± 9.4	45.6–7.7	42.5 ± 8.1	54.6–21.5	**0.000**
BMI ($kg.m^{-2}$)	28.7 ± 4.9	41.3–18.8	30.3 ± 5.7	44.7–16.2	**0.045**
Legs' lean mass (kg)	21.8 ± 2.9	30.0–15.1	15.7 ± 2.5	20.8–9.6	**0.000**
HRmax (beats. min^{-1})	187.4 ± 12.3	206.6–134.0	185.8 ± 13.1	212.0–149.0	0.443
VO_2max (mL. kg^{-1}. min^{-1})	38.0 ± 9.7	60.8–20.6	27.5 ± 7.5	47.6–12.6	**0.000**
VO_2max (mL. kg LLM^{-1}. min^{-1})	154.5 ± 26.9	224.8–106.3	137.9 ± 24.1	201.4–82.8	**0.000**
Wmax (W)	256.0 ± 42.4	380.0–157.3	170.5 ± 33.9	257.8–87.0	**0.000**
MHC I (%)	42.1 ± 15.1	76.6–16.5	53.5 ± 11.4	71.2–37.5	**0.013**
MHC IIa (%)	44.8 ± 11.8	70.3–15.7	36.3 ± 6.5	44.5–26.2	**0.015**
MHC IIx (%)	13.1 ± 10.3	39.9–0.0	10.2 ± 6.3	20.6–0.0	0.339

HRmax: maximal heart rate; VO_2max, maximal oxygen uptake; Wmax: maximal intensity during the incremental exercise test to exhaustion; LLM: lean mass of the lower extremities; MHC, myosin heavy chain composition (n = 57 for males, and 13 for females). P values based on two-tailed unpaired t-tests.

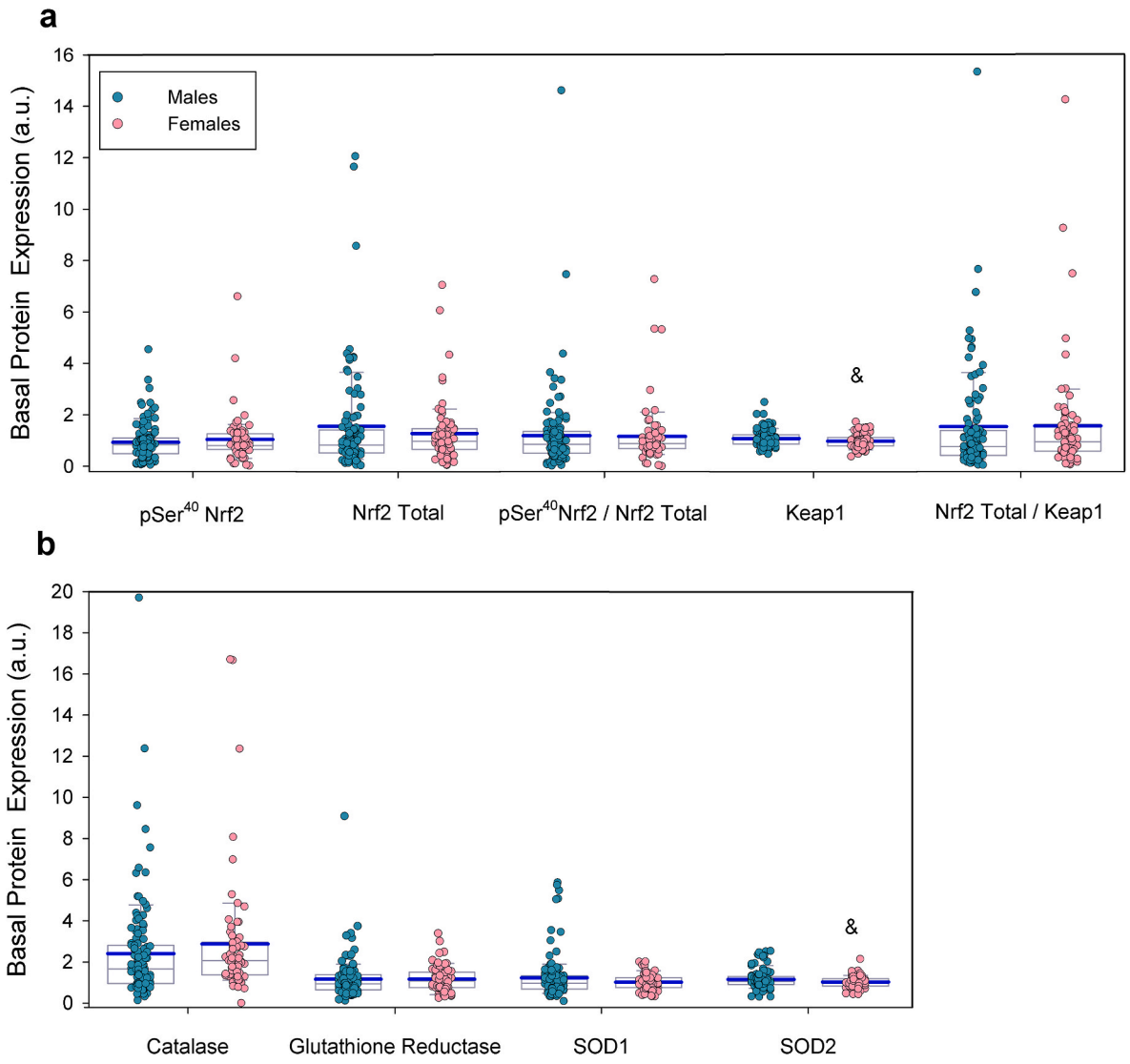

Fig. 1. Sex-related differences in protein expression levels of: pSer^{40}-Nrf2, total Nrf2, pSer^{40}-Nrf2/Nrf2 ratio, Keap1, and Nrf2/Keap1 ratio (a); catalase, glutathione reductase, superoxide dismutase 1 (SOD1), and superoxide dismutase 2 (SOD2) (b). n = 189 (120 males and 69 females) expressed in arbitrary units (a.u.). Box and whisker plots: the extremes of the whiskers represent the limits of the 5th and 95th percentiles, respectively; the thick and thin horizontal lines inside the boxes correspond to the mean and median values, respectively; and the lower and upper limits of the box delimit the 1st and 3rd quartiles, respectively. Male data are presented in green while female data are presented in pink. & $P < 0.05$ males compared to females. Statistical differences were not significant after accounting for differences in age and VO_2max in mL.kg lower extremities lean mass^{-1}.min^{-1}.

A previous study has reported a positive correlation between VO_2max in mL.kg^{-1}.min^{-1} and the skeletal muscle enzymatic activities of SOD and catalase in 12 males aged between 17 and 19 years [25]. These results need confirmation in a larger population sample, including males and females. Moreover, no study has determined if the basal levels of Nrf2 and Keap1 protein expression in human skeletal muscle are linked to VO_2max or are more influenced by other factors such as age, adiposity, or sex. It remains unknown whether there is sexual dimorphism in the expression of antioxidant enzymes in human skeletal muscle, as reported for some tissues in rodents [26]. In support, oestradiol stimulates the expression of enzymatic antioxidants in some cells [27,28], through an Nrf2-depending mechanism [29,30].

Thus, the primary aim of this study was to determine whether higher levels of VO_2max require an increased antioxidant capacity in human skeletal muscle, as assessed by examining the protein expression levels of Nrf2/Keap1 and antioxidant enzymes like SOD, catalase and GR in human skeletal muscle. A secondary aim was to determine whether there is a sexual dimorphism in the expression levels of Nrf2/Keap1 and antioxidant enzymes in human skeletal muscle. For this purpose, we obtained muscle biopsies and measured VO_2max and body composition in 189 volunteers to calculate the VO_2max per kg of lower extremities lean mass (LLM), a variable independent of body size and adiposity. Our central hypothesis is that a greater VO_2max and female sex would be associated with a higher expression of Nrf2 and antioxidant enzymes.

2. Materials and methods

2.1. Subjects

This is a study combining muscle biopsies obtained in previous research projects collected from 189 volunteers, 120 males and 69 females [31–36]. All volunteers were non-smokers. Among them, 55 were healthy university students with varying physical activity levels, while the remaining 148 predominantly led sedentary lifestyles and were either overweight or obese. Within the overweight or obese participants, hypertension (defined as a systolic blood pressure >130 or a diastolic >80 mmHg) was present in 27 of them. Four hypertensives were on diuretics, three on RAS inhibitors/blockers, and one was treated with a

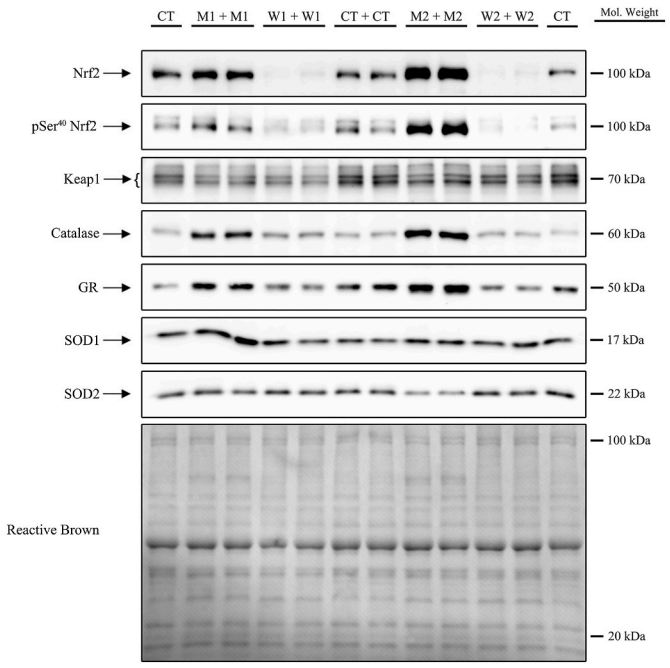

Fig. 2. Representative visual depictions of baseline protein expression levels were obtained using Western Blot analysis for the proteins under investigation. The total protein loaded onto the gels was evaluated through Reactive Brown Staining (last image). The figure represents 2 male and 2 female individuals. To ensure normalization and serve as a control for loading consistency, a non-experimental human sample was included in quadruplicate on each gel. The images presented in a top-to-bottom sequence encompass total Nrf2, $pSer^{40}$-Nrf2, Keap1, catalase, SOD1, SOD2, and Reactive Brown. The reference 'CT' denotes the control non-experimental sample, while 'M' and 'W' respectively indicate samples obtained from male and female participants. Molecular weights are displayed on the right side of the blot.

calcium antagonist. One participant had type 2 diabetes handled with diet and exercise, while two were on statins, as previously reported [37].

All female participants were premenopausal, as pre-established by the inclusion criteria of the original studies. Of these, three were using oral contraceptives. The experiments were conducted per the Declaration of Helsinki, except for registration in a database. All subjects signed a written informed consent before the start of the procedures.

2.2. Main procedures

All volunteers were instructed to abstain from exercise, alcohol, and caffeine intake 48 h before testing and muscle biopsy procedures. Additionally, participants were requested to refrain from taking nutritional supplements or vitamins throughout the study. Before initiating the experiments, the subjects were familiarized with the exercise tests. Subsequently, their anthropometric characteristics were documented, and body composition was assessed using dual-energy X-ray absorptiometry (Lunar iDXA, General Electric, Madison, WI, USA) [38]. Then, their VO_2max and maximal power output (Wmax) were determined using an incremental cycle ergometer exercise test (Lode Corival/Excalibur Sport, Groningen, The Netherlands). The incremental exercise test was customized to the volunteers' profile, with load increments designed to bring exhaustion between 6 and 20 min [39]. Oxygen uptake (VO_2) during all the exercise tests was monitored through open-circuit indirect calorimetry using metabolic carts (Vyntus, Jaeger-CareFusion, Hoechberg, Germany; Vmax N29, Sensormedics, Yorba Linda, CA, USA; COSMED, Rome, Italy; and Jaeger Oxycon Pro, Viasys Healthcare, Hoechberg, Germany) in breath-by-breath mode. Prior to each test, the gas analysers were calibrated following the manufacturer's guidelines. Respiratory variables were recorded breath-by-breath and averaged every 20 s. The peak 20-s averaged VO_2 was retained as the VO_2max and expressed per kg of leg lean mass.

2.3. Muscle biopsies

All participants were asked to refrain from intense physical exertion for a period of 48 h prior to the muscle biopsy, which was conducted following an overnight fast of 10–12 h. They were also instructed to avoid consuming carbonated, caffeinated and alcohol-containing beverages for 24 h before the tests. Muscle biopsies were obtained from the middle section of the muscle *vastus lateralis* muscle using Bergstrom's technique with suction. For this purpose, the skin was disinfected, and the skin and subcutaneous adipose tissue was infiltrated with local anaesthetic (1–2 mL of Lidocaine 2 % without epinephrine). Special care was taken to avoid injecting lidocaine beneath the superficial fascia. After a pause of 10 min, an incision of 6–7 mm was made, and the biopsy needle was inserted 2 cm deep into the muscle belly and 4 cm apart from the point of local anaesthesia. The procured muscle sample, approximately 100 mg, was meticulously rid of debris and fat tissue and subsequently flash-frozen in liquid nitrogen and preserved at −80 °C until further analysis.

Muscle lysates were prepared as described previously [40], and total protein concentration was quantified in triplicate using the bicinchoninic acid assay [41]. For this purpose, 10 mg of muscle were homogenized in urea lysis buffer (6 M urea, 1 % SDS), 50X Complete protease inhibitor and 10X PhosStop phosphatase inhibitor cocktails (Roche). Afterwards, the lysate was centrifuged for 12 min at 25,200 g at 16 °C and the obtained supernatant containing the protein fraction was diluted with electrophoresis loading buffer (160 mM Tris-HCl, pH 6.8, 5.9 % SDS, 25.5 % glycerol, 15 % β-mercaptoethanol- bromophenol blue). The amount of protein required for optimal resolution of the western blot analysis was determined by loading a gradient of control protein extracts (non-interventional human muscle prepared similarly to the experimental samples) in different amounts ranging from 1 to 35 μg, assayed with different antibody concentrations. Equal amounts of protein (1.5–15 μg), corresponding to the middle section linear antigen-antibody response, were loaded and electrophoresed with SDS-PAGE using the system of Laemmli [42]. Then, the proteins were transferred onto the polyvinylidene fluoride (PVDF) membranes for protein blotting (Bio-Rad Laboratories, Hercules, CA, USA). The samples from each subject were run onto the same gel with an equal protein amount from an internal control (same used for linearity optimization) loaded in triplicate or quadruplicate. The densitometric value of the protein of interest was normalized to the mean value of the control sample to account for between-gels variability.

The membranes were subjected to blocking for 1 h using either 4 % bovine serum albumin (BSA) or 2.5–5% non-fat dried milk powder (blotting-grade blocker), diluted in Tris-buffered saline that included 0.1 % Tween 20 (TBS-T). The membranes were incubated overnight at 4 °C with primary antibodies diluted using either the 4 % BSA-blocking buffer or the 5 % Blotto-blocking buffer. Following the incubation with primary antibodies, the membranes were washed and then incubated at room temperature with either an HRP-conjugated anti-rabbit or anti-mouse antibody. In all cases, these were diluted at ratios ranging from 1:5000 to 1:20000 with a 5 % Blotto-blocking buffer. The membranes were then exposed to chemiluminescent visualization with Clarity™ Western ECL Substrate (Bio-Rad Laboratories) using the ChemiDocTM Touch Imaging System (Bio-Rad Laboratories). The optical density of the bands was measured using the Image Lab© software 5.2.1 (Bio-Rad Laboratories). Equal loading and transfer efficiency was verified by staining with Reactive Brown 10 (Sigma-Aldrich, St. Louis, MO, USA) [37].

The Protein Plus Precision All Blue Standards were procured from Bio-Rad Laboratories (Hemel Hempstead Hertfordshire, UK). The antibodies employed in this investigation were obtained from different manufacturers. The corresponding catalogue numbers from Abcam

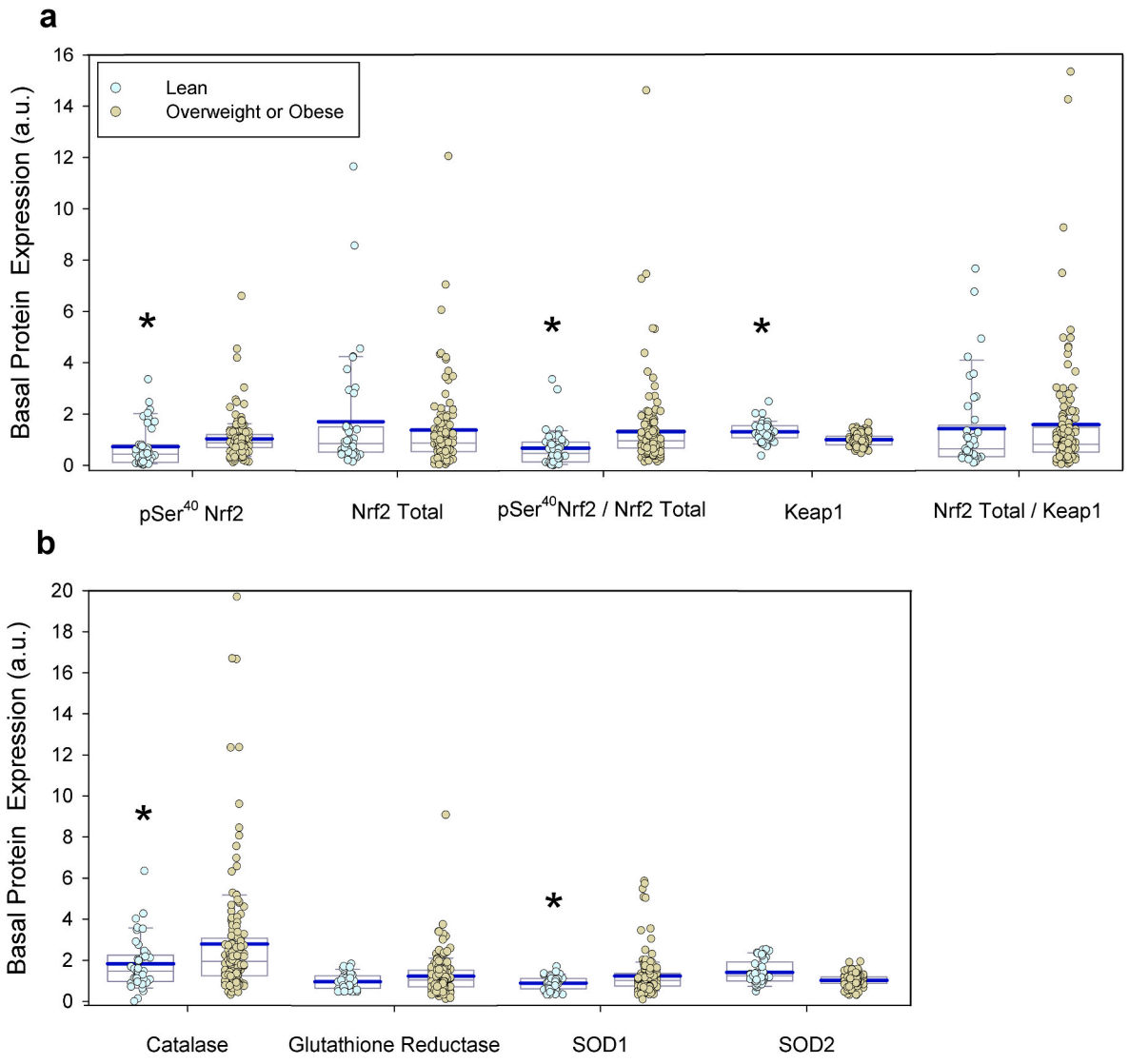

Fig. 3. Protein expression levels in lean and overweight or obese participants. Nrf2 total, pSer^{40}-Nrf2, pSer^{40}-Nrf2/Nrf2 ratio, Keap1, and Nrf2/Keap1 ratio (a); catalase, glutathione reductase, superoxide dismutase 1 (SOD1), and superoxide dismutase 2 (SOD2) (b). n = 189 (41 lean and 148 with overweight or obesity). Box and whisker plots: the extremes of the whiskers represent the limits of the 5th and 95th percentiles, respectively; the thick and thin horizontal lines inside the boxes correspond to the mean and median values, respectively; and the lower and upper limits of the box delimit the 1st and 3rd quartiles, respectively. Data from lean participants are presented in blue while data for overweight or obese participants are presented in yellow. *P < 0.05 lean compared with overweight or obese participants.

(Waltham, MA, USA) were as follows: pSer^{40}-Nrf2 (no. ab76026), Nrf2 (no. ab62352), Keap1 (no. ab119403) and SOD1 (no. ab16831). The antibodies purchased from Cell Signaling Technology (Denver, MA, USA) were catalase (no.14097) and SOD2 (no. 13141). The antibody for GR was purchased from Proteintech (Rosemont, IL, USA) (no. 18257-1-AP). The secondary HRP-conjugated goat anti-rabbit (no. 111-035- 144) and the HRP-conjugated goat anti-mouse (no.115-035-003) antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, USA). Additional secondary HRP-conjugated goat antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX, USA): anti-rabbit (no. sc2004) and anti-mouse (no. sc2031). See Supplementary Table 1 for a more detailed description of the antibodies and procedures.

2.4. Myosin heavy chain analysis

Myosin heavy chain isoform proportions were determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the same aliquots used for western blotting. Experimental and two control samples (7.5–10 μg) were loaded in triplicate onto the same gel. The two control samples served as an internal control to assess the inter-gel variability but were not used for normalization purposes. The experimental samples and controls were processed at 4 °C on an SDS-PAGE gel with a 3 % acrylamide (v/v) phase (stacking gel) for close to 12 h at 70 V, and subsequently, on a 6 % acrylamide (v/v) and 30 % glycerol (v/v) phase (resolving gel) for about 20 h at 350 V. The gels were then stained with Coomassie for roughly an hour, followed by an hour of distaining using a solution of 40 % methanol (v/v) and 10 % glacial acetic acid (v/v). The gels were then submerged in distilled water for around an hour for background subtraction. Finally, the MHC isoform content was determined by scanning the gel (GS-800 Imaging Densitometer, Bio-Rad Laboratories, Hercules, CA, USA) and followed by quantification with the Image Lab© software 5.2.1 (Bio-Rad Laboratories).

2.5. Statistics

The Shapiro-Wilks test was employed to verify the Gaussian distribution of variables. Sex differences for ergometric variables and physical

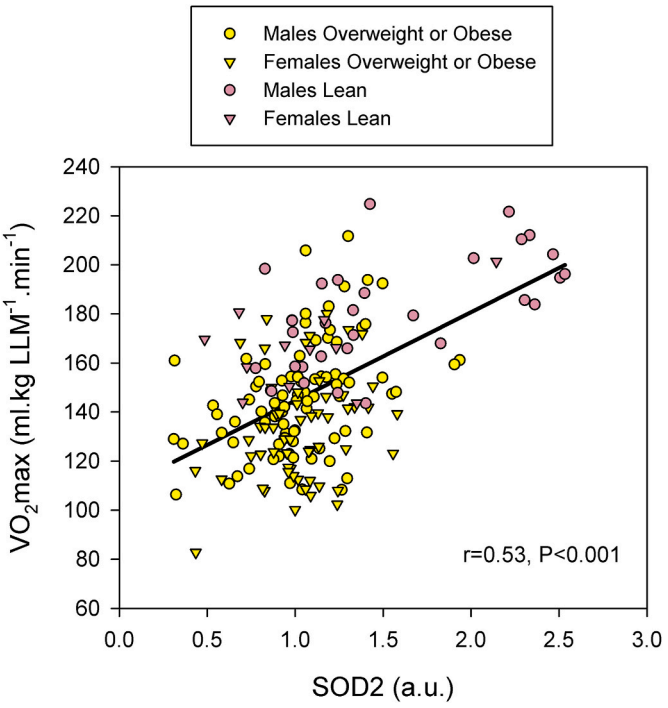

Fig. 4. Relationship between VO_2max in mL.kg of lower extremities lean mass^{-1}.min^{-1} and protein expression of superoxide dismutase 2 (SOD2). n = 188 (41 lean and 147 with overweight or obesity). Data from lean participants are presented in magenta while data for overweight or obese participants are presented in yellow, including a circle shape for males and a triangle shape for females. The values shown are means ± standard errors and expressed in mL.kg LLM^{-1}.min^{-1} and arbitrary units (a.u.). Statistical significance was set at P < 0.05.

characteristics were determined using t-tests for independent samples. Sex differences in protein expression levels were determined by ANOVA, testing the impact of VO_2max in mL.kg LLM$^{-1.min^{-1,}}$ age, and percentage of body fat. Simple and multiple linear regression analyses were used to explore linear relationships between variables. Unless otherwise mentioned, results are presented as the mean ± the standard deviation (SD). A P < 0.05 was considered statistically significant. Statistical analyses were conducted using IBM SPSS Statistics v.29 for Mac (SPSS Inc., Chicago, IL, USA) and Jamovi v1.8.1. (Jamovi project, 2021).

3. Results

3.1. Physical characteristics and performance

Males and females had similar ages, but females had a lower body weight, leg lean mass, and a higher percentage of body fat (Table 1). Males had a 38 and 12 % higher VO_2max per kg of body weight and kg of LLM, respectively (both P < 0.001) (Table 1). Myosin heavy chain composition also differed between sexes, with females exhibiting relatively more MHC I and less MCH IIa (Table 1).

3.2. Sex differences in muscle antioxidant proteins and Nrf2/Keap1

Fig. 1 shows no significant between-sex differences in pSer^{40}-Nrf2, Total Nrf2, pSer^{40}-Nrf2/Total Nrf2 ratio, and Nrf2/Keap1 ratio, catalase, GR and SOD1. Although the Keap1 protein expression was 22 % higher in males than females (P = 0.039) (Fig. 1a), the Nrf2/Keap1 ratio was similar in both sexes. Males also had 13 % higher SOD2 protein expression levels than females (P = 0.032) (see representative immunoblots in Fig. 2). However, the sex difference in Keap1 and SOD2 disappeared after accounting for differences in VO_2max expressed as mL.kg LLM^{-1}.min^{-1}, or after accounting for VO_2max and age, or VO_2max, age and body fat percentage as covariates.

3.3. Age

Age was linearly associated with VO_2max in mL.kg LLM^{-1}.min^{-1} (r = −0.38, P < 0.001, n = 189), Keap1 (r = −0.35, P < 0.001, n = 189), SOD1 (r = 0.25, P < 0.001, n = 189), and SOD2 (r = −0.16, P < 0.001, n = 189). After accounting for the percentage of body fat and VO_2max, the association between age and Keap1 (r = −0.21, P = 0.005, n = 189) and age and SOD1 (r = 0.29, P < 0.001, n = 189) remained statistically significant.

3.4. Overweight and obesity

Among all participants, 148 (90 males and 58 females, were overweight or obese, i.e., BMI ≥ 25 kg m^{-2}), while obesity (BMI ≥ 30 kg m^{-2}) was present in 80 volunteers (43 males and 37 females). No significant differences were observed between participants with or without obesity in Nrf2, Keap1 and the rest of the antioxidant proteins assessed. Catalase protein expression was 9 % higher in the subjects with obesity, after accounting for differences in VO_2max and age (P = 0.046). As illustrated in Fig. 3, when the analysis was factored according to the presence of overweight or obesity, the participants with overweight or obesity had 22 % lower Keap1 and 81 % higher pSer^{40}-Nrf2 protein expression values than their lean counterparts after accounting for differences in age and VO_2max (P < 0.001 and P = 0.012, respectively). SOD1 protein expression was 56 % higher (P = 0.026), and the ratio pSer^{40}-Nrf2/Total Nrf2 was 2.6-fold higher in the group with overweight or obesity (P = 0.014), after accounting for differences in VO_2max and age. In females, the percentage of body fat was associated with the protein expression levels of pSer^{40}-Nrf2 (r = 0.42, P < 0.001, n = 69), Keap1 (r = −0.37, P = 0.002, n = 69), ratio Nfr2/Keap1 (r = 0.28, P = 0.021, n = 69), SOD1 (r = 0.32, P = 0.008, n = 69), catalase (r = 0.35, P = 0.004, n = 69), and GR (r = 0.28, P = 0.021, n = 69). In males, the percentage of body fat was associated with the protein expression levels of Keap1 (r = −0.40, P < 0.001, n = 119) and SOD2 (r = −0.60, P < 0.001, n = 119).

Table 2
Predictive models for VO_2max in mL.kg LLM^{-1}.min^{-1}.

Predictive model for the VO_2max expressed as mL per kg of lower extremities lean mass (LLM)													
			95 % Confidence Interval					95 % Confidence Interval			Model fit measures		
Predictor	Estimate	SE	Lower	Upper	t	p	Stand. Estimate	Lower	Upper	Model	R	R^2	*P*
Intercept	100.83	6.972	87.072	114.59	14.46	<.001							
SOD2 (a.u.)	29.49	4.189	21.229	37.76	7.04	<.001	0.436	0.3136	0.558	1	0.53	0.28	**< .001**
Keap1 (a.u.)	15.75	5.359	5.176	26.32	2.94	0.004	0.179	0.059	0.3	2	0.56	0.32	**< .001**
Total Nrf2 (a.u.)	1.62	0.703	0.237	3.01	2.31	0.022	0.138	0.0202	0.256	3	0.58	0.34	**< .001**
Sex (1 = male; 2 = female)	−10.86	3.354	−17.479	−4.25	−3.24	0.001	−0.4	−0.6437	−0.156	4	0.61	0.37	**< .001**

SOD2, superoxide dismutase 2; Keap1, kelch-like ECH-associated protein 1; Nrf2, nuclear factor (erythroid-derived 2)-like 2.

3.5. VO_2max and antioxidant enzymes

VO_2max expressed as mL.kg $LLM^{-1}.min^{-1}$ was linearly associated with SOD2 ($r = 0.53$, $P < 0.001$) (Fig. 4), Keap1 ($r = 0.30$, $P < 0.001$), and total Nrf2 ($r = 0.21$, $P = 0.003$). However, no significant association was observed between VO_2max expressed as mL.kg $LLM^{-1}.min^{-1}$ and SOD1 ($r = 0.14$, $P = 0.062$). Multiple regression analysis indicates that the VO_2max in mL.kg $LLM^{-1}.min^{-1}$ can be predicted from the levels of SOD2, total Nrf2 and Keap1 ($r = 0.58$, $P < 0.001$), with SOD2 being the main predictor explaining 28 % of the variance in VO_2max, while Nrf2 and Keap1 explained around 3 % of the variance each (Table 2). Sex explained 3.6 % of the variance in VO_2max.

3.6. Associations between $pSer^{40}$-Nrf2 and the Nrf2/Keap1 ratio and antioxidant enzymes

Significant associations were observed between $pSer^{40}$-Nrf2 and catalase ($r = 0.64$, $P < 0.001$), GR ($r = 0.33$, $P < 0.001$), and Keap1 ($r = -0.25$, $P < 0.001$). Likewise, significant associations were observed between Nrf2/Keap1 ratio and catalase ($r = 0.65$, $P < 0.001$) and GR ($r = 0.41$, $P < 0.001$). There was also an association between the percentage of MHC I + MHC IIa and the protein expression levels of GR ($r = 0.27$, $P = 0.025$, $n = 70$), catalase ($r = 0.24$, $P = 0.045$, $n = 70$), and SOD2 ($r = 0.24$, $P = 0.048$, $n = 69$).

4. Discussion

In the present investigation, the Nrf2/Keap1 and the Nrf2-regulated antioxidant enzymes (SOD1-2, catalase, GR) expression levels in skeletal muscle have been determined for the first time in a large sample of males and females with marked differences in adiposity, cardiorespiratory fitness, and age. The present findings show that Nrf2 and the main antioxidant enzymes in human skeletal muscle are expressed in equal amounts in males and females when sex differences in VO_2max, age and adiposity are accounted for. We have also demonstrated that a greater aerobic fitness (i.e., a higher VO_2max per kg LLM) associates with increased basal expression of total Nrf2, Keap1 and SOD2. Our findings also show that SOD2 is the strongest predictor of VO_2max in human skeletal muscle, while SOD1 protein expression increases with ageing after accounting for differences in VO_2max and body fat percentage. Finally, we have also shown that a BMI $\geq$ 25 kg m^{-2} is associated with increased levels of $pSer^{40}$-Nrf2, $pSer^{40}$-Nrf2/Total Nrf2 ratio and SOD1 protein expression levels, after accounting for differences in age and VO_2max normalized to the lean mass of the lower extremities.

4.1. Why are some antioxidant enzymes associated to VO_2max?

Superoxide (O_2^-) and H_2O_2 can be generated in at least 10 different mitochondrial sites during aerobic metabolism and at several extramitochondrial locations by nicotinamide adenine dinucleotide phosphate oxidases (NADPH Oxidases or NOXs), Xanthine Oxidase, Phospholipase A2 (PLA2), Lipoxygenases and Cyclooxygenases [43,44]. Although the relative importance of these sources of O_2^- and H_2O_2 during exercise has yet to be established, it is thought that at low and moderate exercise intensities most O_2^- and H_2O_2 have a mitochondrial origin, while at high exercise intensities, extramitochondrial sources may predominate [45]. In contrast to the general belief, the mitochondrial production of O_2^- and H_2O_2 is lower in conditions mimicking moderate or intense aerobic exercise than at rest [43]. Thus, performing low or moderate-intensity exercise should not require more antioxidant capacity than needed for resting conditions. However, experiments carried out in humans using electron paramagnetic resonance spectroscopy have detected an increase in biomarkers of oxidative stress in muscle biopsies [46], which should not have been detected had the ROS production remained below the resting values observed in isolated mitochondria tested in biochemical environments mimicking exercise conditions [43]. Thus, either mitochondrial O_2^- and H_2O_2 production is increased during aerobic exercise, or there is a large increase in extramitochondrial O_2^- and H_2O_2 production, as suggested by experiments showing reduced oxidative stress and blunted ROS-mediated signalling when exercise is performed after the administration of allopurinol, a xanthine oxidase inhibitor [47,48]. Increased O_2^- production during aerobic exercise is also supported by the fact that several studies have reported increased skeletal muscle SOD2 expression in endurance-trained athletes [49,50] and in response to endurance training [2,3,51,52]. However, other studies have reported no effects of training on antioxidant enzymes or glutathione status in skeletal muscle [53,54]. The level of muscle expression of SOD2 may also determine exercise capacity since heterozygous SOD2 gene–knockout mice, which have 30–80 % lower expression of SOD2, have reduced exercise capacity [55].

The present investigation has shown that SOD2, a mitochondrial antioxidant enzyme, is the antioxidant enzyme with the highest predictive value for VO_2max. This could indicate that an increased capacity to quench the free radicals produced during mitochondrial respiration is likely a critical factor for a higher VO_2max. This interpretation aligns well with the fact that animals with exceptional VO_2max possess remarkably increased levels of SOD in their skeletal muscles [56]. In agreement, it has been reported that conditional knockout of Mn-SOD (SOD2) targeted to type IIB skeletal muscle fibres in mice increases oxidative stress and is sufficient to alter muscle strength and aerobic exercise capacity [57]. In contrast, SOD2 overexpression reduces fibrosis and pro-apoptotic signalling in ageing hearts [58]. However, some studies have reported a lack of SOD increase with endurance [53,54,59] or sprint interval training [60] despite improvements in VO_2max. This divergence between studies could be explained by the variability of measurements combined with the small number of participants, the training programme, and the fitness levels of the participants in training studies.

4.2. Nrf2 is associated with VO_2max

The present investigation shows for the first time an association between Nrf2 total protein expression and VO_2max per kg of lower extremities lean mass. This manner of expressing VO_2max gives a better assessment of the intrinsic capacity of active muscles to uptake and utilize O_2. Thus, the observed association between VO_2max per kg of active muscle suggest that the local expression of Nrf2 is linked to changes in skeletal muscle that facilitate aerobic energy production. Accordingly, abrogation of Nrf2 expression reduces muscle performance [8], while the opposite is observed with Nrf2 overexpression in mice [6,61]. Increased levels of Nrf2 may improve endurance by increasing the antioxidant capacity [61], which, in turn, may counteract the pro-fatigue action of O_2^- and H_2O_2 during prolonged or fatiguing muscle contractions [62]. Besides, a sustained basal elevation of Nrf2 may promote mitochondrial biogenesis and enhance mitochondrial density [63,64]. Increased mitochondrial density is a pivotal feature of endurance-trained muscles and has been associated with increased O_2 extraction capacity and more capacity for fatty acid oxidation [65].

4.3. Age is associated with increased SOD1 protein expression in human skeletal muscle, regardless of VO_2max and body fat percentage

In most previous research with humans, markers of oxidative stress have been determined primarily in the circulation. Due to the necessity of muscle biopsies, skeletal muscle oxidative stress has been less studied in humans. Skeletal muscle oxidative stress has been reported with age [66–68], hypertension [69], and obesity in women [59] and men [2], and patients with chronic pulmonary obstructive disease (COPD) [70]. In these conditions, the oxidative stress is due to excessive production of RONS since the antioxidant mechanisms are unchanged or insufficiently increased to fully counteract the increased RONS production [5].

In the present cohort, a positive association between age and SOD1 protein expression has been observed, which remained present after accounting for the percentage of body fat and VO_2max. In agreement, a 92 % higher expression of SOD1 has been observed in m. *vastus lateralis* of ten physically active elderly males (mean age 71 years old) compared to ten physically active younger males (mean age 23 years old) [71]. The present finding confirms the association between ageing and SOD1 in a much larger cohort, including males and females. It is shown that this association is not explained by the reduction of VO_2max and the increase of the percentage of body fat with ageing. It has been proposed that the lifespan may be determined by the accumulation of oxidative damage to critical structures [72,73], among other factors [74]. The increase of SOD1 with ageing may reflect a counterregulatory response to balance an increased rate of O_2^- production [75], which seems necessary even after accounting for differences in VO_2max, as shown in the present research.

4.4. Being overweight or obese is associated with increased expression of $pSer^{40}$-Nrf2, its phosphorylation ratio and SOD1, while Keap1 protein expression is reduced

The present findings indicate that RONS production is likely elevated in obesity, which triggers adaptative mechanisms mediated by the activation of Nrf2 signalling, facilitated by its higher phosphorylation level and the reduction of its inhibitory factor Keap1. This results in an increased expression of SOD1, which is the most abundant antioxidant enzyme in skeletal muscle [5].

In agreement with our findings, increased expression of SOD1 has been reported in 12 middle-aged women [59] and 9 obese men [2] compared to lean peers. Also, in concordance with the present results, no elevation of SOD2 or catalase was seen in obese patients in these two previous studies [2,59]. After a 12-week endurance training programme that improved VO_2max (expressed normalized to the whole-body lean mass), SOD1 protein expression was normalized in the female participants, despite no change in body fat percentage [59]. In contrast, after 12-week endurance training, SOD1 remained elevated in the obese males, despite improvements in VO_2max and no change in the percentage of body fat [2]. Superoxide dismutase 2 was increased after 12 weeks of endurance training in males [2] but not in females [59]. In the present investigation, VO_2max was related to SOD2 protein expression in males and females ($r > 0.50$).

The reason why Nrf2 signalling and the antioxidant enzymes are increased in the *vastus lateralis* with obesity, even after accounting for the confounding effects of age and the percentage of body fat, remains unknown. The most plausible explanation for enhanced Nrf2 signalling in the skeletal muscle of patients with obesity is the low-grade chronic inflammation that develops with obesity, which by multiple mechanisms may cause oxidative stress [76]. In favour of this explanation, several studies have reported higher levels of oxidative damage markers (especially protein carbonyls and 4-hydroxy-2-nonenal, HNE adducts) in the skeletal muscle of obese patients compared to lean counterparts [2,59]. It could also be that the mechanical overload caused by obesity explains a similar response to that described in subjects doing strength training [77]. Another mechanism could be the activation of Nrf2 signalling mediated by NFκB, which in turn responds to pro-inflammatory cytokines increased in the circulation in obese patients [78].

4.5. Sex differences in skeletal muscle antioxidant enzymes, Keap1 and Nrf2

In the present investigation, no significant differences in the protein expression levels of Nrf2, Keap1 and antioxidant enzymes were observed after accounting for age, body composition and VO_2max differences. Only one previous study has assessed the antioxidant enzyme status in human skeletal muscle biopsies obtained from males and females undergoing orthopaedic surgery (57 males and 63 females, aged from 17 to 91 years old) [79]. In agreement with our results, no sex differences were observed in SOD1, SOD2, catalase and glutathione peroxidase activities in muscle biopsies obtained from the *rectus abdominis*, *vastus lateralis* and *gluteus maximus* [79]. The collection conditions and time to freezing are not reported, nor are the specific characteristics of the subjects (underlying disease, medications, body composition, and physical activity/fitness), which limits the interpretation of the results. Barreiro et al. did not find significant sex differences in SOD2 or catalase in healthy volunteers' *external intercostalis* and *vastus lateralis* (12 males and 12 females) [67].

4.6. Limitations

This study has focused on assessing the changes in the protein expression levels of the main antioxidant enzymes and their main regulatory factors, i.e., Keap1 and Nrf2. Thus, future studies should also evaluate enzymatic activities and the ratio of total enzyme protein expression to enzymatic activity to check for potential enzyme deactivation, which should be minimal at rest in a healthy muscle. Although we have taken care to account for differences in physical fitness, body composition and age as confounding factors, we did not control for the diet. A diet rich in natural polyphenols may have anti-inflammatory and antioxidant effects [80,81]. Although daily physical activity was not measured, we determined VO_2max directly and normalized its value to the lower extremities lean mass, which allows a direct comparison between males and females or participants with different ages and levels of physical activity since muscle mass and VO_2max are primary outcome variables modified by physical activity. Although all females were premenopausal, they were assessed disregarding the potential influence of the menstrual cycle phase. In plasma, SOD and catalase activities are lower in the luteal than the follicular phase of the menstrual cycle [82]. So far, no study has determined whether skeletal muscle antioxidant enzymes fluctuate with the menstrual cycle in women. Although the most abundant antioxidant enzymes in skeletal muscle were measured [83], other antioxidant enzymes such as glutathione peroxidase, peroxiredoxins, thioredoxins, thioredoxin reductases, and glutaredoxins were not determined. Finally, the present results do not exclude potential differences in non-enzymatic antioxidants, which were not assessed.

In summary, this is the first study demonstrating an association between VO_2max per kg of lean mass of the lower extremities and the expression of some antioxidant enzymes in human skeletal muscle. We have shown that, among the antioxidant enzymes in human skeletal muscle, SOD2 protein expression is the stronger predictor of VO_2max and that SOD1 protein expression increases with ageing after accounting for differences in VO_2max and percentage of body fat. Moreover, our current observations indicate that males and premenopausal females have similar protein expression levels of Nrf2 and antioxidant enzymes in human skeletal muscle. Finally, this investigation shows that overweight and obesity are associated with elevated protein expression levels of $pSer^{40}$-Nrf2, $pSer^{40}$-Nrf2/Total Nrf2 ratio and SOD1, after accounting for differences in age and VO_2max. Overall, at the group level, higher aerobic fitness is associated with elevated basal expression of certain muscle antioxidant enzymes, which may explain some of the benefits of regular exercise.

Disclosure summary

The authors have nothing to disclose.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was financed by grants from the Ministerio de Economía y Competitividad (DEP2015-71171-R; DEP2017-86409-C2-1-P; PID2021-125354OB-C21; PI14/01509), University of Las Palmas de Gran Canaria (ULPAPD-08/01–4), Agencia Canaria de Investigación, Innovación y Sociedad de la Infomación (ProID2017010106), FEDER, Swedish Olympic Committee (Ref: 070–4058960), Consejo Superior de Deportes (EXP_75097), European Union NextGenerationEU, Gobierno de España, Ministerio de Cultura y Deporte, Plan de Recuperación, Transformación y Resilencia. Authors would like to acknowledge Cabildo de Gran Canaria (grant 12/22) and FDCAN (Fondo de Desarrollo de Canarias) for the economic support to this research work. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript. The technical assistance by Jose Navarro de Tuero is greatly appreciated.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2023.10.393.

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