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REVIEW

Electrical pulse stimulation of skeletal muscle in vitro: A systematic review of the methods used to mimic exercise

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Abstract

Background: The use of electrical pulse stimulation (EPS) as a method to induce contractions in cultured skeletal muscle has experienced rapid growth in recent years. Despite evidence demonstrating its utility in replicating some acute and chronic adaptations to exercise observed in vivo, there is a lack of evidence concerning the optimal parameters necessary to induce specific molecular adaptations. Methods: This systematic review adhered to the Preferred Reporting Systems for Systematic Reviews. Eligible studies were published in English within peer-reviewed articles. Searches were executed in three databases, with an additional search for grey literature conducted on Google Scholar. Studies investigating chronic EPS in C2C12, and primary human muscle cells were incorporated. Results: One hundred and twenty-seven studies spanning over twenty years of research fulfilled the criteria of this review. Outcome measures were categorised into distinct groups: Morphology, gene expression, protein abundance and/or activity, mitochondrial content/function, contractile function, metabolic changes, and cell viability. Conclusions: EPS is effective in inducing a generalised exercise-like response in cultured muscle cells. However, unlike exercise in vivo, the observed molecular responses divergent in endurance/resistance exercises do not occur. Instead, both AKT/mTOR and AMPK/PGC-1α molecular pathways are activated concurrently across a wide range of stimulation durations and intensities. Therefore, EPS in culture is not yet suitable for investigating specific endurance/resistance training adaptations, which may be more readily observed in human and animal models.

Keywords Electrical Pulse Stimulation (EPS) · Muscle · *in vitro* exercise · Endurance training · Resistance training

Introduction

Exercise is an effective way to improve health. Substantial evidence underscores the beneficial effects of exercise on cardiometabolic, neurological and skeletal muscle health (Gordon et al. 2018; Mandsager et al. 2018; Bennie et al. 2020). Physical exercise provides substantial improvements beyond reductions in morbidity and mortality, with improvements in aerobic capacity, muscular strength, hypertrophy, and power commonly observed following systematic exercise training (Gibala and Jones 2013; Suchomel et al. 2018). These outcomes are often used to assess the enhancement of health and sports performance (Joyner and Coyle 2008; Suchomel et al. 2016). It is well documented that the intensity and duration of exercise influence the subsequent adaptive response (Romijn et al. 1993; van Loon et al. 2001). As a simple example, high-force contractions with a low number of repetitions typically induce strength and hypertrophic responses, while lower-force contractions with a higher number of repetitions over longer exercise sessions elicit endurance-like aerobic adaptations (Hughes et al. 2018). Key exercise-induced stressors that underpin the adaptive response are increased substrate utilisation, activation of mechanosensors, reactive oxygen and nitrogen species production and increased calcium flux (van Loon et al. 2001; Hood 2009; Liu and Latham 2009). Our understanding of the adaptive response to exercise training has

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been gained through in vivo human and animal models, as well as in vitro cellular models. However, challenges such as participant health/training status, intervention duration, and limited sample collection can make it challenging to achieve mechanistic insights from in vivo research. Cell and in vitro models have been routinely used to elucidate the molecular mechanisms of exercise-induced adaptations, providing a highly controlled system for investigating skeletal muscle physiology. Thus, the development and availability of valid biomimetic models of skeletal muscle and exercise are desirable.

Skeletal muscle contraction is permitted by the interaction between actin thin filaments and highly conserved motor proteins known as myosin heavy chains (MyHCs). Myosin isotype expression is associated with many of the wide range of biochemical and physiological differences observed within muscle, with isotype expression correlated to fibre colour, ATPase activity and contractile force (Bottinelli et al. 1994; Harridge et al. 1996; Reggiani et al. 2000; Schiaffino and Reggiani 2011). The specificity of exercise has been shown to drive distinct MyHC fibre type shifts with high load resistance training protocols (>70% one-repetition max), leading to a more glycolytic, type II muscle phenotype and typical endurance training programmes leading to a more oxidative, type I muscle phenotype (Adams et al. 1993; Carroll et al. 1998; Plotkin et al. 2021).

Electrical pulse stimulation (EPS) of cultured skeletal muscle cells has emerged as a useful model for identifying the molecular signalling cascades that occur following skeletal muscle contraction (Nikolić et al. 2017). EPS involves the delivery of programmed electrical stimulation to elicit an electrochemical response in specific tissue types, including skeletal muscle. The use of EPS has increased dramatically in recent years, with 24,180 results for 'electrical pulse stimulation' in PubMed alone at the time of writing this review. This rise in usage can likely be attributed to both the utility of EPS as a method of inducing exercise-like responses and the production of commercially available stimulators. Early investigations into the effects of EPS in muscle cell culture demonstrated profound changes in total skeletal muscle myosin accumulation, protein synthesis and calcium handling (Brevet et al. 1976; Thelen et al. 1997). Seminal work from Marotta et al. (2004) demonstrated the effects of an 'exercise-like' EPS protocol and found reductions in total glycogen content and increased lactate production and glycogen mobilisation following ninety minutes of continuous EPS (3 Hz, 50 V, 30 ms). The serine/threonine protein kinase mammalian target of rapamycin (mTOR) is a 'master-regulator' of protein synthesis, and senses changes in nutrient availability, growth factors and oxygen to promote cell growth (Liu and Sabatini 2020). mTOR phosphorylation is known to mediate changes in anabolic protein signalling in whole muscle, leading to skeletal muscle hypertrophy following intense 'resistance-like' training protocols (Deldicque et al. 2005). Moreover, changes in mTOR activity have been demonstrated following EPS in both human and murine differentiated skeletal muscle cells (Tarum et al. 2017; Beiter et al. 2018; Khodabukus et al. 2019; Valero-Breton et al. 2020). Moreover, changes in the energy sensor AMP-activated protein kinase (AMPK) have also been shown to increase following EPS of cultured muscle cells (Evers-van Gogh et al. 2015; Gong et al. 2016; Beiter et al. 2018; Al-Bayati et al. 2019; Chang and Kong 2020; Tamura et al. 2020a, b). This signalling molecule detects changes in the energy status of the cell, i.e. rising AMP/ATP and ADP/ATP ratio, and is associated with 'endurance-like' adaptations, the hallmark of which is mitochondrial biogenesis (Jornayvaz and Shulman 2010). However, despite clear changes in both endurance and resistance-like molecular responses, there is a paucity of evidence surrounding optimal parameters to elicit specific exercise-like responses in skeletal muscle cell models.

This systematic review aimed to 1) assess whether programmed electrical stimulation protocols can be used effectively to replicate acute and chronic exercise responses in vitro and 2) determine whether optimal parameters exist that stimulate in vitro the specific molecular responses to 'endurance' and 'resistance' exercise.

Methods

Search procedure

Due to the study criteria involving in vitro cell culture experiments, this review was not eligible for registration on PROSPERO, which is restricted to systematic reviews of human or in vivo animal studies. However, a detailed review protocol was developed a priori in accordance with PROSPERO registration requirements and is provided as Supplementary Material (S1). Deviations from the original protocol occurred as planned quantitative synthesis (meta-analysis) and formal assessment of publication bias were not feasible due to substantial heterogeneity in study designs, stimulation protocols, and outcome measures. As such, a narrative synthesis approach was adopted.

A systematic search was performed in September 2024 using three electronic bibliographic databases: PubMed, Scopus, and Web of Science. A grey literature search was conducted using Google Scholar to identify unpublished works and minimise publication bias (Haddaway et al. 2015). Any grey literature located was scrutinised according to the inclusion and exclusion criteria of the review.

A Boolean search strategy was used using the operators AND, OR, NOT with journal articles published in English between 01/01/2004 and the date of the last search included in the review. The initial date was chosen because to our knowledge, the first published article on continuous electrical stimulation in skeletal muscle cells as a model of specifically inducing exercise-like adaptations was published in 2004 (Marotta et al. 2004). The search strategies for each database are detailed in **(S2)** as supplementary information, with a link to one of the database searches as per Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher et al. 2009). The PRISMA flow diagram detailing the procedure can be found **(**Fig. 1**)**.

Data extraction

All search results were exported to Covidence (Covidence, Melbourne, Australia), enabling central review and collection of all texts before and after further manual screening. All search results were exported to the same software and duplicates were removed. All titles and abstracts were screened by a panel of independent researchers, with each study reviewed by two researchers. The titles and abstracts were initially screened for relevance, and approved studies underwent a full-text review. Studies in the full-text review were also independently screened for relevance by four researchers, and any disagreements were resolved in a meeting involving all four researchers. Study data were then extracted, including stimulation protocols (e.g., frequency, duration), cell characteristics (e.g., cell type), and outcome measures (e.g., myotube hypertrophy, mitochondrial content). Additionally, texts which passed full-text review were searched for additional eligible texts within the reference list, and additional manuscripts meeting the inclusion criteria were also included. All data included was complete and agreed upon for inclusion by all authors. Authors of any paper that raised concerns regarding lack of access to data were contacted to provide further context for any incomplete data. Selective outcome reporting was considered when assessing the study data and its potential impact on the results.

Study selection

Studies were eligible if they contained the EPS of differentiated human skeletal muscle cells (HSMC) or the differentiated C2C12 mouse cell-line. The C2C12 cell line was included due to the resemblance of differentiated C2C12

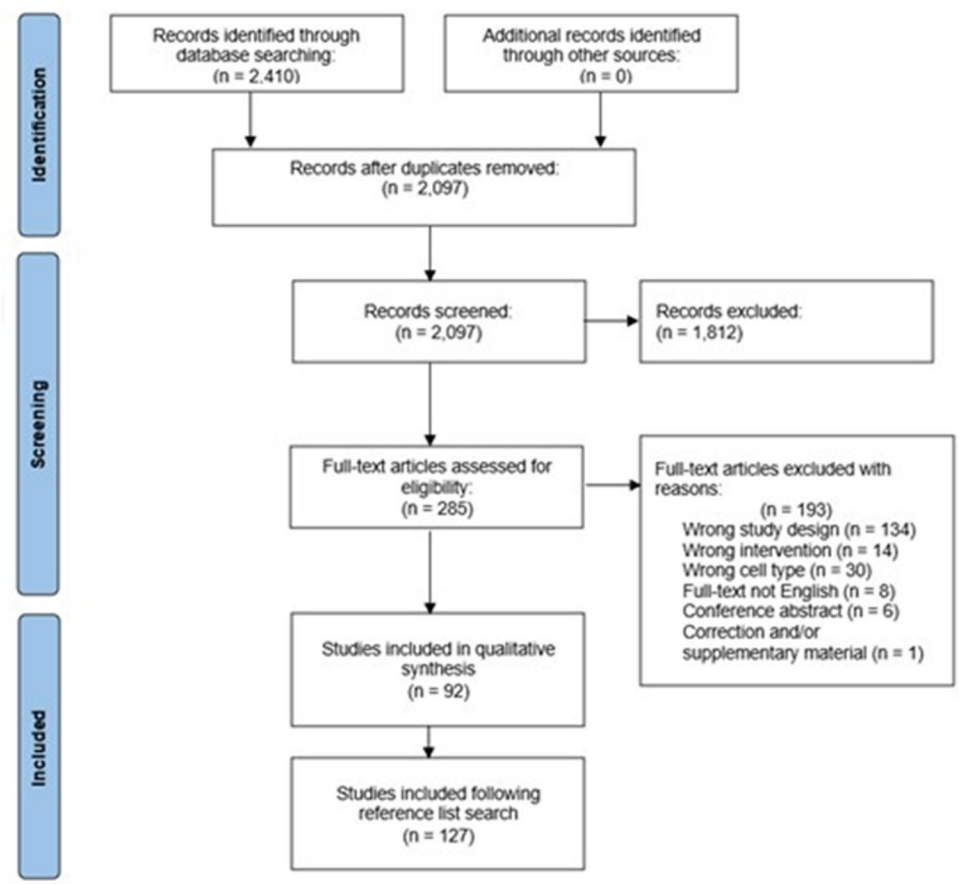

Fig. 1 PRISMA flow diagram. This is line with systematic review recommendations which suggest wide general search screening, followed by the removal of duplicates. Remaining studies then undergo a title and abstract screen, and then a subsequent full text screen. Reference lists of included texts were also screened for eligibility and inclusion. Reasons for exclusion are listed and the remaining studies (96) were then included in qualitative synthesis (Moher et al. 2009)

cells to human muscle tissue in terms of myosin content and glycogen storage, thereby making them suitable for studies of exercise/stress responses alongside HSMCs (Abdelmoez et al. 2020). Included studies investigated EPS in both 2D monolayers (adhered to a flask/plate) and tissue constructs (3D cell structures), and included comparison against an appropriate, unstimulated control. Studies that included multiple cell lines and/or treatments were acceptable if cell-specific results were available and/or accessible. Only studies which stimulated differentiated myotubes were included. Moreover, the stimulation protocol had to be classed as continuous (i.e., delivery of repeated electrical pulses over hours or days). Studies based on isolated electrical impulses to measure force production were discounted. Finally, measures without statistical analysis (e.g., images) were excluded from the study to minimise bias and author's interpretation.

EPS of other muscle cell lines (such as Rat L6) were excluded from this review primarily due to their inability to contract following electrical pulse delivery and limited translation to human muscle (Manabe et al. 2012; Abdelmoez et al. 2020). Furthermore, other typically stimulated cell types (fibroblasts, cardiomyocytes), and stimulation studies involving animals and humans were also excluded from the review.

Methodological quality and risk of bias assessment

Due to the in vitro nature of the included studies, standard risk of bias tools designed for clinical or in vivo research (e.g., randomisation, allocation concealment, and participant blinding) were not directly applicable. No validated risk of bias instrument currently exists for EPS studies conducted in cultured skeletal muscle models. Therefore, methodological quality and potential sources of bias were assessed qualitatively during data extraction and synthesis. This assessment considered key domains relevant to in vitro experimental designs, including: **(1)** cell model selection and characterisation (e.g., C2C12 vs. human primary cells), **(2)** differentiation status of myotubes prior to stimulation, **(3)** clarity and reproducibility of stimulation protocols (frequency, voltage, duration), **(4)** outcome measurement validity and reporting transparency, and **(5)** completeness of reported data and potential selective outcome reporting.

Studies were not excluded on the basis of methodological quality; however, identified limitations were considered during interpretation and synthesis of findings, with greater emphasis placed on studies demonstrating clear and reproducible methodologies. This approach is consistent with methodological guidance for systematic reviews of in vitro research, where no standardised risk of bias tools exists and qualitative domain-based assessments are commonly adopted (Rooney et al. 2014; Samuel et al. 2016; Hammel et al. 2022).

Results

Despite the dramatic increase in the use of EPS in recent years, there is little consensus regarding the overall effectiveness of EPS in generating exercise-like adaptations in vitro. Moreover, it is currently unknown whether there exist optimal parameters that dictate exercise-like divergent molecular responses to EPS. Consequently, this raises questions about the effectiveness of EPS in eliciting both the acute post-exercise responses and chronic exercise adaptations. This review aimed to address these knowledge deficits and to characterise where EPS has been previously shown to be effective, as well as where future studies should focus their investigation.

General results

One hundred and twenty-seven studies were accepted for final inclusion. To optimise data clarity, each paper was subcategorised into outcome sections including morphology (twenty-five included papers), gene expression (sixty-nine included papers), protein abundance and/or activity (ninety-two included papers), mitochondrial content/function (eighteen included papers), contractile function (ten included papers), metabolic changes (forty-four included papers, and cell viability (twenty-two included papers). A table of each eligible study alongside the stimulation protocol used and their outcome sub-catagories were included (see Table 1). In all included studies electrical current was generally applied via electrodes placed into the growth medium so that an electrical field was generated across the cultured cells. There were one hundred and two included studies which used the C2C12 cell line, and twenty-seven papers electrically stimulating human primary cells. Furthermore, only twenty-seven studies used 3D tissue constructs compared to one hundred and two papers that were performed using cell monolayers. There exists no absolute conformity within the literature surrounding optimal stimulation parameters, and as such, there are large differences regarding the stimulation duration, frequency and intensity between studies. This disparity between stimulation protocols exists in both stimulated monolayers and tissue constructs, indicating a lack of consensus within the literature regarding an optimal stimulation procedure. The most common stimulation protocol used within the literature, however, was 1 Hz, 11.5 V, 2 ms, which was first used by (Lambernd et al. 2012) and subsequently in eleven other papers (Lambernd et al. 2012; Görgens et al. 2013, 2016; Raschke et al. 2013; Evers-van

Table 1 Meta-data table highlighting author, cell type, model dimension, stimulation protocol, duration and outcome measures included in this review

Lead Study Author and Date	Cell Type	2D/3D	Stimulation Protocol	Stimulation Duration	Outcome Measures						
					Morphological	Gene Expression	Protein Abundance and/or/ Activity	Mitochondrial Content/Function	Contractile Function	Metabolic Changes	Viability
Ahadian (2012)	C2C12	3D	Protocol 1: (1 Hz, 0.5 V, 10 ms) Protocol 2: (1 Hz, 6 V, 10 ms)	Protocol 1: 72 h Protocol 2: 24 h	X	X					
Ahadian (2013)	C2C12	3D	1 Hz, 10 V, 10 ms duration	48 h		X					
Ahadian (2014)	C2C12	3D	1 Hz, 8 V, 10 ms	48 h	X	X					
Al-Bayati (2019)	Human Primary	2D	Protocol 1: 2 Hz, 5 V, 24 ms Protocol 2: 0.2 Hz, 5 V, 24 ms	Each protocol was performed for 1 h alternating for a total stimulation time of 16 h			X			X	
Banan Sadeghian (2018)	C2C12	3D	1 Hz, (3, 4 & 4.5 V), 0.5 ms	12 h	X	X					
Basturkman (2022)	C2C12	2D	3 Hz, (0.15 and 0.3 V/mm), 1 ms	5 min per day for 5 days	X						X
Bayol (2005)	C2C12	2D	2 Hz, 0.3 V/mm	(24, 48 & 72 h)		X					
Beiter (2018)	C2C12	2D	1 Hz, 14 V, 2ms	90 min		X	X			X	
Broholm (2011)	Human Primary	2D	1 Hz, 40 V, 1 ms	3 h			X				
Brown (2015)	Human Primary	2D	Protocol 1: 2 Hz, 5 V, 24ms Protocol 2: 0.2 Hz, 5 V, 24ms	1 h alternation between protocol 1 and 2 for (4, 16 and 24 h)			X			X	X
Burch (2010)	C2C12	2D	50 Hz (ON;1s/ OFF; 1s), 14 V, 1 ms	Protocol 1: 90 min Protocol 2: 90 min for 4 consecutive days Protocol 3: 24 h		X					
Carter (2012)	C2C12	2D	5 Hz, 9 V	Protocol 1: 3 h/ day for 4 consecutive days Protocol 2: 2 h followed by 2 h rest	X		X	X			

Table 1 (continued)

Lead Study Author and Date	Cell Type	2D/3D	Stimulation Protocol	Stimulation Duration	Outcome Measures						
					Morphological	Gene Expression	Protein Abundance and/or/ Activity	Mitochondrial Content/Function	Contractile Function	Metabolic Changes	Viability
Lead Study Author and Date	Cell Type	2D/3D	Stimulation Protocol	Stimulation Duration	Outcome Measures						
					Morphological	Gene Expression	Protein Abundance and/or/ Activity	Mitochondrial Content/Function	Contractile Function	Metabolic Changes	Viability
Chang (2020)	C2C12	2D	Protocol 1: 1 Hz, 11.5 V, 2ms Protocol 2: 99 Hz, 11.5 V, 1ms for 20 pulses every 20 s	24 h			X				
Chen (2019)	C2C12/ Human Primary	2D	1 Hz, 20 V, 4 ms	16 h		X	X				
Chien (2020)	C2C12	2D	1 HZ, 11.5 V, 2 ms	16 h			X	X		X	
Christensen (2015)	Human Primary	2D	1 Hz, 40 V, 2ms	6 h			X				X
Danilov (2017)	C2C12	2D	1 Hz, 40 V, 10 ms	(30, 60, 120 & 240 min)						X	X
Davis (2019)	Human Primary	3D	5 Hz, 100 V, 5 ms pulse with a 50 ms delay between pulses	30 & 60 min				X	X		
Donnelly (2010)	C2C12	2D & 3D	Protocol 1: ([2D] 10 Hz 4 × 0.3 ms pulses, 400 ms train with 3.6 s recovery) Protocol 2: ([2D] 100 Hz, 40 × 0.3 ms pulses, 400 ms train with 3.6 s recovery). Protocol 3: ([3D] 10 Hz, 4 × 0.1 ms pulses, [1.25, 2.5 & 5 V/mm] 400 ms train with 3.6 s recovery)	Protocol 1: 3 h Protocol 2: 30 min Protocol 3: 7 days			X		X		
Ducharme (2023)	C2C12	2D	1 Hz, 12 V, 2 ms	8 h	X		X				
Erlich (2018)	C2C12	2D	5 Hz, 11 V	Protocol 1: 2 h Protocol 2: 5 h		X	X				
Evers Van Gogh (2015)	C2C12	2D	1 Hz, 11.5 V, 2 ms	24 h		X	X				

Table 1 (continued)

Lead Study Author and Date	Cell Type	2D/3D	Stimulation Protocol	Stimulation Duration	Outcome Measures						
					Morphological	Gene Expression	Protein Abundance and/or Activity	Mitochondrial Content/Function	Contractile Function	Metabolic Changes	Viability
Fachada (2023)	C2C12	2D	1 Hz, 12 V, 2 ms	24 h			X				
Lead Study Author and Date	Cell Type	2D/3D	Stimulation Protocol	Stimulation Duration	Outcome Measures						
					Morphological	Gene Expression	Protein Abundance and/or Activity	Mitochondrial Content/Function	Contractile Function	Metabolic Changes	Viability
Farmawati (2013)	C2C12	2D	0.1–1 Hz, 0.67 V/mm, 2 ms	24 h		X	X			X	
Feng (2015)	Human Primary	2D	1 Hz, 30 V, 2ms	48 h		X	X	X		X	
Fernandez-Verdejo (2017)	C2C12	2D	1 Hz, 20 V, 2ms	4 h		X	X				
Fukushima (2021)	C2C12	2D	(1 & 10 Hz), 20 V, 5 ms	24 h		X					
Fujita (2010)	C2C12	3D	1 Hz, 1 V/mm, 10 ms	Protocol 1: 24 h Protocol 2: 4 days							X
Fujita (2021)	C2C12	2D	1 Hz, 1 V/mm, 2 ms	4 days followed by an 8 h rest and then a subsequent 4 h		X					
Furuichi (2018)	C2C12	2D	1 Hz, (15, 20, 25 & 30 mA), (3, 20, 30 & 50 ms)	1 h			X				
Gao (2020)	C2C12	2D	1 Hz, 15 V, 2 ms	1 h			X	X			
Gong (2016)	C2C12	2D	(1,4,10 & 30 Hz), 30 V, 11ms	(1, 3, 5, 10 & 15 min)			X			X	
Gonzalez-Franquesa (2021)	C2C12	2D	1 Hz, 11.5 V, 2 ms	6 & 24 h			X				
Görgens (2013)	Human Primary	2D	1 Hz, 11.5 V, 2 ms	24 h		X	X				
Görgens (2016)	Human Primary	2D	1 Hz, 11.5 V, 2 ms	24 h		X	X				
Goto-Inoue (2016)	C2C12	2D	1 Hz, 15–20 mA, 30 ms	3 h			X				X
Grande (2023)	C2C12	2D	Overnight stimulation (4–12 h) at 0.5 Hz, 10 V, 4 ms and then treatment with EPS at 1 Hz, 10 V, 10 ms	0.5, 1, 2 & 4 h			X				X

Table 1 (continued)

Lead Study Author and Date	Cell Type	2D/3D	Stimulation Protocol	Stimulation Duration	Outcome Measures						
					Morphological	Gene Expression	Protein Abundance and/or Activity	Mitochondrial Content/Function	Contractile Function	Metabolic Changes	Viability
Guigni (2019)	C2C12	2D	1 Hz, 20 V, 12 ms	1 h each day for 3 days		X	X	X		X	
Guo (2022)	C2C12	2D	1 Hz, 20 V, 2 ms	12 h		X	X				
Lead Study Author and Date	Cell Type	2D/3D	Stimulation Protocol	Stimulation Duration	Outcome Measures						
					Morphological	Gene Expression	Protein Abundance and/or Activity	Mitochondrial Content/Function	Contractile Function	Metabolic Changes	Viability
Heden (2022)	C2C12	2D	0.5 Hz, 20 V, 2 ms	1 h			X			X	
Horie (2015)	C2C12	2D	1 Hz, (14, 20 & 40 V), 2 ms	1, 3 & 6 h		X	X			X	X
Hoshino (2020)	C2C12	2D	(2 & 20 Hz), 50 V, 3 ms	2, 5, 15, 30 & 60 min		X	X			X	
Hosseini (2012)	C2C12	3D	1 Hz, 22 mA, 2 ms	48 h	X						
Hu (2018)	C2C12	2D	1 Hz, 20 V, 24ms	20, 40 and 60 min			X				
Hu (2024)	C2C12	3D	1 Hz, (0.01, 0.033, 0.1 and 0.33 V/mm)	4 h per day for 4 days total	X	X	X				X
Ikeda (2016)	C2C12	3D	1 Hz, 0.3 V/mm, 4.0 ms	72 h	X				X		
Irrcher (2004)	C2C12	2D	Protocol 1: 5 Hz, 65 V Protocol 2: 5 Hz, 55 V	Protocol 1: 5, 15, 30, 60 min Protocol 2: 180 min–12 h or 1–4 successive 3-h bouts of contractile activity with 21 h rest between bout		X	X				
Ishiuchi (2018)	C2C12	2D	1 Hz, 0.6667 V/mm, 2 ms	24 h		X	X				
Ito (2014)	C2C12	3D	(0.5, 1, 2 Hz), (0.3, 0.5 V/mm), (4, 10 ms)	3 days and 10 days	X		X		X		
Jan (2021)	Human Primary	2D	1 Hz, 30 V, 2ms	48 h		X	X				
Jo (2017)	C2C12	2D	1 Hz, 5 V, 10 ms	4 h per day for 3–7 days		X					

Table 1 (continued)

Lead Study Author and Date	Cell Type	2D/3D	Stimulation Protocol	Stimulation Duration	Outcome Measures						
					Morphological	Gene Expression	Protein Abundance and/or/ Activity	Mitochondrial Content/Function	Contractile Function	Metabolic Changes	Viability
Karvinen (2022)	C2C12	2D	1 Hz, 12 V, 2 ms	16 and 24 h						X	
Kemler (2020)	C2C12	2D	6 Hz, 10 V, 25 ms every 5 s	1 h		X	X				
Khodabukus (2012)	C2C12	3D	10 Hz 0.4 s with 3.6 s rest, (0.5, 1 & 2 V/mm)	24 h			X		X		
Lead Study Author and Date	Cell Type	2D/3D	Stimulation Protocol	Stimulation Duration	Outcome Measures						
					Morphological	Gene Expression	Protein Abundance and/or/ Activity	Mitochondrial Content/Function	Contractile Function	Metabolic Changes	Viability
Khodabukus (2015)	C2C12	3D	Protocol 1: 10 Hz, 0.4 s train and 3.6 s rest Protocol 2: (10 & 20 Hz), 0.6 s train and 0.4 s rest	Protocol 1: 24 h Protocol 2: 14 days Protocol 2 was performed immediately after protocol 1					X		
Khodabukus (2015)	C2C12	3D	Protocol 1: 10 Hz, 0.4s with 3.6 s rest Protocol 2: 10 Hz, (0.6, 6, 60 & 600 s)	Protocol 1: 24 h Protocol 2: 14 days Protocol 2 was performed immediately after protocol 1			X	X	X		
Khodabukus (2019)	Human Primary	3D	Protocol 1: 1 Hz, 70 mA, 2 ms Protocol 2: 10 Hz 0.5s every 5 s, 70 mA, 2 ms	1 h ON and 7 h OFF for 7 days	X	X	X	X	X	X	
Kono (2022)	C2C12	2D	1 Hz, (1.25, 1.75 and 2.25 V/mm) 2 ms	24 h		X	X				X
Lambernd (2012)	Human Primary	2D	1 Hz, 11.5 V, 2 ms	2, 4, 8, 14, 24 h		X	X	X		X	X
Langelaan (2011)	C2C12	2D & 3D	2 Hz, 0.4 V/mm, 6 ms	48 h		X					
Laurens (2016)	Human Primary	2D	0.1 Hz, 2ms	24 h		X				X	
Laurens (2020)	Human Primary	2D	Protocol 1: 0.5 Hz, 10 V, 24 ms Protocol 2: 0.1 Hz, 10 V, 2 ms	Protocol 1: 5 min–3 h Protocol 2: 24 h		X	X			X	

Table 1 (continued)

Lead Study Author and Date	Cell Type	2D/3D	Stimulation Protocol	Stimulation Duration	Outcome Measures						
					Morphological	Gene Expression	Protein Abundance and/or Activity	Mitochondrial Content/Function	Contractile Function	Metabolic Changes	Viability
Lautoaja (2021)	C2C12	2D	1 Hz, 12 V, 2 ms	24 h			X	X		X	
Lautaoja-Kivipelto (2024)	C2C12	2D	1 Hz, 12 V, 2 ms	24 h		X	X				
Lee (2018)	C2C12	2D	1 Hz, 0.3 V/mm, 4.0 ms	96 h	X					X	
Lee (2019)	C2C12	2D	1 Hz, 2.5 V/mm, 5 ms	1, 2,3 & 6 h			X				
Lee (2022)	C2C12	2D	1 Hz, 11.5 V, 2 ms	24 h		X	X				
Lead Study Author and Date	Cell Type	2D/3D	Stimulation Protocol	Stimulation Duration	Outcome Measures						
					Morphological	Gene Expression	Protein Abundance and/or Activity	Mitochondrial Content/Function	Contractile Function	Metabolic Changes	Viability
Leon-Salas (2013)	C2C12	2D	20 Hz, 15 V, pulse train 50 ms with individual pulses 1 ms, periodicity of 10 min. Stimulated at 60–72 h and again 96–108 h after differentiation induction	24 h total over 2 × 12 h bouts	X	X	X				
Li (2018)	C2C12	2D	1 Hz, 20 V, 24ms	1 h			X			X	
Liao (2008)	C2C12	3D	1 Hz, 20 V, 10 ms	Protocol 1: 1 h with 5 h rest each day from day 0–14 Protocol 2: 1 h with 5 h rest each day from day 4–14 Protocol 3: 1 h with 5 h rest each day from day 7–14	X						
Liu (2018)	C2C12	3D	1 Hz, (0.05–0.15 V/cm), 10 ms	24 h with 1 h ON: 1 h OFF	X						
Liu (2021)	C2C12	2D	5 Hz, 45 V, 20 ms	2 h						X	X

Table 1 (continued)

Lead Study Author and Date	Cell Type	2D/3D	Stimulation Protocol	Stimulation Duration	Outcome Measures						
					Morphological	Gene Expression	Protein Abundance and/or/ Activity	Mitochondrial Content/Function	Contractile Function	Metabolic Changes	Viability
Liu (2022)	C2C12	2D	1 Hz, 20 V, 24 ms	1 h			X			X	
Liu (2022)	C2C12	2D	1 Hz, 20 V, 24 ms	1 h		X	X			X	
Løvsletten (2020)	Human Primary	2D	0.1 Hz, 10 V, 2 ms	24 h		X		X		X	
Manabe (2012)	C2C12	2D	1 Hz, 50 V, 3 ms	60 min			X			X	X
Manabe (2014)	C2C12	2D	1 Hz, (10, 12, 30 and 50 V), 3 ms	1, 3, 6 & 24 h			X				X
Marotta (2004)	C2C12	2D	3 Hz, 50 V, 30 ms	90 min			X			X	
Marš (2021)	Human Primary	2D	1 Hz, 30 V, 2ms	48 h	X	X	X			X	
Martin (2017)	C2C12	3D	10 Hz, 1 V/mm, 5 bipolar 1 ms pulses with 3.5 s rest period	24 h	X				X		
Masson (2021)	C2C12	2D	2 Hz, 40 V, 10 ms	30–60 min			X				
Matthews (2009)	C2C12	2D	2 Hz, 18 V, 10 ms	120 min		X	X			X	X
Lead Study Author and Date	Cell Type	2D/3D	Stimulation Protocol	Stimulation Duration	Outcome Measures						
					Morphological	Gene Expression	Protein Abundance and/or/ Activity	Mitochondrial Content/Function	Contractile Function	Metabolic Changes	Viability
Melouane (2019)	C2C12	2D	Protocol 1: 1 Hz, 20 V Protocol 2: 50 Hz, 14 V	Protocol 1 on day 1: 8 h Protocol 2 on day 2: 16 h		X					
Mengeste (2022)	Human Primary	2D	Protocol 1: 0.1 Hz, 10 V, 2 ms Protocol 2: 1 Hz, 30 V, 2 ms	Protocol 1: 24 h Protocol 2: 48 h		X	X			X	
Menzies (2013)	C2C12	2D	5 Hz, 7 V	Protocol 1: 3 h bout with 21 h rest Protocol 2: 3 h/day for 4 consecutive days			X	X		X	

Table 1 (continued)

Lead Study Author and Date	Cell Type	2D/3D	Stimulation Protocol	Stimulation Duration	Outcome Measures						
					Morphological	Gene Expression	Protein Abundance and/or Activity	Mitochondrial Content/Function	Contractile Function	Metabolic Changes	Viability
Mesbah Moosavi (2017)	C2C12	2D	5 Hz, 9 V	3 h/day for 4 consecutive days			X	X			
Mestre (2019)	C2C12	3D	(0.1, 1, 5, 10 Hz), 1 V/mm, 1 ms	1 h day for 4 days		X					
Miyatake (2016)	C2C12	2D	1 Hz, 20 V, 2 ms	1, 2, 3, 4 & 8 h		X	X				X
Murata (2023)	C2C12	2D	Protocol 1: 1 Hz, 10 V, 2 ms Protocol 2: 30 Hz pulse train every 30 s, 10 V, 2 ms	24 h	X	X	X				X
Nedachi (2008)	C2C12	2D	1 Hz, 0.667 V/mm, 2 ms	24 h		X	X			X	X
Nedachi (2009)	C2C12	2D	(0.1 & 1 Hz), 0.666 & 0.8 V/mm), 2 ms	3 & 24 h		X	X				
Nieuwoudt (2017)	C2C12	2D	1 Hz, 1.5 V/mm, 6 ms	16 h			X			X	
Nikolić (2012)	Human Primary	2D	Protocol 1: 100 Hz for 200 ms every 5 s, 30 V Protocol 2: 1 Hz, 30 V, 2 ms	Protocol 1: 60 min Protocol 2: 24 & 48 h		X	X	X		X	
Lead Study Author and Date	Cell Type	2D/3D	Stimulation Protocol	Stimulation Duration	Outcome Measures						
					Morphological	Gene Expression	Protein Abundance and/or Activity	Mitochondrial Content/Function	Contractile Function	Metabolic Changes	Viability
Orfanos (2016)	C2C12	2D	Protocol 1: (Twitch) 1 Hz, 10 V, 20 ms Protocol 2: (Tetanic) 15 Hz (ON; 5 s/OFF 5s) followed by 5 Hz (ON; 5s/OFF 5s), 10 V, 20 ms	1, 3 and 5 h	X						
Ostrovidov (2017)	C2C12	3D	1 Hz, 0.171 V/mm, 1ms	48 h	X	X					
Pan (2012)	C2C12	2D	5 Hz, 45 V, 20 ms	45, 60, 75, 90 & 120 min		X	X			X	

Table 1 (continued)

Lead Study Author and Date	Cell Type	2D/3D	Stimulation Protocol	Stimulation Duration	Outcome Measures						
					Morphological	Gene Expression	Protein Abundance and/or/ Activity	Mitochondrial Content/Function	Contractile Function	Metabolic Changes	Viability
Park (2008)	C2C12	3D	Multiple EPS combinations used: (1 Hz, 0.2 V/mm), (1 Hz, 0.5 V/mm), (1 Hz 0.7 V/mm), (2 Hz, 0.2 V/mm), (2 Hz, 0.5 V/mm), (2 Hz, 0.7 V/mm)	5 days		X	X			X	
Park (2019)	Human Primary	2D	1 Hz, 11.5 V, 2 ms	24 h			X			X	X
Parousis (2018)	C2C12	2D	5 Hz, 9 V	3 h/day for 4 consecutive days		X	X	X			
Patel (2019)	C2C12	2D	2 Hz, 0.05 V/mm, 2ms	1 h & 1 h per day for 3 days			X			X	X
Pattamaprapan-ont (2016)	C2C12	2D	1 Hz, 30 V, 2ms	30 min		X					
Philp (2011)	C2C12	2D	1 Hz, 40 V, 0.3 ms	3 h		X					
Pourteymour (2017)	Human Primary	2D	1 Hz, 11.5 V, 2 ms	1, 2, 4, 6 & 24 h		X	X				
Pribil Pardun (2024)	C2C12	2D	50 Hz, (2.5–10 V), 10 ms, 0.3 s/3 s	1 h/day for 4 days			X	X		X	X
Ramón-Azcón (2013)	C2C12	3D	1 Hz, 8 V, 10 ms	48 h		X					
Raschke (2013)	Human Primary	2D	1 Hz, 11.5 V, 2 ms	4, 8, 12 & 24 h		X	X				
Scheler (2013)	Human Primary	2D	5–30 Hz, 14 V, 2 ms	2, 4, 8 & 24 h		X	X				
Sidorenko (2018)	C2C12	2D	1 Hz, 40 V, 10 ms	2 h		X	X			X	
Lead Study Author and Date	Cell Type	2D/3D	Stimulation Protocol	Stimulation Duration	Outcome Measures						
					Morphological	Gene Expression	Protein Abundance and/or/ Activity	Mitochondrial Content/Function	Contractile Function	Metabolic Changes	Viability
Small (2020)	C2C12	2D	1 Hz, 30 V, 2 ms	30 min		X	X				
Son (2019)	C2C12	2D	10 Hz, 11.5 V, 2 ms	1 h		X	X			X	

Table 1 (continued)

Lead Study Author and Date	Cell Type	2D/3D	Stimulation Protocol	Stimulation Duration	Outcome Measures						
					Morphological	Gene Expression	Protein Abundance and/or Activity	Mitochondrial Content/Function	Contractile Function	Metabolic Changes	Viability
Sugimoto (2022)	C2C12	3D	66 Hz, 13 V, 2 ms, on: 5 s/off: 5 s	3 h			X			X	
Sylow (2016)	C2C12	2D	1 Hz, 24 ms	1 h			X				
Takahashi (2022)	Human Primary	2D	1 Hz, 0.8 V/mm, (1, 2, 4 or 6 ms)	Protocol 1: 4 & 8 h Protocol 2: 16 h with a 1 h rest interval after 8 h Protocol 3: 24 h with a 1 h rest interval after 8 and 16 h		X	X				
Tamura (2020)	C2C12	2D	1 Hz, 35 V, 25 ms	3 h			X			X	X
Tamura (2020)	C2C12	2D	Protocol 1: (Twitch) 2 Hz, 13 V, 2 ms Protocol 2: (Tetanic) 66 Hz, (ON: 5s/OFF: 5s), 13 V, 2 ms duration	3 h and 3 h per day for 3 days	X	X	X	X		X	
Tarum (2017)	Human Primary	2D	1 Hz, 12 V, 2 ms	1, 4 & 8 h	X		X				
Terrie (2022)	Human Primary	2D/3D	75 ± 2 Hz, 5 ± 1 mV, 2 ± 0.2 mT, 1.3 ms	2 & 22 h per day for 4–16 days	X	X					
Tejedera-Villafranca (2023)	Human Primary	3D	1 Hz, 1 V/mm, 1 ms	15 min & 24 h	X				X		
Uguccioni (2011)	C2C12	2D	5 Hz, 9 V	3 h/day for 4 consecutive days		X	X	X			
Valero-Breton (2020)	Human Primary	2D	100 Hz, 15 V, 0.4ms with 30 s rest between each interval	Protocol 1: 30 min Protocol 2: 30 min for 3 days			X				
Lead Study Author and Date	Cell Type	2D/3D	Stimulation Protocol	Stimulation Duration	Outcome Measures						
					Morphological	Gene Expression	Protein Abundance and/or Activity	Mitochondrial Content/Function	Contractile Function	Metabolic Changes	Viability
Vechetti (2021)	Human Primary	2D	1 Hz, 12 V, 2 ms	8 h	X	X					

Table 1 (continued)

Lead Study Author and Date	Cell Type	2D/3D	Stimulation Protocol	Stimulation Duration	Outcome Measures						
					Morphological	Gene Expression	Protein Abundance and/or/ Activity	Mitochondrial Content/Function	Contractile Function	Metabolic Changes	Viability
Vianney (2014)	C2C12	2D	1–5 Hz, 20–25 V, 30 ms	30 min, 90 min, 12 h, 24 h & 48 h			X				
Wang (2010)	C2C12	2D	Protocol 1: 12 Hz, 13 V Protocol 2: 100 Hz, 13 V	Protocol 1: 11 & 90 min Protocol 2: 11 min		X	X				
Whitham (2012)	C2C12	2D	1 Hz, 0.667 V/mm, 10 ms	4 h		X	X				
Yi (2023)	C2C12	3D	1 Hz, 30 V, 2 ms	24 h ON: 24 h OFF for 7 days			X				
Yue (2020)	C2C12	2D	1 Hz, 20 V, 24 ms	60 min			X			X	
Yue (2021)	C2C12	2D	1 Hz, 20 V, 24 ms	60 min		X	X			X	

Gogh et al. 2015; Pourteymour et al. 2017; Park et al. 2019; Chang and Kong 2020; Chien et al. 2020; Gonzalez-Franquesa et al. 2021; Lee et al. 2022a, b). However, voltage alone simply describes electrical potential and does not inform the electric field strength that the cells are exposed to. Consequently, nominal applied electrical field strength was estimated for included studies (a few were exempt due to lack of voltage data) and the most frequently utilised electrical field strength was 0.985 V/mm. A comprehensive list of relevant findings from each eligible paper, along with their corresponding stimulation modalities, including estimated nominal field strength, is available in **(S3)** as supplementary information. Variability in methodological quality across studies, particularly in the reporting of stimulation protocols and outcome measures, contributed to heterogeneity and was considered when interpreting the consistency of findings across outcome domains.

The misapplication of electrical stimulation can result in harmful side effects due to electrochemically induced tissue damage (Khodabukus and Baar 2012). While applying intense EPS protocols may be useful as models for inducing skeletal muscle damage, subsequent tissue damage can be detrimental to measuring contraction-induced adaptations to exercise. Therefore, initially determining the safety of EPS is important prior to quantifying the efficacy of in vitro exercise models. The enzyme lactate dehydrogenase (LDH) is often used as a marker of viability due to its release from the cytoplasm into the extracellular environment following cellular damage (Kaja et al. 2017). The finding of this review supports the safety of EPS, as LDH release was unchanged when EPS was applied to both C2C12 and human primary cells, at low frequencies (0.2–2 Hz), and after both acute and chronic stimulation durations (1–24 h) (Matthews et al. 2009; Lambernd et al. 2012; Manabe et al. 2012, 2014; Brown et al. 2015; Christensen et al. 2015; Goto-Inoue et al. 2016; Miyatake et al. 2016; Tamura et al. 2020a, b). Additionally, while most studies only report voltages, we attempted to estimate nominal applied field strength for each study based on the reported sizes of commercially available stimulators, to determine whether the applied electrical activity delivered to the cells differed between experiments. The estimated nominal applied field strength was 0.2463 to 1.97 V/mm for the studies quantifying LDH release, further suggesting that EPS of cultured muscle is well tolerated. While not a viability change per se, EPS has also been shown to cause an increase in sarcomeric lesions, which are microscopic areas of tissue damage which cross over multiple sarcomeres, and may be contribute to post-exercise weakness (Orfanos et al. 2016; Grande et al. 2023). EPS has been shown to increase the presence of sarcomeric lesions after both a twitch (1 Hz, 10 V, 20 ms) and tetanic (15 Hz [ON; 5 s/OFF 5s] followed by 5 Hz [ON;

5s/OFF 5s], 10 V, 20 ms) protocol in C2C12 cells (Orfanos et al. 2016). Therefore, while EPS doesn't seem to cause significant damage to the cell membrane, it is causing some minor structural damage to the contractile apparatus of the cell, which is likely evidence of recapitulation of the normal contractile responses to exercise.

Acute exercise

This systematic review aimed to address whether EPS can effectively mimic the acute and chronic adaptations commonly observed following exercise in vivo. During prolonged muscular contraction, metabolic demand substantially increases to resynthesise ATP. This demand is predominantly met by oxidative metabolism of carbohydrates and lipids (Romijn et al. 1993; van Loon et al. 2001). Additionally, prolonged contraction leads to changes in the cellular microenvironment, including an increased ratio of AMP: ATP and ADP: ATP, an increase in Pi, the release of Ca^{2+} from the sarcoplasmic reticulum, and reactive oxygen species (ROS) production from both mitochondrial and non-mitochondrial sources (Hardie et al. 2006; Allen et al. 2008; Henríquez-Olguin et al. 2019). AMPK is a critical energy sensor and central regulator of energy homeostasis and is acutely activated following the onset of exercise in vivo by increased AMP concentrations (Stephens et al. 2002). AMPK is a heterotrimeric enzyme complex which contains a catalytic α-subunit, together with regulatory β and γ subunits (Xiao et al. 2013). The γ subunit can bind molecules of AMP which increases its enzymatic activity through increasing allosteric activation and protecting the Thr-172 site from dephosphorylation (Carling et al. 1989; Davies et al. 1995). Upon its activation, AMPK can subsequently phosphorylate a plethora of downstream targets regulating carbohydrate and lipid metabolism, protein synthesis, autophagy and mitochondrial biogenesis (Herzig and Shaw 2018). Twenty-eight studies demonstrated EPS induced AMPK phosphorylation in vitro (Matthews et al. 2009; Uguccioni and Hood 2011; Lambernd et al. 2012; Manabe et al. 2012; Menzies et al. 2013; Brown et al. 2015; Christensen et al. 2015; Evers-van Gogh et al. 2015; Gong et al. 2016; Goto-Inoue et al. 2016; Miyatake et al. 2016; Beiter et al. 2018; Erlich, Brownlee et al. 2018; Hu, Li et al. 2018; Li et al. 2018; Al-Bayati et al. 2019; Lee et al. 2019; Park et al. 2019; SonLee et al. 2019; Chang and Kong 2020; Gao et al. 2020; Hoshino et al. 2020; Tamura et al. 2020a, b; Yue et al. 2020, 2021; Mengeste et al. 2022; Sugimoto et al. 2022). Moreover, the EPS induced activation of AMPK in cultured skeletal muscle cells appears particularly efficacious as evidenced by observed increases at intensities of 0.1–99 Hz and durations of 15 min to 96 h, suggestive of a general stimulation-induced change in energy homeostasis (Hardie et al. 2012). Depletions in cellular ATP and glucose serve as key metabolic signals which activate AMPK and lead to subsequent bioenergetic adaptations. An increased cellular bioenergetic demand as a consequence of EPS induced contraction is supported by seven papers which demonstrate reductions in cellular ATP and glycogen content post-stimulation (Marotta et al. 2004; Nedachi et al. 2008; Lambernd et al. 2012; Manabe et al. 2012; Nikolić et al. 2012; Li et al. 2018; Park et al. 2019; Laurens et al. 2020; Liu et al. 2021). These changes in ATP and glycogen concentrations occurred in both human primary and C2C12 across an extensive range of stimulation intensities (0.1–100 Hz) and following both acute and chronic stimulation (1–24 h). Unsurprisingly, thirteen studies also observed either an associated increase in the uptake of glucose from the cell culture media, or a reduction in the glucose concentrations within the cell medium, with these occurring in both cell types, following both low and high frequencies (0.2–30 Hz) and both acute and chronic stimulation durations (15–48 h) (Lambernd et al. 2012; Manabe et al. 2012; Nikolić et al. 2012; Brown et al. 2015; Gong et al. 2016; Nieuwoudt et al. 2017; Li et al. 2018; Al-Bayati et al. 2019; Khodabukus et al. 2019; Chien et al. 2020; Tamura et al. 2020a, b; Yue et al. 2020; Lautaoja et al. 2021; Liu et al. 2022a, b). Additionally, during prolonged low-intensity exercise, following the progressive decrease in cellular glycogen stores, there is a compensatory shift within the bioenergetic fuel mixture away from glucose/glycogen towards fatty acids (Holloway et al. 2006). EPS has also shown to be efficacious in increased lipid oxidation rates with eight studies demonstrating increases in lipid oxidation in both saturated (palmitic) and unsaturated (oleic) fatty acids (Lambernd et al. 2012; Nikolić et al. 2012; Feng et al. 2015; Laurens et al. 2016, 2020; Løvsletten et al. 2020; Marš et al. 2021; Mengeste et al. 2022). This progressive depletion of glucose and glycogen alongside an associated increase in fatty acid oxidation altogether suggest EPS is depleting intracellular carbon sources and ultimately causing an acute bioenergetic stress response.

Upregulation of AMPK leads to the recruitment of the transcription co-activator peroxisome proliferator-activated receptor-γ (PPARγ) coactivator-1α (PGC-1α) through the phosphorylation of sites Thr-177 and Ser-538, which are necessary for the binding of PGC-1α to its promoter (Jäger et al. 2007). PGC-1α is a master-regulator of cellular energy metabolism through its interactions with transcription factors and nuclear receptors, including oestrogen-related receptor-α (Puigserver et al. 1998; Huss et al. 2002, 2004), thyroid receptor (Puigserver et al. 1998), nuclear respiratory factor-1 (NRF-1) (Wu et al. 1999), NRF-2 (Mootha et al. 2004), and myocyte enhancer factor 2 (MEF2) (Michael et al. 2001; Handschin et al. 2003). Consequently, these

transient increases in PGC-1α activity, resulting from habitual exercise, lead to the coordinated regulation of nuclear- and mitochondrial-encoded genes required for contractile and metabolic adaptations in skeletal muscle (Pilegaard et al. 2003; Lira et al. 2010). This review notes eight studies which demonstrated an increase in PGC-1α gene expression following EPS (Burch et al. 2010; Philp et al. 2011; Uguccioni and Hood 2011; Lambernd et al. 2012; Scheler et al. 2013; Parousis et al. 2018; Guigni et al. 2019; Melouane et al. 2019). Moreover, this increase was preserved in both C2C12 and human primary cells, at both low and high frequencies (1–50 Hz), and during both acute and chronic stimulation (3–24 h) (Burch et al. 2010; Philp et al. 2011; Scheler et al. 2013). However, very low-frequency EPS protocols (0.1 Hz) and very acute EPS durations (30–90 min) seemed to have little effect in inducing changes in PGC-1α gene expression (Burch et al. 2010; Pattamaprapanont et al. 2016; Beiter et al. 2018; Løvsletten et al. 2020). PGC-1α has been shown to be very sensitive to acute endurance exercise in vivo with transient increases in mRNA expression observed after only 45 min and peaking at around 2–3 h post-exercise, with its protein nuclear localisation occurring immediately following exercise (Pilegaard et al. 2003; Mathai et al. 2008; Egan et al. 2010; Little et al. 2010a, b; Norheim et al. 2014). Burch et al. (2010) found that a single bout of 90 min EPS in C2C12 myotubes (50 Hz [ON;1s/ OFF; 1s], 14 V, 1 ms) was ineffective at increasing PGC-1α mRNA expression, however both increasing the EPS duration (24 h) and the total number of stimulation bouts (90 min for 4 consecutive days) resulted in increases in PGC-1α gene expression. Scheler et al. (2013) similarly found in human primary myotubes that while 24 h EPS (5 Hz, 14 V, 2 ms) led to changes in PGC-1α mRNA expression, no changes occurred after 2, 4–8 h suggesting that EPS duration may help dictate PGC-1α sensitivity. Additionally, it is possible that the lack of observed changes in some EPS protocols which have utilised longer stimulation durations (24–48 h) could be due to the pulsatile timing of PGC-1α gene expression rather than a total lack of PGC-1α activity, as this review reports eleven studies demonstrating protein activation of PGC-1α following EPS durations of 1–96 h and between frequencies of 1–10 Hz (Uguccioni and Hood 2011; Carter and Hood 2012; Menzies et al. 2013; Khodabukus et al. 2015; Mesbah Moosavi and Hood 2017; Erlich, Brownlee et al. 2018; Parousis et al. 2018; Guigni et al. 2019; Son, Lee et al. 2019; Gao et al. 2020; Tamura et al. 2020a, b). Therefore, while there seems to be some agreement within the literature regarding the ability for EPS to modulate the acute post-exercise increases in PGC-1α mRNA and protein activity, differences in the stimulation durations, intensities and sampling times may dictate the magnitude of the observed response, and higher intensities (≥1 Hz) and longer durations (~3 h) seem to be particularly effective.

The serine/threonine kinase mTOR is a master regulator of cellular anabolic and catabolic signalling (Bolster et al. 2003). mTOR acts to sense changes in the intracellular environment and coordinates diverse cellular processes, including cell growth, differentiation, autophagy, and metabolism (Laplante and Sabatini 2012). mTOR acts as a catalytic subunit within distinct multiprotein complexes known as mTOR complex 1 (mTORC1) and mTORC2 (Liu and Sabatini 2020). These protein complexes differ in their cellular functions with mTORC1 governing cellular responses to nutritional availability and subsequent anabolism/catabolism, and mTORC2 regulating cytoskeletal behaviour and cell survival pathways (Liu and Sabatini 2020). Resistance training is a well-known activator of the mTOR pathway, with mTOR playing a significant role in governing mechanical load-induced muscle hypertrophy (Baar and Esser 1999; Bodine et al. 2001; Drummond et al. 2009; Goodman et al. 2011). During muscle force production, muscle fibres experience mechanical tension which can be detected by several candidate mechanoreceptors within the cell including integrins, focal adhesion kinases (FAK), and phospholipase D (Wackerhage et al. 2019). Mechanical force mediated activation of integrin–FAK signalling can lead to downstream activation of Phosphoinositide 3-kinase (PI3K), which can drive Phosphoinositide-dependent kinase-1 (PDK1) mediated phosphorylation and activate of Akt (Burkholder 2007; Boppart and Mahmassani 2019). Akt is tightly linked to mTOR activity, as it drives the activation of mTORC1 through the inhibition its negative regulator TSC1–TSC2 complex, but AKT itself is activated by Ser-473 phosphorylation via mTORC2 (Inoki et al. 2002; Sarbassov et al. 2005). This review highlights ten studies that suggest EPS is capable of increasing AKT phosphorylation across a range of stimulation durations (30–96 h) (Manabe et al. 2012; Miyatake et al. 2016; Nieuwoudt et al. 2017; Beiter et al. 2018; Hu, Li et al. 2018; Sidorenko et al. 2018; Khodabukus et al. 2019; Hoshino et al. 2020; Valero-Breton et al. 2020; Liu et al. 2022a, b). However, there is some discordance within the literature regarding AKT responsiveness to EPS, but this could be due to differences in expression between AKT phosphorylation sites. AKT has two main sites of phosphorylation, threonine-308 within its catalytic domain via PDK1, which can be upregulated following mechanical activation of integrins and FAK, and serine-473 on the C-terminal hydrophobic motif through mTORC2 activation, however this second pathway is less understood (Riaz et al. 2012; Thompson et al. 2013; Manning and Toker 2017). AKT^{Thr308} phosphorylation has been reported in several studies ranging from stimulation durations of 40–90 min (Manabe et al. 2012; Miyatake et al. 2016; Beiter et al.

2018; Hu, Li et al. 2018). However, AKT^{Ser473} is more conflicted as Hu, Li et al. (2018) found that 40 min EPS (1 Hz, 20 V, 24ms) increased AKT^{Thr308} phosphorylation with no change in AKT^{Ser473}, whereas Hoshino et al. (2020) found increases in AKT^{Ser473} (and not AKT^{Thr308}) occurred following 60 min EPS at 20 Hz (50 V, 3 ms) with no changes at 2 Hz. However, while the frequency is lower in Hu, Li et al. (2018) the duty cycle (the percentage of time that a device is operating over a specified period) is 300% higher following 1 Hz, 20 V, 24ms (2.4% duty cycle) when compared to 2 Hz, 50 V, 3 ms (0.6% duty cycle). Moreover, studies into the AKT phosphorylation found that AKT^{Thr308} significantly correlated with the phosphorylation of 3 AKT downstream targets (PRAS40, TSC2 and TBC1D4) whereas there was no correlation with AKT^{Ser473} phosphorylation, suggesting that Thr-308 is the better marker of AKT activity (Vincent et al. 2011). Therefore, EPS does seem efficacious in driving AKT phosphorylation at the Thr-308 site which is in line with its reported activation following contraction induced signalling pathways.

However, the ability of EPS to induce changes in mTOR itself currently seems less conclusive as there is conflicting evidence within the literature as to whether mTOR phosphorylation can be consistently augmented within both C2C12 and human primary cells (Carter and Hood 2012; Christensen et al. 2015; Tarum et al. 2017; Beiter et al. 2018; Khodabukus et al. 2019; Patel et al. 2019; Hoshino et al. 2020; Valero-Breton et al. 2020; Yi et al. 2023; Pribil Pardun et al. 2024). This may be in part due to some studies quantifying changes in mTOR activity via changes in mTOR^{Ser2448} phosphorylation as a marker of mTOR activity (Carter and Hood 2012; Christensen et al. 2015; Valero-Breton et al. 2020). Phosphorylation of mTOR at Ser-2448 has historically been used as a metric to infer mTOR activation following resistance exercise in vivo alongside exposure to other anabolic stimuli such as amino acids or insulin, due to its suggested role as a direct AKT phosphorylation target site (Reynolds et al. 2002; Holz and Blenis 2005; Farnfield et al. 2009). However, caution should be taken in interpreting mTOR^{Ser2448} phosphorylation alone as a signifier of mTOR activity as it has also been shown to be induced via the activation of the downstream mTOR target S6K1 through a regulatory feedback loop mechanism and consequently may not necessarily correlate with mTOR functional activity. (Chiang and Abraham 2005; Holz and Blenis 2005). Nevertheless, there is some evidence that more intense protocols are effective in driving an increase in mTOR ^{Ser2448} activity in human primary cells, with 30 min of 100 Hz, 15 V, 0.4 ms (with a 30 s rest between intervals) causing an increase in phosphorylated mTOR^{Ser2448} immediately post EPS alongside increases in phosphorylation of the mTOR downstream targets pS6K1^{Thr389} and ribosomal protein S6 (pS6$^{Ser240/244}$) 1 h post EPS (Valero-Breton et al. 2020). Moreover, Christensen et al. (2015) utilised a longer protocol in human primary cells with a lower pulse frequency (1 Hz, 40 V, 2 ms) for 6 h which also caused an increase in phosphorylated mTOR^{Ser2448} and further phosphorylation of downstream p70S6K^{Thr389}. While not the most commonly measured phosphorylation site within the context of post-exercise adaptations, Ser-2481 represents an mTOR autophosphorylation site within the mTOR catalytic region and can be a measure of mTOR intrinsic kinase activity (Peterson et al. 2000). However, caution should also be taken in the interpretation of changes in mTOR^{Ser2481} given that mTORC1 and mTORC2 respond to different molecular signals and therefore the measurement of total mTOR^{Ser2481} autophosphorylation could obscure any changes occurring to individual mTOR complexes (Soliman et al. 2010). Nevertheless, EPS has been shown to drive changes in mTOR^{Ser2481} phosphorylation, with increases occurring after only 90 min (1 Hz, 14 V, 2 ms) (Beiter et al. 2018). Alternatively, the phosphorylation of downstream mTOR effecters S6K1 and 4E-BP1 are a more direct proxy for mTOR activation in response to exercise-like stimulation (Figueiredo et al. 2017). Limited studies have attempted to quantify changes in both these downstream mTOR target proteins with little agreement amongst the literature regarding their responsiveness (Scheler et al. 2013; Christensen et al. 2015; Tarum et al. 2017; Guigni et al. 2019; Khodabukus et al. 2019; Hoshino et al. 2020; Tamura et al. 2020a, b; Valero-Breton et al. 2020; Yi et al. 2023). Some studies using human primary cells have found acute bouts of EPS of as little as 6 (1 Hz, 40 V, 2 ms) and 8 h (1 Hz, 12 V, 2 ms) were enough to induce increases in pS6K1 and p4E-BP1 respectively (Christensen et al. 2015; Tarum et al. 2017). However, these changes were not observed in other studies utilising multiple bouts of EPS over several days (24 h ON:24 h OFF for 7 days) which may reflect the transient nature of these changes in phosphorylation state of mTOR effector proteins (Yi et al. 2023). A possible explanation for the more inconclusive mTOR response may lie in the effectiveness of EPS in augmented AMPK activity. AMPK can phosphorylate the mTOR scaffolding protein RAPTOR at Ser-792, and this binding results in 14-3-3 binding to RAPTOR and subsequent inhibition of the mTORC1 complex (Gwinn et al. 2008). The EPS of C2C12 myotubes for 2 h (5 Hz, 9 V) was enough to cause a transient increase phosphorylated Raptor^{Ser792}, which can lead to a diminished mTOR response (Gwinn et al. 2008; Carter and Hood 2012). Considering the efficacy of EPS in activating AMPK signalling cascades due to a contraction mediated reduction in ATP: AMP, it is possible that EPS drives a more 'endurance-based' phenotype even at higher stimulation intensities, which in turn will limit mTOR activity. However, mTOR

activation is transient and very few studies have examined downstream mTOR target proteins, so future studies should aim to examine the role of EPS in driving changes in these effector proteins and whether these are better correlated with contractile activity.

MyHC isoforms are the primary contractile proteins in skeletal muscle, determining its functional properties, including peak power output (Gilliver et al. 2009), rate of force development (Harridge et al. 1998) and mechanical efficiency (Sargeant and de Haan 2006). Muscle fibres can be classified based on their contractile and metabolic characteristics into distinct fibre types. The types of muscle fibre type can differ between mammalian species with rodents containing type I (oxidative slow-twitch), type IIa (oxidative-glycolytic fast-twitch), type IIx (intermediate oxidative-glycolytic fast-twitch), and type IIb (predominantly glycolytic fast-twitch) (Schiaffino and Reggiani 2011). However, muscle fibres containing MyHC type IIb are absent in most skeletal muscles of humans (Schiaffino and Reggiani 2011). The overall process of myogenesis is a highly coordinated signalling cascade with temporal changes in gene expression occurring throughout myotube differentiation (Vicente-García et al. 2022). However, fibre types are not static and adult skeletal muscle is also capable of conversion between fibre types in response to exercise, with endurance training resulting in a fast-to-slow transition and resistance training resulting in a slow-to-fast transition (Schiaffino and Reggiani 2011; Yan et al. 2011; Ehlers et al. 2014). *MYH1/Myh1* is the gene that encodes MyHC-IIx/d, the fastest MyHC isoform (Schiaffino and Reggiani 2011). There is conflicting evidence surrounding the effectiveness of EPS at increasing *MYH1/Myh1*, with five studies across C2C12 and human primary cells demonstrating no change in *MYH1/Myh1* expression following EPS treatment (Langelaan et al. 2011; Beiter et al. 2018; Son, Lee et al. 2019; Marš et al. 2021; Yi et al. 2023). However, *MYH1/Myh1* expression seems to be more responsive following EPS treatment to 3D muscle constructs when compared to traditionally used monolayers, with seven studies in 3D models demonstrating increases in expression, suggesting a potential role for more biologically valid models in the analysis of skeletal muscle adaptations (Ahadian et al. 2012, 2014; Ahadian, Ramón-Azcón et al. 2013; Ramón-Azcón et al. 2013; Ostrovidov et al. 2017; Banan Sadeghian et al. 2018; Terrie et al. 2022). However, *Myh2*, which encodes MyHC-IIa, is particularly responsive to EPS within C2C12 myotubes with nine studies demonstrating increases following EPS at a range of stimulation durations (1 h- 4 days) in both 2D and 3D models (Langelaan et al. 2011; Ahadian et al. 2012; Ahadian, Ramón-Azcón et al. 2013; Ostrovidov et al. 2017; Beiter et al. 2018; Mestre et al. 2019; Son, Lee et al. 2019; Fukushima et al. 2021; Lautaoja-Kivipelto et al. 2024). Despite the ability for EPS treatment to promote transcription of fast-twitch MyHC isoforms, the effects of EPS on *MYH7/Myh7*, which encodes slow MyHC, was conflicting, with no effect on human primary cells and mixed evidence regarding C2C12s across the nine studies which investigated it (Burch et al. 2010; Nikolić et al. 2012; Feng et al. 2015; Beiter et al. 2018; Son, Lee et al. 2019; Fukushima et al. 2021; Marš et al. 2021; Yi et al. 2023; Lautaoja-Kivipelto et al. 2024).

The expression of different MyHC transcripts within C2C12 cells are upregulated at different stages of differentiation, and broadly fall into 2 temporal stages; early (*Myh3*, *Myh8*, *Myh7*) and late (*Myh2*, *Myh1* and *Myh4*) (Brown et al. 2012). Consequently, the increased responsiveness of *Myh2* to EPS when compared to *MYH1/Myh1* may highlight a role for EPS in promoting myotube development. Skeletal muscle maturation is highly coordinated and fast-twitch fibre development normally involve early expression of embryonic and perinatal MyHC genes, followed by *Myh2* and then finally *MYH1/Myh1* (Schiaffino et al. 2015). The expression of faster twitch MyHC genes following EPS may subsequently reflect improved maturation of incomplete skeletal muscle development. Interestingly, *Myh8* (which encodes the perinatal MyHC isoform) has also been shown to be responsive to EPS (Ahadian et al. 2012; Ahadian, Ramón-Azcón et al. 2013; Ostrovidov et al. 2017; Lautaoja-Kivipelto et al. 2024). The expression of *MYH8 in vivo* decreases substantially in early life and its expression within cultured muscle highlights either; the biological immaturity of the myotubes themselves, heterogeneity within the culture system as there are (at least in monolayers) likely still some undifferentiated myoblasts remaining, or suggest structural remodelling and maturation of the in vitro system (Periasamy et al. 1984; Schiaffino et al. 2015). Additionally, EPS can also lead to increases in myogenic transcription factors (Irrcher and Hood 2004; Ahadian et al. 2012; Ahadian, Ramón-Azcón et al. 2013; Ahadian et al. 2014; Jo et al. 2017; Ostrovidov et al. 2017; Son, Lee et al. 2019; Hu et al. 2024a, b). The contribution of myogenesis to in vivo exercise adaptations is very small. Therefore, these broad changes in myogenesis-related genes observed during muscle stem cell growth alongside expression of early MyHC isoforms do suggest overall biological immaturity within these cell culture models. Another factor which may be responsible for the variance between studies is the differentiation status of the myotubes upon stimulation, which will differ substantially between monolayer and 3D constructs. Moreover, given many studies do not report images of the myotubes upon initiation of EPS, it is possible that the cultured muscle cells may be at fundamentally different stages of development which will also impact MyHC responsiveness.

Chronic exercise

The increase in size and number of mitochondria known as mitochondrial biogenesis serves as a well-known chronic adaptation to habitual endurance exercise (Freyssenet et al. 1996). The signalling cascade induced following PGC-1α activation can result in the increased expression of Transcription Factor A, Mitochondrial (TFAM), which translocates to the mitochondria and is essential for mitochondrial DNA maintenance and replication (Kang 2018, Chu et al. 2018). There were six studies which quantified TFAM gene and protein expression, of which four demonstrated an increase in expression levels (Burch et al. 2010; Ugucconi and Hood 2011; Carter and Hood 2012; Mesbah Moosavi and Hood 2017). Moreover, the protein citrate synthase (CS) which catalyses the initial rate-limiting step of the tricarboxylic acid cycle (TCA), is located within the mitochondrial matrix and often used to as a surrogate marker to quantify total mitochondrial content (Pallotti and Lenaz 2007). This review documented 5 studies that demonstrated EPS-induced increases in mitochondrial content as measured by CS activity, alongside more direct markers of mitochondrial content such as MitoTracker Green or MitoBright Red fluorescent intensity (Nikolić et al. 2012; Menzies et al. 2013; Feng et al. 2015; Khodabukus et al. 2015; Tamura et al. 2020a, b). However, 3 studies demonstrated no change in mitochondrial content despite having similar frequencies (1–2 Hz) and durations (1–3 h total stimulation time) (Guigni et al. 2019; Gao et al. 2020; Tamura et al. 2020a, b). This disagreement may be explained in part due to mitochondria being inherently dynamic organelles which are maintained via a multitude of quality control processes included selective mitochondrial degradation termed mitophagy (Ding and Yin 2012). Gao et al. (2020) demonstrated that 1 h of EPS (1 Hz, 15 V, 2 ms) resulted in an increase in the expression of the outer mitochondrial membrane protein FUNDC1, alongside a subsequent colocalization of FUNDC1 with the autophagy marker LC3, which contributes to autophagosome assembly (Ding and Yin 2012). Therefore, changes in static mitochondrial content measures may not truly reflect the dynamic restructuring that mitochondria can undergo following exercise-like stimuli (Guan et al. 2019). Additionally, the overall pattern of contraction also matters as Tamura et al. (2020a, b) found that while both twitch (2 Hz, 13 V, 2 ms) and tetanic (66 Hz, [ON: 5s/OFF: 5s], 13 V, 2 ms duration) protocols were able to cause significant increases in mitochondrial content after 3 h of EPS each day for 3 days, the increase in citrate synthase activity or Mitobright Red signal were higher in the tetanic condition compared to the twitch. Moreover, Khodabukus et al. (2015) compared similar protocols changing only the contraction duration but keeping active time constant at 60%, and 60–600 s durations were able to induce greater changes in the mitochondrial electron transporter cytochrome c when compared to 0.6–6 s. Conversely, studies which demonstrate no effect may not be adding enough physiological stress to the cells as 1 h EPS (1 Hz, 20 V, 12 ms) per day for 3 days was shown to be ineffective at driving changes in mitochondrial content, but when calculated the period of EPS accounts for only around 4% of the total experimental time, so treatment times may need to be large enough to drive an adaptive response (Guigni et al. 2019). Importantly, the absence of changes in mitochondrial biogenesis does not preclude remodelling of the existing mitochondrial machinery resulting in increases efficiency of existing mitochondria (Bennett et al. 2022).

The mitochondrial respiratory chain is responsible for the trafficking of electrons and generation of ATP during oxidative phosphorylation, and this process consists of four multimeric protein complexes (Complexes I-IV) which are responsible for the trafficking of electrons to produce the electrochemical gradient required for ATP production via complex V. The protein complexes within the ETC drive the transfer of electrons, which is couple to proton pumping from the mitochondrial matrix to the intermembrane space which generates an electrochemical gradient known as mitochondrial membrane potential ($\Delta\Psi m$). $\Delta\Psi m$ refers to the voltage differential across the mitochondrial matrix and the inner membrane space and is approximately −150 to −180 mV. Moreover, $\Delta\Psi m$ is required for a multitude of mitochondrial processes which contribute to mitochondrial function including the production of ATP via ATP synthase, mitochondrial protein import and Ca^{2+} uptake (Zorova et al. 2018). $\Delta\Psi m$ was only measured in three studies, within which there was no consensus (Lambernd et al. 2012; Tamura et al. 2020a, b; Liu et al. 2021). However, this disparity may be due to the timing of measurement, as exercise is known to increase ROS as a consequence of increased oxidative phosphorylation (Wang et al. 2021). Moreover, mild depolarisation of the inner mitochondrial membrane can serve as a method of attenuating increased ROS production in an attempt to limit oxidative stress (Sack 2006). Therefore, measurements immediately post EPS may demonstrate some mild uncoupling when compared to measurements following periods of rest. There appears to be evidence suggesting that EPS can modulate the activity of respiratory proteins in line with what is observed in vivo although these changes do not extend to every protein complex equally (Gusdon et al. 2017). NADH: ubiquinone oxidoreductase (complex I) which is the site of NADH oxidation and it is therefore highly coupled to redox balance (NADH: NAD ratio) seems to have mixed responses to EPS, with some studies demonstrating robust increases in activity (Tamura et al. 2020a, b; Pribil Pardun et al. 2024),

and others not demonstrating any differences (Lambernd et al. 2012; Løvsletten et al. 2020). However, both studies that demonstrated increases in complex I were performed in C2C12 cells and at a higher stimulation frequency (10–66 Hz) compared to the studies in HSMCs, which demonstrated no effect (0.1–1 Hz), suggesting either a decreased responsiveness in HSMCs or a requirement for higher stimulation frequencies. However, succinate dehydrogenase (complex II) and cytochrome c oxidase (complex IV) seem to be very responsive to EPS in 2D and 3D constructs in both C2C12 and HSMCs (Lambernd et al. 2012; Khodabukus et al. 2015; Tamura et al. 2020a, b; Pribil Pardun et al. 2024). The efficacy of EPS in driving changes in complex II is unsurprising given that it forms a dual role in both trafficking electrons through the ETC alongside oxidising succinate to fumarate within the TCA cycle. Complex II is therefore uniquely placed relative to other ETC complexes in that its functional capacity to produce ATP through electron transport will also directly dictate the capacity to generate reducing equivalents ($FADH_2$). There is also substantial evidence suggesting that EPS on monolayers can induce increases in ATP synthase activity (complex V) in C2C12 cells (Uguccioni and Hood 2011; Carter and Hood 2012; Menzies et al. 2013; Mesbah Moosavi and Hood 2017; Parousis et al. 2018; Tamura et al. 2020a, b). The effects of EPS on coenzyme Q: cytochrome c–oxidoreductase (complex III) are more mixed, however, with only four studies investigating the post EPS changes on complex III, of which three report no effect (Khodabukus et al. 2019; Løvsletten et al. 2020; Tamura et al. 2020a, b; Pribil Pardun et al. 2024). However, given that complex III sits at the nexus of electron flow from both complex I and II, it therefore possesses substantial spare reserve capacity allowing it to quickly upregulate activity to respond to dynamic changes in electron flux (Sriskanthadevan et al. 2015). Therefore, it is possible that complex III requires greater stimuli to drive compensatory adaptations when compared to other respiratory complexes. It should be noted however, that the mitochondrial complexes do not exist as separate units and instead operate as an interconnected network of protein complexes termed supercomplexes (Novack et al. 2020). The non-uniformity in ETC complex content/function is also observed following exercise training in vivo as 12 weeks of high-intensity interval training has been shown to increase complex I, II and IV but not III (Wyckelsma et al. 2017). Additionally, while 4 months of exercise training in vivo has also been shown to cause a significant increase in complex I, complex I was also almost exclusively found assembled in supercomplexes (Greggio et al. 2017). The organisation of mitochondrial supercomplexes has been shown to increase protein stability and proximity to other complexes which may help facilitate improved electron flow (Vartak et al. 2013). Subsequently, the lack of consistent increases amongst the different ETC components may not indicate a lack of adaptation but instead reflect mitochondrial remodelling which is improving ETC component stability in the absence of changes to total content. This is supported by an ETC mediated increase in mitochondrial ATP production and overall OXPHOS which occurred following both acute and chronic EPS bouts ranging from 3 to 48 h (Feng et al. 2015; Chien et al. 2020; Tamura et al. 2020a, b). Therefore, it is entirely possible that while individual complex protein and/or activity remains unchanged there are still changes in the structural organisation of complexes into different configurations to increase respiratory capacity and/or reduce ROS (Novack et al. 2020). However, to data no study has attempted to examine mitochondrial supercomplex formation following EPS treatment.

The growth and maintenance of skeletal muscle are desirable factors in both sporting settings and within certain clinical populations such as cancer cachexia and are hallmarks of chronic adaptations to resistance training protocols (English and Paddon-Jones 2010; Roelofs et al. 2015). Prolonged endurance exercise alone has also been shown to induce some skeletal muscle hypertrophy (Konopka and Harber 2014). This review identified that EPS of cultured skeletal muscle myotubes can effectively mimic the chronic hypertrophic response to exercise, with increases in myotube length, width, diameter, and cross-sectional area reported (Ahadian et al. 2012; Leon-Salas et al. 2013; Martin et al. 2017; Ostrovidov et al. 2017; Tarum et al. 2017; Banan Sadeghian et al. 2018; Khodabukus et al. 2019). Eleven studies analysed myotube diameter/width following EPS treatment, of which six reported increases post stimulation (Liao et al. 2008; Carter and Hood 2012; Hosseini, Ahadian et al. 2012; Ikeda et al. 2016; Martin et al. 2017; Tarum et al. 2017; Khodabukus et al. 2019; Marš et al. 2021; Vechetti et al. 2021; Terrie et al. 2022; Ducharme et al. 2023). Three studies documented EPS-induced changes in myotube area occurring within both C2C12 and human primary monolayers/3D constructs (Leon-Salas et al. 2013; Ostrovidov et al. 2017; Khodabukus et al. 2019). Furthermore, changes in myotube area also occurred following both low and high frequency protocols (across 1–20 Hz) and from 7 to 48 h total stimulation time suggesting EPS mediated hypertrophy (Leon-Salas et al. 2013; Ostrovidov et al. 2017; Khodabukus et al. 2019; Hu et al. 2024a, b). Interestingly, the delivery of EPS prior to myotube formation seems to have a detrimental effect on myotube development. Liao et al. (2008) demonstrated that EPS (1 Hz, 20 V) for 1 h each day, prior to myotube assembly, decreased the percentage of striated myotubes, whereas EPS post-differentiation induced contractile activity and subsequently increased myotube striation. Therefore, changes in the timing of EPS

onset (pre/post myotube formation) may dictate the effectiveness of EPS treatment and therefore, care should be taken to ensure adequate myotube formation prior to EPS delivery. Mature skeletal muscle in vivo exists as large, tubular multinucleated cells, and consequently their organisation and alignment can influence their force production (Sicherer et al. 2025). Resistance training in vivo not only causes changes in skeletal muscle hypertrophy but can also cause changes in muscle architecture, albeit typically fascicle length and pennation angle rather than at the myofibre level (Blazevich 2006). Nevertheless, the development of more mature in vitro models which contain more aligned myotubes is a desirable metric in tissue bioengineering. EPS has been shown to increase myotube alignment within C2C12 3D constructs (Liao et al. 2008; Ahadian et al. 2012; Ostrovidov et al. 2017). Moreover, Khodabukus et al. (2019) has also shown that both 1 Hz, 70 mA, 2 ms and 10 Hz (0.5 s every 5 s), 70 mA, 2 ms produced an increase in cross-striation frequency suggesting more organised and mature muscle constructs. Consequently, while morphological data is limited compared to other measured metrics, in part because 3D models are required to delineate between some measures, there is evidence to suggest EPS can drive distinct 'exercise-like' morphological adaptations.

Recent advances in skeletal muscle bioengineering systems have allowed for the easier development of biologically relevant three-dimensional models of skeletal muscle, derived from primary myoblasts or cell lines (Powell et al. 2002; Martin et al. 2013). A major benefit of these constructs over traditionally used monolayers is the ability to measure aspects of skeletal muscle contractile force production which would be otherwise impossible due to the non-uniaxial nature of 2D cell culture. This review notes eight studies which demonstrated improvements in muscle force production following EPS treatment (Donnelly et al. 2010; Khodabukus and Baar 2012, 2015; Ito et al. 2014; Khodabukus et al. 2015, 2019; Ikeda et al. 2016; Martin et al. 2017). These responses were all observed following chronic EPS delivery as the minimum stimulation duration was 24 h. One study demonstrated no difference in contractile function post EPS; however, the contraction duration was substantially shorter at only 30 min (Davis et al. 2019). Interestingly, this increase in force production extends towards both twitch and tetanic force production, as 1 Hz (70 mA, 2 ms) and 10 Hz (0.5s every 5 s, 70 mA, 2 ms) protocols were both effective when applied 1 h per day for 7 days (Khodabukus et al. 2019). However, not only does EPS seem capable of modulating total contractile force, but it also appears to improve skeletal muscle fatigue responses. Khodabukus et al. (2019) also found that while 1 h per day for 7 days EPS (10 Hz 0.5s every 5 s, 70 mA, 2 ms) did not impact time to peak tension, it reduced both half-relaxation time and subsequently fatigue resistance.

Specific attempts to delineate the types of skeletal muscle force production have also been observed with twitch force production shown to improve in two studies (Ikeda et al. 2016; Khodabukus et al. 2019), and tetanic force production has been shown to improve in three studies (Khodabukus et al. 2015, 2019; Ikeda et al. 2016). Interestingly, during 14 days chronic low frequency EPS (10 Hz), increasing the contraction duration from 0.6 to 60 or 600 secs was associated with a fast to slow phenotype shift as demonstrated by reductions in MyHC-IIx and a slower contraction duration (as measured by time to peak tension) (Khodabukus et al. 2015). However, all contraction durations induced changes in proteins associated with mitochondrial biogenesis and activity such as PGC-1α, carnitine palmitoyltransferase 1 (CPT-1) and Beta-hydroxyacyl-CoA dehydrogenase (β-HAD), suggesting efficacy in EPS generating a more 'endurance-like' adaptive response (Khodabukus et al. 2015). Overall, while there may be subtle differences in the responsiveness of exercise markers to different EPS modalities, it does seem that broadly EPS can induce both 'resistance-like' and 'endurance-like' adaptive contractile responses, but these seem to be present across similar stimulation parameters **(see** Fig. 2**)**.

Discussion

Can EPS replicate endurance and resistance exercise in vivo?

While exercise intensity and duration clearly dictate the in vivo adaptive response, the concept of an AKT/AMPK 'master switch' is likely too simplistic to fully explain the molecular cascades that underpin the responses to both endurance and resistance exercise (Fyfe et al. 2014). Seminal work by Atherton et al. (2005) found that high intensity (6 × 10 3-s repetitions at 100 Hz for 20 min) stimulation of rats induced an increase in anabolic AKT/mTOR signalling with no effect on the AMPK/PGC-1α axis. However, Atherton et al. (2005) also found that 3 h low intensity stimulation (10 Hz) led to an increase in AMPK/PGC-1α and a subsequent decrease in AKT/mTOR signalling. Yet despite this, concomitant exercise using both resistance and endurance exercise seems to result in no molecular interference in vivo (Carrithers et al. 2007; Donges et al. 2012; Apró et al. 2013). However, within in vitro systems following EPS, there does seem to be complexity within the literature regarding the inhibitory role these two 'master systems'. Beiter et al. (2018) found increases in both $AMPK^{Thr172}$ and $mTOR^{Ser2481}$ phosphorylation following 90 min of EPS

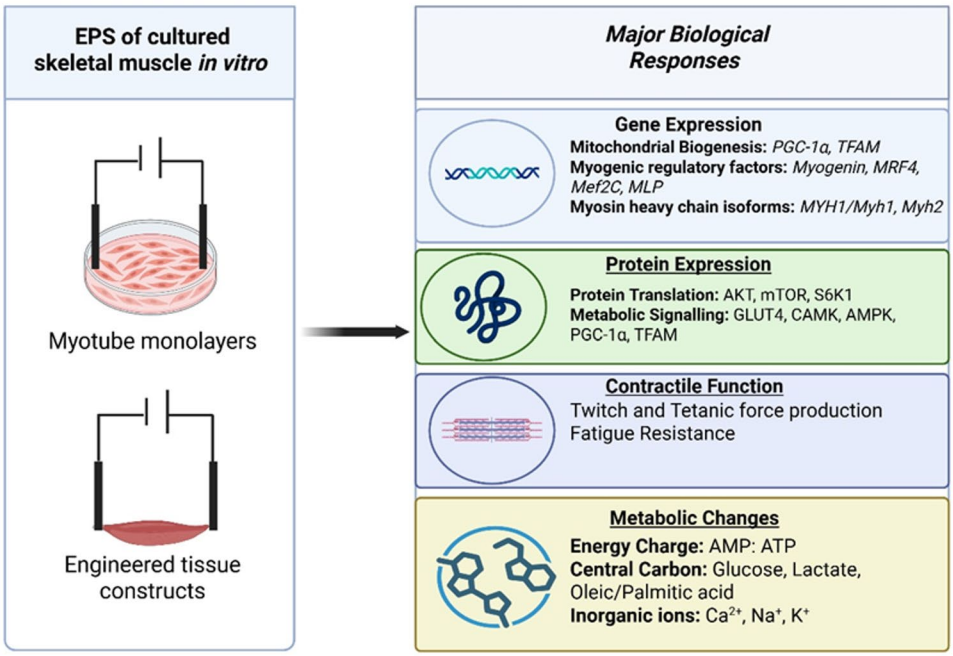

Fig. 2 Overview of observed changes following EPS delivery to cultured skeletal muscle in vitro. Image created using BioRender (BioRender, Canada)

(1 Hz, 14 V, 2 ms). However, while this suggests a concomitant activation of both major signalling pathways, it cannot be ruled out that there is still some reciprocal inhibition from AMPK on mTOR via the phosphorylation of RAPTOR, and this inhibitory effect may not be adequately reflected by measuring the mTOR autophosphorylation site at Ser-2481. Moreover, this AMPK mediated inhibition was examined by Carter and Hood (2012) who speculated that there existed a possible inhibitory role for AMPK post EPS due to its phosphorylation of $RAPTOR^{Ser792}$. AMPK has shown to be particularly sensitive to EPS, and its responsiveness exists across at a range of pulse frequencies. Chang and Kong (2020) for instance found AMPK phosphorylation increased after both low (1 Hz, 11.5 V, 2ms) and high frequency (99 Hz, 11.5 V, 1ms for 20 pulses every 20 s) stimulation. This promiscuous increase in AMPK is likely due to a combination of factors including the depletion of substrate and increased energetic demand driving higher AMP: ATP concentrations, and changes in intracellular calcium (Salt et al. 1998; Woods et al. 2005). While it is often overlooked, AMPK activation is also driven by increased Ca^{2+} flux which drives CAMK family member activation (specifically CaMKKβ). The activation of CAMK family members can lead to Threonine-172 phosphorylation of the catalytic α subunit of AMPK and importantly this Ca^{2+} mediated activation of AMPK can occur independently of changes to AMP: ATP ratios (Woods et al. 2005). Additionally, the process of pumping calcium back into the sarcoplasmic reticulum via sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) also consumes ATP, therefore changes in calcium alone can also contribute to a reduction in cellular energy charge independent of contraction mediated reductions in ATP (Valentim et al. 2022). Calcium is present within standard DMEM media at a concentration of 1.8 mM, with an additional ~ 2.5–3 mM total calcium contained within serum (although this can differ between lots). The depolarisation caused by the delivery of electrical stimulation can drive increased calcium uptake through excitation-coupled Ca^{2+} entry (ECCE) via the skeletal L-type Ca^{2+} channel further contributing to the Ca^{2+}/calmodulin-dependent pathways capable of promoting AMPK activation (Cherednichenko et al. 2004; Bannister et al. 2009). Therefore, changes in calcium homeostasis may be contributing to the sensitivity of AMPK to EPS alongside the known changes in ATP: AMP ratios, and this might explain why EPS seems particularly effective in driving changes in AMPK activity. Consequently, while PI3K/AKT/mTOR adaptations do occur following EPS, these same stimulation protocols are also driving changes in AMPK signalling. Overall, this suggests that while adaptations do occur following EPS, the intensity-specific adaptations commonly observed in vivo are not observed. Instead, EPS appears to induce a generalised adaptive response in cells in vitro, which can be likened loosely to the induction of exercise training in a sedentary individual, where aerobic and hypertrophic adaptations can occur simultaneously (Konopka and Harber 2014).

A limitation of this review is the wide range of literature using EPS to form small parts of larger experiments; it is entirely possible that some relevant studies have not been included in the review and may have been missed during the screening process. However, the screening process was as broad as possible whilst retaining subject relevance to

mitigate this. Another limitation of this review is the absence of a standardised, validated risk of bias tool for in vitro EPS studies. As such, methodological quality was assessed qualitatively rather than through a formalised scoring system. This approach may limit direct comparison of study quality across the included literature; however, it reflects current limitations within the field and aligns with previous reviews of mechanistic in vitro research. Finally, due to the study criteria involving cell culture experiments, this review was not eligible for pre-registration on PROSPERO.

To our knowledge, this is the first systematic review aimed at determining whether EPS can successfully mimic acute and chronic exercise responses and whether EPS modalities can be tailored specifically to replicate endurance or resistance exercise in vivo. In summary, while there appears to be evidence suggesting that EPS can be used to simulate acute and chronic responses to exercise in vitro, it cannot be used to differentiate between the specific molecular responses to 'endurance-like' or 'resistance-like' exercise. Moreover, unlike traditional in vivo exercise, there do not seem to be divergent adaptations, with the AKT/mTOR and AMPK/PGC-1α molecular pathways occurring simultaneously across a wide range of stimulation durations and intensities. This is perhaps explained by both the biological immaturity of the cell model and the lack of limitations commonly linked to systemic factors such as oxygen transport (determined by arteriovenous oxygen difference or cardiac output) which would dictate overall exercise capacity. As such, EPS is likely showing a generalised response to muscle contractility and consequently has diminished specificity of adaptations when compared to exercise in vivo. Therefore, while the EPS of cultured muscle may be beneficial for inducing a more generalised exercise-like response, it is not currently an appropriate substitute for in vivo experiments when investigating specific endurance/resistance training adaptations. Moreover, for the purpose of quantifying acute and chronic exercise-like responses, it is likely that a range of stimulation durations would be sufficient in inducing an appropriate response. There also exists considerable methodological variability across studies, including inconsistencies in stimulation protocols, cell models, and outcome reporting, limited direct comparability between studies and precluded quantitative synthesis. These factors were considered during interpretation of the evidence and may partially explain inconsistencies observed across outcome domains.

Finally, there is little consensus regarding experimental standardisation and the nomenclature surrounding EPS parameters used within the literature, leading to discrepancies between researchers. To that end, these suggested recommendations for undertaking electrical stimulation experiments to elicit exercise-like adaptations are advised to promote a better understanding amongst researchers.

- The term electrical pulse stimulation (EPS) is used when describing the delivery of sustained electrical activity to myotubes. This is the most used term within the literature; therefore, it is logical that it remains the accepted terminology.
- Images of myotubes upon the induction of EPS should be included. Given that EPS delivery prior to the formation of myotubes can lead to decreases in the post-EPS adaptive responses (Liao et al. 2008), researchers should aim to include representative images to confirm myotube differentiation.
- Studies should attempt to include V/mm measurements given that these better reflect the electrical stimulus experienced by the cells over voltage alone. At a minimum, papers should include information on electrode distance so this can be calculated during data interpretation.
- Acute experiments should be undertaken within 10 h of the onset of stimulation, in line with previous work demonstrating the time-course of post-exercise transcriptional responses (Kuang et al. 2022).
- Low-frequency stimulation should be classed as ≤ 10 Hz as higher frequencies can induce a tetanic contraction and should be more appropriately labelled high-frequency stimulation (Orfanos et al. 2016).
- If a generalised stimulation protocol is required, then 1 Hz, 11.5 V (0.567 V/mm estimated nominal field strength assuming 20.3 mm electrode spacing), 2 ms is the most commonly used protocol within the literature and has been shown to induce some of the gene, protein and metabolic changes commonly observed following exercise in vivo (Lambernd et al. 2012; Görgens et al. 2013, 2016; Raschke et al. 2013; Evers-van Gogh et al. 2015; Park et al. 2019; Chang and Kong 2020; Chien et al. 2020).

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Declarations

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