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Exercise-responsive phosphoproteins in the heart

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ABSTRACT

Endurance exercise improves cardiac performance and affords protection against cardiovascular diseases but the signalling events that mediate these benefits are largely unexplored. Phosphorylation is a widely studied post-translational modification involved in intracellular signalling, and to discover novel phosphorylation events associated with exercise we have profiled the cardiac phosphoproteome response to a standardised exercise test to peak oxygen uptake (VO2peak).

Male Wistar rats (346 ± 18 g) were assigned to 3 independent groups (n = 6, in each) that were familiarised with running on a motorised treadmill within a metabolic chamber. Animals performed a graded exercise test and were killed either immediately (0 h) after or 3 h after terminating the test at a standardised physiological end point (i.e. peak oxygen uptake; VO2peak). Control rats were killed at a similar time of day to the exercised animals, to minimise possible circadian effects. Cardiac proteins were digested with trypsin and phosphopeptides were enriched by selective binding to titanium dioxide (TiO2). Phosphopeptides were analysed by liquid chromatography and high-resolution tandem mass spectrometry, and phosphopeptides were quantified by MS1 intensities and identified against the UniProt knowledgebase using MaxQuant (data are available via ProteomeXchange, ID PXD006646).

The VO2peak of rats in the 0 h and 3 h groups was 66 ± 5 ml kg^{-1} min^{-1} and 69.8 ± 5 ml kg^{-1} min^{-1}, respectively. Proteome profiling detected 1169 phosphopeptides and one-way ANOVA found 141 significant ($P < 0.05$ with a false discovery rate of 10%) differences. Almost all (97%) of the phosphosites that were responsive to exercise are annotated in the PhosphoSitePlus database but, importantly, the majority of these have not previously been associated with the cardiac response to exercise. More than two-thirds of the exercise-responsive phosphosites were different from those identified in previous phosphoproteome profiling of the cardiac response to β_1-adrenergic receptor stimulation. Moreover, we report entirely new phosphorylation sites on 4 cardiac proteins, including S81 of muscle LIM protein, and identified 7 exercise-responsive kinases, including myofibrillar protein kinases such as obscurin, titin and the striated-muscle-specific serine/threonine kinase (SPEG) that may be worthwhile targets for future investigation.

1. Introduction

Exercise has an irrefutable role in preventing heart failure and cardiac diseases, for example acute exercise has cardio-protective effects similar to ischaemic preconditioning [1] and chronic exercise training results in physiological cardiac hypertrophy [2] and a heart phenotype that affords protection against pathological insults such as ischaemia/reperfusion injury [3]. Although the physiological benefits of exercise are clear, less is known about the molecular mechanisms that underlie these effects. Yet greater molecular understanding could enable the benefits of exercise to be further optimised or personalised and could suggest new targets for more effective modes of diagnosis, prevention or rehabilitation of debilitating cardiac diseases.

Previous work has investigated discrete signalling events activated in response to exercise, for example in the context of acute cardiac preconditioning [1] or adaptive versus maladaptive cardiac hypertrophy [2]. The IGF-1 receptor/PI3K (p110α)/Akt1 pathway is perhaps the most well-explored regulatory pathway associated with exercise-induced cardiac hypertrophy but it is unlikely that a biological phenomenon as complex as cardiac growth is entirely mediated by a single pathway and more often integrated networks of molecules across

Abbreviations: AR, adrenergic receptor; VCO2, carbon dioxide production; ESI, electrospray ionization; FDR, false discovery rate; HCD, High-energy collision-induced dissociation; MS, mass spectrometry; VO2, oxygen uptake; VO2peak, peak oxygen uptake; MS/MS, tandem mass spectrometry; S, serine; TiO2, titanium dioxide; T, threonine; Y, tyrosine.

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multiple pathways are required to achieve physiological adaptations to environmental stimuli [4]. Therefore, events outside of the canonical IGF-1R/PI3K (p110α)/Akt1 pathway are likely to also contribute to exercise-induced cardiac adaptations and remain to be discovered.

Vigorous exercise is associated with significant elevations in cardiac work and myocardial contractility which are driven by the chronotropic and inotropic effects of beta-adrenergic receptor (AR) signalling (sympathetic drive) as well as local metabolic responses and mechanical strain. In addition to driving acute increases in cardiac output, the molecular events associated with exercise also instigate adaptive processes that alter the cardiac proteome [5] and increase the capacity for work (i.e. VO2peak). Phosphorylation networks are recognised widely in the literature and are known to transduce signals involved in the skeletal muscle response to exercise in humans [6] but until now the cardiac phosphoproteome response to exercise has not been reported. Phosphoproteome profiling is a useful approach to discover the pathways and signalling events involved in physiological processes, and a key advantage of this technique is its non-targeted approach that it is not biased by preconceptions about which pathways or events may be of greatest importance.

Due to the implausibility of sampling human cardiac tissue in the context of exercise physiology, models are required that simulate exercise prescription in humans while allowing access to the heart for molecular investigation. The exercise stimulus is a composite of 3 interrelated variables, i.e. exercise intensity, duration and frequency, and the cardio-protective of exercise is intensity-dependent [1]. Therefore, to control and standardise exercise intensity we [5] have used indirect calorimetry and an incremental protocol of exercise on a motorised treadmill to measure peak oxygen uptake (VO2peak) of rats in a manner that is equivalent to best practice in human studies (e.g. [7].). During the VO2peak test the animal's respiratory gases are monitored and the test is terminated when the animal reaches its peak aerobic capacity (this intensity of exercise is attainable even by previously sedentary animals). By using this physiological end-point we minimise the influence of acute stress induced by an unrealistic exercise load. Such, standardisation is important because differences in exercise capacity exist even within a colony of animals exposed to identical environmental conditions. Therefore, exposure to the same relative exercise stimulus represents an optimised model with the best chances of successfully identifying the key regulatory networks that mediate exercise-induced adaptation.

2. Methods

2.1. Graded treadmill test of peak oxygen uptake

Experiments were conducted under the British Home Office Animals (Scientific Procedures) Act 1986 and according to UK Home Office guidelines. Male Wistar rats were bred in-house in a conventional colony and the environmental conditions controlled at 20 ± 2 °C, 45–50% humidity with a 12-h light (1800–0600) and dark cycle. Water and food (containing 18. 5% protein) were available ad libitum.

Exercise sessions were conducted during the animals' dark period. All rats (n = 18) completed a 14-day familiarization procedure encompassing daily bouts (15 min duration) at various belt speeds and inclines on a motorised treadmill within a metabolic chamber (Columbus Instruments, OH). On the 15th day the VO2peak of animals (n = 12) assigned to the exercise groups was measured using an incremental test, as described previously [5,8]. Briefly, a warm-up (5 min running at 6 m min^{-1}, 0° incline) was completed followed by a series of 3 min stages of alternating increases in speed (increments of 2 m min^{-1}) and incline (increments of 5°; maximum incline 25°). Air pumped (2.5 l min^{-1}) through the chamber was analysed for concentrations of oxygen and carbon dioxide (Oxymax system; Columbus Instruments, OH; calibrated to an external standard) and a metal grid at the rear of the treadmill belt, which delivered a maximum of 3 electric stimuli (0.1 mA, 0.3 s duration), was used to encourage the animals to achieve their VO2peak. Independent groups (n = 6, in each) of animals were killed by cervical dislocation either immediately (0 h) after cessation of the exercise test or 3 h after completing the exercise test. Hearts were isolated from the exercised animals and from control rats (n = 6) that completed the familiarization training but did not perform an incremental exercise test. Hearts were rapidly isolated, cleaned and weighed before being stored at −80 °C. To minimise the influence of circadian differences, control rats were killed at a time of day coinciding with the incremental exercise test.

2.2. Sample preparation

Left ventricles were pulverized in liquid nitrogen and an accurately weighed portion (100 mg) homogenized on ice in 10 volumes of 8 M urea, 4% w/v CHAPS, 40 mM Tris base including protease and phosphatase inhibitor cocktails (Roche Diagnostics, Lewes, UK) at 4 °C. After centrifugation at 20,000*g*, 4 °C for 45 min the supernatant was decanted and the protein concentration measured using a modified 'microtitre plate' version of the Bradford assay (Sigma, Poole, Dorset, UK).

Aliquots containing 2 mg protein were reduced with 2.5 mM dithiothreitol for 1 h at room temperature then alkylated with 5 mM iodoacetamide for 45 min in the dark at room temperature. Samples were diluted with 50 mM ammonium bicarbonate to bring the concentration of urea to 1 M and sequencing-grade trypsin (Promega) was added at a substrate to enzyme ratio of 50:1. After 4 h, samples were diluted threefold with 50 mM ammonium bicarbonate containing additional trypsin, and the digestion was allowed to proceed overnight. After acidification to a final concentration of 1% (v/v) formic acid, the peptide solutions were desalted using disposable Toptip C18 columns (Glygen) and lyophilized to dryness. Phosphopeptides were selectively enriched by binding to titanium dioxide (TiO2)-coated magnetic beads (Pierce) according to the manufacturer's instructions, as described in previously [9]. Briefly, peptides were resuspended in 200 μl 80% acetontirile, 2% formic acid and incubated for 1 min with 10 μl of slurry containing TiO2 magnetic beads. Unbound peptides and supernatant were decanted and the beads were washed three times with 200 μl binding buffer (supplied with the kit). After final decanting, the beads were incubated for 10 min with 30 μl elution buffer and the eluate was carefully removed and dried prior to mass spectrometry analysis.

2.3. Mass spectrometry analysis

Tryptic peptide mixtures were analysed by nano-scale high-performance liquid chromatography (Proxeon EASY-Nano system) and online nano electrospray ionization (ESI) tandem mass spectrometry (LTQ-Orbitrap Velos mass spectrometer; Thermo Fisher Scientific). Samples were loaded in aqueous 0.1% (v/v) formic acid via a trap column constructed from 25 mm of 75 μm i.d. silica capillary packed with 5 μm Luna C18 stationary phase (Phenomenex). The analytical column was constructed in a 100 mm × 75 μm i.d. silica capillary packed with 3 μm Luna C18 stationary phase. Mobile phase A, consisted of 5% acetonitrile and 0.1% formic acid, and organic phase B contained 95% acetonitrile and 0.1% formic acid. Reverse phase separation was performed over 120 min at a flow rate of 300 nl/min, rising to 6% B in 1 min then from 6% to 24% B over 89 min followed by a 16 min gradient to 100% B, which was held for 5 min prior to re-equilibration to 0% B over 9 min. Eluted peptides were sprayed directly in to an LTQ-Orbitrap Velos mass spectrometer using a nanospray ion source (Proxeon). Tandem mass spectrometry (MS/MS) was performed using high-energy collision-induced disassociation (HCD) and 10 MS/MS data-dependent scans (7500 resolution) were acquired in centroid mode alongside each profile mode full-scan mass spectra (30,000 resolution), as reported previously [9]. The automatic gain control (AGC) for MS scans was 1×10^6 ions with a maximum fill time of 250 ms. The AGC for MS/MS scans was 3×10^4, with 150 ms maximum injection time, 0.1 ms