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Nrf2 deficiency induces skeletal muscle mitochondrial dysfunction: a proteomics/bioinformatics approach

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Intracellular redox homeostasis is crucial for maintaining healthy skeletal muscle. Substantial redox imbalance due to excess reactive oxygen species (ROS) production is consequently associated with muscle fatigue, functional decline, metabolic dysfunction and even muscle wasting. The nuclear factor erythroid-derived 2 (Nrf2)/Kelch ECH-associated protein 1 (Keap1) complex is a redox-sensitive transcriptional regulatory system pivotal for the regulation of redox homeostasis, where the cytoplasmic repressor protein, Keap1, senses intracellular ROS and electrophiles, and the transcription factor, Nrf2, promotes antioxidant and other cytoprotective (e.g. anti-inflammation, metabolism) gene expression. In the resting state, Nrf2 is sequestered in the cytoplasm by Keap1 and targeted for proteasomal degradation and thus antioxidant expression remains low. However, in response to cellular stress, intracellular ROS levels increase and several Keap1 cystine residues become oxidised, facilitating the liberation of Nrf2 from Keap1. Nrf2 therein translocates to the nucleus where it binds to antioxidant response elements, in turn promoting the transcription of antioxidant enzyme genes. Targeted analytical research has shown that exercise stress induces Nrf2-mediated antioxidant expression in mouse skeletal muscle (Li *et al.* 2015) and, conversely, that Nrf2-deficient mice display higher intramyocellular ROS levels and attenuated antioxidant enzyme abundance (Miller *et al.* 2012). Consequently, impaired Nrf2/Keap1 function has been implicated in skeletal myopathy and disease and the Nrf2/Keap1 complex has been suggested as a promising therapeutic target against such pathologies. However, despite growing evidence for the Nrf2/Keap1 complex as a powerful intramuscular antioxidant system, the precise biological implications of Nrf2 in skeletal muscle tissue remain poorly defined.

In a recent article published in *The Journal of Physiology* (Gao *et al.* 2020), the authors aimed to address this current research aperture by applying untargeted proteomic analysis to Nrf2-deficient or Nrf2-overexpressed mouse skeletal muscle tissue in order to identify a broad spectrum of novel targets and downstream pathways of Nrf2. For this purpose, two skeletal muscle-specific transgenic mouse models were produced: (i) iMS-*Nrf2*$^{flox/flox}$, a model for Nrf2 deficiency, and; (ii) iMS-*Keap1*$^{flox/flox}$, a model for Nrf2 overexpression. At 12 weeks of age, intraperitoneal injection of tamoxifen (or vehicle control) was administered for five consecutive days to activate Cre-recombination and thus induce selective Nrf2 (*Nrf2*-KO) or Keap1 (*Keap1*-KO) gene inactivation. At 32 weeks of age, mice were assigned to two cohorts for functional assessment and proteomic/bioinformatic analysis of skeletal muscle. Functional assessment included maximal exercise capacity via treadmill running, *in situ* muscle contractility under 2% isoflurane anaesthesia via tetanic stimulation, targeted protein content via immunoblotting, glutathione content via glutathione assays and mitochondrial function via high resolution respirometry and citrate synthase activity. Proteome-wide expression was quantified using mass spectrometry, with the resultant data being subjected to downstream proteomic/bioinformatic analyses.

Gao *et al.* (2020) reported many interesting and novel findings. First, *Nrf2*-KO mice displayed significantly impaired maximal running speed, distance, duration and reduced muscle force-generating capacity (compared with vehicle, i.e. *Nrf2*-WT mice), whereas these performance parameters were improved in *Keap1*-KO mice, suggesting that Nrf2 has potent effects on skeletal muscle function. This is perhaps to be expected given the mechanisms of action of the Nrf2/Keap1 complex: specifically, that reduced Nrf2 (i.e. *Nrf2*-KO) leads to reduced antioxidant expression (either in the rested state and/or in response to exercise stress) and therein a redox imbalance, in turn negatively affecting muscle function. Second, differential protein-level analysis revealed 114 differentially expressed proteins in *Nrf2*-KO mouse muscle (11 upregulated, 103 downregulated) and 117 differentially expressed proteins in *Keap1*-KO mouse muscle (108 upregulated, nine downregulated), of which 10 proteins were found to be commonly regulated. Given this overall lack of commonality, the authors suggest that regulated proteins can be distinguished into two distinct categories: (i) upregulated proteins in *Keap1*-KO muscle are molecular targets activated by a high level of Nrf2, and; (ii) downregulated proteins in *Nrf2*-KO muscle are Nrf2 targets, which rely on basal Nrf2 activity. Downstream Gene Ontology enrichment analysis and Ingenuity Pathway Analysis (IPA) of differentially expressed proteins expectedly revealed that proteins upregulated by *Keap1*-KO were involved in well-known Nrf2 functions (e.g. detoxification, antioxidant defence). However, *Nrf2*-KO regulated proteins were found to be involved in (e.g.) purine ribonucleoside triphosphate metabolism, of which the implications are currently unclear. A particularly striking finding was that *Nrf2*-KO downregulated proteins were associated with many aspects of mitochondrial dysfunction, including abnormal mitochondrial morphology (COQ7), volume, length and coupling of the mitochondria (AK1), transmembrane potential of mitochondria (PHB and YWHAE), permeability transition of mitochondria (PPID) and mitochondrial complex I deficiency (NDUFS6) – implicating a role for, and candidate mechanistic targets of, Nrf2-related regulation of muscle mitochondrial health/remodelling. Mitochondrial disturbance was subsequently confirmed at the functional level. Specifically, citrate synthase activity and electron transport chain (ETC) complex protein expression

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were reduced in *Nrf2*-KO mouse muscle, indicative of reduced muscle mitochondrial content. Further, coupled mitochondrial respiration of the soleus muscle was lower in *Nrf2*-KO mice (compared with *Nrf2*-WT, *Keap1*-KO and *Keap1*-WT mice), with no changes observed in *Keap1*-KO mice (*vs.* *Keap1*-WT mice). Thus, it would be logical to postulate that reductions in mitochondrial respiration may be a cause/consequence of reduced ETC protein content and/or a more globally occurring dysregulation of mitochondrial function-related proteins, as indicated by untargeted proteomics.

When interpreting the results presented by Gao *et al.* (2020), a number of important caveats/limitations are worthy of mention. First, as the authors themselves highlight, the sample size available for mass spectrometry-based analysis was small ($n = 3$/group), perhaps increasing the likelihood of false-negative and/or false-positive results. Larger group sizes would have also better facilitated the incorporation of more holistic bioinformatic tools into the analysis pipeline. One example is weighted co-expression network analysis (Pei *et al.* 2017), wherein molecular complexity is accounted for by modelling interactions among all molecules contained within a large-scale dataset as a function of expression similarity. Such a data-driven form of network modelling could have further extended novel mechanistic insight into the biological relevance of Nrf2 in skeletal muscle beyond knowledge-based pathway modelling of individually regulated proteins (e.g. IPA); namely, by providing a framework to derive entirely new pathways of interacting proteins (dys)regulated by *Nrf2*-KO/*Keap1*-KO, the key 'hub' components of such pathways and, importantly, novel pathways that might be directly associated with the observed functional consequences of *Nrf2*-KO/*Keap1*-KO (Uddin & Singh, 2017). It is also worthwhile mentioning that *Nrf2*-KO/*Keap1*-KO protein changes have been generalized to be changes in skeletal muscle *per se*. Yet, the presented volcano plots (Figure 3*A*) suggest that *Nrf2*-KO protein changes are in fact driven by changes in the extensor digitorum longus (EDL), whereas *Keap1*-KO protein changes are instead driven by changes in the soleus. It is well established that these two particular muscles are markedly different from one another in terms of fibre-type distribution (i.e. EDL is predominantly composed of fast-twitch fibres whereas the soleus is predominantly composed of slow-twitch fibres) and, as a consequence, also differ in their mitochondrial capacity (i.e. lower mitochondrial capacity in EDL *vs.* soleus). Such a caveat is important to consider in relation to the authors' interpretation of Nrf2 function in muscle, since it is plausible that the overall functions of/specific mitochondrial regulation by Nrf2 in skeletal muscle may partly depend on muscle/fibre type, as the proteomic data suggest. Further demonstrating this point, the authors found Troponin T protein, which is specific to fast-twitch fibres, to be downregulated in *Nrf2*-KO muscle, which we expect is probably driven by *Nrf2*-KO-induced changes in the EDL (as opposed to skeletal muscle *per se*). Therefore, the biological role/mechanisms of Nrf2 may be distinct in different muscles owing to differences in fibre-type distribution (and thus the underlying metabolic and molecular characteristics), a hypothesis worthy of further investigation. Finally, whilst a thorough and well-conducted investigation has been conducted by Gao *et al.* (2020), it must be remembered that these findings are based on data obtained from transgenic mice, thus precluding direct translation to humans.

As with any novel experiment, numerous follow-up questions and future research avenues directly arise from the findings observed by Gao *et al.* (2020). For example, where might the onset of muscle mitochondrial dysfunction occur in the context of *Nrf2*-KO? The adoption of a systems biology approach (i.e. integrated genomics, transcriptomics and proteomics) into a data-driven network framework, a feat rightly becoming more popular within physiology, should help identify such (e.g.) transcriptional reprogramming under said conditions and thus help answer such fundamental biological questions.

In summary, Gao *et al.* (2020) advance our current understanding on the biological relevance of Nrf2 in skeletal muscle, with a key finding being that Nrf2 deficiency leads to muscle mitochondrial dysfunction. Further, by delving into the analytical world of proteomics and bioinformatic analysis, Gao *et al.* highlight mitochondrial targets of Nrf2, which may in turn represent promising candidates for therapeutic intervention.

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Additional information

Competing interests

No competing interests declared.

Author contributions

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Keywords

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