## Reply to TA Schiffer et al.

Dear Editor:

We appreciate the interest that Schiffer et al. have shown in our recent work (1), where we investigated the potential mechanisms through which inorganic nitrate and nitrite may influence skeletal muscle energetics.

The authors of this letter expressed concern regarding the use of the standard bolus addition of ADP to determine phosphate:oxygen (P:O) ratios in our respiratory measurements of mitochondrial efficiency. We acknowledge that although the alternative method to administer ADP by infusion, as used by Larsen et al. (2), represents a desirable incremental step towards in vivo–like conditions in such in vitro experiments, it would be unlikely to have had a significant impact on the overall findings (3). Although results may differ quantitatively, either method will detect P:O ratio changes associated with altered proton leak, should they occur. We note that our other key respiratory measurements relevant to mitochondrial efficiency, proton leak rates, and respiratory control ratio values, were performed in a similar manner to those in Larsen et al. (2), yet still showed no difference between supplementation and controls, consistent with our P:O ratio findings.

The authors of the letter also suggested that our measured P:O values are unusual by being lower than their expected value of 3. We would like to point out that for complex I substrates, the theoretical maximum P:O ratio for 100% coupled mammalian mitochondria is 2.7 but will be lower than this in practice owing to proton leak activity and other protonmotive force–driven processes [expected experimental values are ∼2.5, see (4)]. As such, our measured values of ∼2.4 are what one should expect.

The authors of the letter go on to highlight that the P:O ratio of mitochondria from mice is reported to decrease in response to nitrate administration (5), in contrast to what they had observed in humans, concluding that this difference disqualifies mice as a suitable model. Yet, we and others (6) found no evidence to suggest that dietary nitrate/nitrite altered mitochondrial efficiency in humans, similar to our findings in mice. Until the reasons for those inconsistencies in both human and murine studies are resolved, we believe this conclusion to be premature.

With regards to our human studies, the authors highlighted the divergent methods we employed with respect to their own: we acknowledged this in our article, reporting the pharmacokinetics of plasma nitrate and nitrite in human plasma over 24 h after bolus intravenous administration of nitrite. However, it is worth noting that *1*) bio-absorption of nitrate/nitrite can be quite rapid, with plasma concentrations of nitrate and nitrite increasing ∼30 min after oral ingestion, with similarly rapid tissue diffusion (7); and *2*) not all effects of nitrite/nitrate track plasma concentrations, as demonstrated by us recently in the context of long-lasting, delayed, vascular effects of inorganic nitrite (8). In addition, the authors' concern that a 30-min infusion of nitrite may not be expected to affect protein concentrations 24 h later is at odds with earlier observations in rats of widespread alterations in both expression and posttranslational modification of the cardiac mitochondrial proteome after a single intraperitoneal bolus application of nitrite the day before (9). That said, the extensive use of $\beta$-blockers within our patient cohort may have attenuated any effect of nitrite that we hoped to see (10). Given our results and others, we agree that more research into these areas is warranted.

Lastly, our study includes work that demonstrates a lack of change in the expression of human proteins that had previously been implicated in altered mitochondrial efficiency in response to nitrate by others (2). Hence, our title and conclusions are based on data obtained from humans as well as mice.

In conclusion, we would welcome further investigations in this field, particularly with regards to whether or not divergent effects of inorganic nitrate and nitrite on mitochondrial function occur between rodents and humans, and the mechanisms responsible for this.

Author disclosures: The authors report no conflicts of interest.

Maria Ntessalen
Nathan EK Procter
Martin Feelisch
Paul G Crichton
Michael P Frenneaux

From the Institute of Medical Sciences, University of Aberdeen, Aberdeen, United Kingdom (MN, e-mail: m.frenneaux@uea.ac.uk; MPF); Norwich Medical School, University of East Anglia, Norwich, United Kingdom (NEKP, PGC, MPF); and Clinical & Experimental Sciences, Faculty of Medicine, University of Southampton, Southampton General Hospital, Southampton, United Kingdom (MF).

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Supported by Medical Research Council grant G1001340 (to MPF).

 

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doi: https://doi.org/10.1093/ajcn/nqz317.