The following is an abstract of the article discussed in the subsequent letter:

Drew, Barry and Christiaan Leeuwenburgh. Method for measuring ATP production in isolated mitochondria: ATP production in brain and liver mitochondria of Fischer-344 rats with age and caloric restriction. Am J Physiol Regul Integr Comp Physiol 285: R1259–R1267, 2003; 10.1152/ajpregu.00264.2003.—The production of ATP is vital for muscle contraction, chemiosmotic homeostasis, and normal cellular function. Many studies have measured ATP content or quantitative changes in ATP production, but few have quantified ATP production in vivo in isolated mitochondria. Because of the importance of understanding the energy capacity of mitochondria in biology, physiology, cellular dysfunction, and ultimately, disease pathologies and normal aging, we modified a commercially available bioluminescent ATP determination assay for quantitatively measuring ATP content and rate of ATP production in isolated mitochondria. The bioluminescence assay is based on the reaction of ATP with recombinant firefly luciferase and its substrate luciferin. The stabilities of the reaction mixture as well as relevant ATP standards were quantified. The luminescent signals of the reaction mixture and a 0.5 μM ATP standard decreased linearly at rates of 2.16 and 1.39% decay/min, respectively. For a 25 μM ATP standard, the luminescent signal underwent a logarithmic decay, due to intrinsic deviations from the Beer-Lambert law. Moreover, to test the functionality of isolated mitochondria, they were incubated with 1 and 5 mM oligomycin, an inhibitor of oxidative phosphorylation. The rate of ATP production in the mitochondria declined by 34 and 83%, respectively. Due to the sensitivity and stability of the assay and methodology, we were able to quantitatively measure in vivo the effects of age and caloric restriction on the ATP content and production in isolated mitochondria from the brain and liver of young and old Fischer-344 rats. In both tissues, neither age nor caloric restriction had any significant effect on the ATP content or the rate of ATP production. This study introduces a highly sensitive, reproducible, and quick methodology for measuring ATP in isolated mitochondria.

Mitochondrial ATP measurements

To the Editor: I read with interest the recent paper by Drew and Leeuwenburgh (5) that, under the heading “Innovative Methodology,” reported a method for measuring ATP concentration and production rate in isolated mitochondria from rat brain and liver. The authors have obviously put a lot of effort into establishing this method in their laboratory. However, the purpose of this letter is to point out that method presented is not as novel or innovative as portrayed and there are some limitations.

The issue of novelty is addressed first. A full paragraph of the introduction is devoted to describing the principle of ATP monitoring with luciferin/luciferase. Yet, surprisingly, the authors fail to acknowledge the prior application of bioluminescent technology to mitochondrial studies. The concept of measuring mitochondrial ATP production rate (MAPR) using luciferin/luciferase has been around for more than 30 years (9). However, for various reasons, not the least of which was the availability of high-quality luciferase/luciferin preparations, the method was not fully developed until 1990 when Wibom et al. (20) published the first paper that presented a readily applicable, reproducible technique. In that paper, Wibom et al. (20) demonstrated how MAPR could be measured in mitochondria isolated from rat skeletal muscle using several different substrate combinations to probe different components of the oxidative-phosphorylation pathways. A luminometer with an automated routine was used to simultaneously measure MAPR in up to 25 reaction tubes. Drew and Leeuwenburgh cite this paper only in reference to the pyruvate concentration used. They also cite a paper by our group in which we measured MAPR in liver and muscle of caloric-restricted rats using Wibom’s method (13). There are many published papers in which luciferin/luciferase has been used to measure MAPR. Wibom has performed studies of MAPR on mitochondria from rat muscle (22) rat heart (4), rat pancreatic islets (23), and human muscle (1, 10, 18, 19). Our research group has used Wibom’s method to measure MAPR in mitochondria from rat heart, liver, and muscle (12–14) and human muscle (15), as have many others (at least 9 other papers from 8 research groups). The point is that several groups have been using the bioluminescent method to measure MAPR for more than a decade, yet Drew and Leeuwenburgh only mention that luciferin/luciferase has been used to detect bacterial contamination. By ignoring the prior development and use of the bioluminescent MAPR assay, the authors have greatly overstated the novelty of the method they present. It is also reasonable to expect that a methodology paper would compare the relative merits and limitations of the new method to prior techniques. Such a comparison was not given.

There are also some technical considerations that deserve mention. The first issue is the quantification of ATP concentration. Drew and Leeuwenburgh used an external ATP standard curve to estimate the ATP concentration in each MAPR reaction tube at multiple measurement times. This approach has been previously used (2, 3, 6). In the method of Wibom (20), however, an ATP standard is added directly to each reaction cuvette after following the reaction for several minutes. This eliminates the need for running a separate set of standards. A more important advantage is that an internal standard accounts for the different degree of light quenching from the various compounds that could be used in the assay. For example, when measuring MAPR with different substrates, light quenching is much less with pyruvate and malate than with either glutamate or succinate. To accurately calculate the ATP concentration using the standard curve approach, a separate curve would be needed for each set of compounds tested to account for differential light quenching. Although this might be feasible, it could make the measurements much more time consuming, expensive, and potentially less accurate.

The second issue concerns equipment and reagents. Drew and Leeuwenburgh used a single cuvette machine for their measurements, as have other groups before them (7, 11, 16). They also used a luciferin/luciferase preparation that is suited for making sensitive rapid measurements of ATP concentration but demonstrates a relatively rapid signal decay. These two factors could explain why the MAPR reactions were followed for such a brief time (30 s). Such an approach might be reasonable if laboratory resources are limited but better alternatives are available. An ideal luminometer should accommodate multiple samples, control temperature, and perform mixing and microinjections. For example, the BioOrbit 1251 (BioOrbit Oy, Turku, Finland) machine has been available for many years and can perform a fully automated MAPR assay on 25 reaction tubes in 20–30 min. Because of the growing popularity of luminescence chemistry, there are new machines entering the market regularly that may also be suitable. There are also luciferin/luciferase preparations available that are better suited for continuous ATP monitoring. For example,
BioThema ATP Reagent SL (BioThema, Haninge, Sweden) is formulated to produce a sustained stable light output (light decay rates <0.5%/min). This makes it ideal for measuring linear rates of MAPR over many minutes and eliminates the need to adjust for curvilinear signal decay. This reagent was developed by Dr. Arne Lundin, a recognized expert in luminescent measurements for more than 30 years.

Third, Drew and Leeuwenburgh claim (page R1261) that ATP production rate should be directly proportional to ADP concentration. However, as Ribom demonstrated in his original paper (20), there are background ATP-producing reactions, particularly adenylate kinase, that result in a nonlinear relationship between ADP concentration and ATP production, especially at lower ADP concentrations. For this reason a tube containing mitochondria and ADP but no exogenous pyruvate or other substrates should be included as a blank that is subtracted from other measurements. More recently, Ribom has reported that the adenylate kinase reaction can be effectively inhibited (17).

Fourth, Drew and Leeuwenburgh measured ATP concentration in the isolated mitochondrial preparations. I have concern about the validity and interpretation of these measurements because during the isolation process the concentrations of the individual adenine nucleotides will change due to the presence of many enzymes that can convert these compounds. It would be more appropriate to measure the ATP concentration of a whole cell lysate either using luciferase (21) or HPLC.

Fifth, in the preparation of liver mitochondria, the speeds used for centrifugation were 1,600 g to pellet the cell debris and 18,000 g to pellet mitochondria. However, it has been demonstrated that when preparing liver mitochondria, use of high speeds (10,000 g or greater) can result in high contamination with other cell components such as microsomes and lysosomes (8). In fact, to achieve 95% pure mitochondria may require centrifugation speeds as low as 3,000 g (8). My concern is that the MAPR rates were expressed per unit of protein in the final pellet, which probably reflects an unknown amount of nonmitochondrial protein that could vary under different physiological conditions, such as caloric restriction.

A final comment is that in both the abstract and the first paragraph of the discussion, it is written that ATP production was measured in vivo. Because isolated mitochondria were used, the measurements were performed in vitro.

In conclusion, the authors of this paper provided a reasonable amount of detail about the bioluminescent MAPR assay that established, but this work is not particularly novel or innovative because it has been used and modified for more than a decade and credit should have been given to previous developers and users of the method. Readers who are considering performing this assay should also be aware of alternate approaches that provide more flexibility, ease of use, and similar or greater reproducibility (17, 20).

REFERENCES

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**REPLY**

*To the Editor:* We appreciate the comments addressed by Dr. Kevin Short. We would like to add that we highly respect the studies done by Dr. Short and his coworkers and that data and conclusions from their research (1, 21, 23) were helpful in explaining some of the results we obtained in our aging and caloric-restricted studies. However, we feel it necessary to address some of the comments he made of our paper “Method for measuring ATP production in isolated mitochondria: ATP production in brain and liver mitochondria of Fischer-344 rats with age and caloric restriction” (5).

First was the issue of novelty. Nowhere in the paper do we mention that the method is novel in any way or that we are the first to propose luciferin/luciferase as a method for measuring ATP production. What we do address as original is the selection of tissues in which we measured the effects of age and caloric restriction on ATP production. Moreover, as a result of the interest in our previous findings (6) on ATP production in skeletal muscle and heart, we have been inundated with requests on a working method to measure ATP production in isolated mitochondria. Although the methodology given in our paper (5) is not novel (10–14, 24, 25), it is a thorough method detailing an assay we used and can be used simply as an in-house protocol because it has the protocol and methodology perfectly spelled out and the reasons why each was chosen.

Second was the issue of an internal standard vs. a standard curve. Adding a known concentration of ATP (internal standard) is ideal for measuring ATP content when an absolute or static concentration is present within the mitochondria. However, there are several limitations when using this method to measure ATP production. First, le Chatelier’s principle (2) states that any introduction of a product into a chemical reaction will shift that reaction toward the reactants. Hence any introduction of ATP as in internal standard will alter the rate of reaction and lead to an inherent error in the analysis. Specifically, an introduction of an external ATP source will result in an increased and competing hydrolysis reaction to form ADP. Additionally, the presence of a free phosphate can convert an inactive precursor(s) into an active enzyme(s), or vice versa, which can also compete with the production of ATP. Moreover, the considerable free energy (~9.7 kcal/mol) (22) that is released due to the removal of a phosphate from the ATP can also drive competing reactions and have great effects on the kinetics and reaction rates governing ATP production. Wibom et al.’s (25) use of an ATP standard is not without its own limitations. The hydrolysis of ATP is both kinetically and thermodynamically favored over the reverse reaction (10^7 ADP produced for every 1 ATP molecule). Additionally, the hydrolysis reaction is very rapid, producing 40–600 ADP per second for F1-ATPase (7). Hence any determination of the rate of ATP production in mitochondria without accounting for the change in ATP concentration as a result of the reverse reaction or taking some step to inhibit ATP hydrolysis introduces an inherent error when using an internal standard as the primary means for the quantification of ATP production. The only situation where an internal standard may be beneficial when measuring rate of production for any enzyme are cases where the reaction rate is very slow or the competing reactions are negligible relative to the pathway of interest. Because the competing reverse reaction is strongly favored both kinetically and thermodynamically, the use of an internal standard to measure the rate of ATP production would be inclusive of potentially significant inherent error. Although we agree that the addition of an internal standard would account for the different degrees of light quenching by the various compounds and substrates, this effect would be negligible compared with the intrinsic errors due to the competing reverse reactions. However, as mentioned in the manuscript, background corrections were made for all substrates and chemicals used in the reaction mixture as well as an identical concentration of ADP (10 μM of 2.5 mM ADP) used to determine ATP production and the corresponding luminescence subtracted from the rates of ATP production in isolated mitochondria. Hence this should account for any light quenching by competing substrates or chemicals within the solution. However, any matrix interaction between additional mitochondrial enzymes and the luciferase/luciferin complex is not known and is assumed to be minimal relative to ATP.

There is no disagreement that a fully automated luminometer that is capable of running multiple samples would not have been better by improving precision and saving time. I agree that this would have minimized much of the luminescent decay that occurred while running samples in triplicate one sample at a time and, as a result, improved the intra-assay coefficient of variation (see Table 3 in original manuscript). Because the amount of light produced is proportional to the amount of ATP produced or present, we took great care to run control standards between each successive sample throughout the experiment and normalized the ATP standard curve so as to minimize any loss of signal decay associated with the reduced activity of the luciferase enzyme over time. The time constraint of single sample analysis is one of the inherent drawbacks of a single-channel luminometer; however, we feel that the normalization of the standard curve for each sample greatly minimizes this weakness so that the results are comparable to a multichannel system.

Another concern by Dr. Short is the measurement of ATP production over a 30-s period relative to a longer time point. The short time point was due to our interest in a near instantaneous rate of ATP production (i.e., what is available for immediate use) instead of a steady-state potential. The production of ATP through ADP has always been considered a first-order reaction, but recent literature assures that this is at least a second-order reaction (8, 9). This was evident when we were testing the protocol, because doubling the concentration of exogenous ADP led to a greater than twofold production of ATP. As a result of a nonlinear or higher-order kinetic system...
associated with ATP production, instantaneous and average ATP production are far from equal. Hence we were more interested in the rate of ATP production available for immediate use (i.e., near-instantaneous rate) vs. a sustained average rate of production. Due to the time dependency, we chose to use 30-s intervals for measuring the rate of ATP production as this period of time corresponded to the part of the nonlinear curve that appeared to be most linear (greatest tangency, see Fig. 1 in original manuscript). Moreover, we chose a physiologic concentration of ADP (20). With regards to contributing reactions, the contribution of ATP production by adenylate kinase should be considerably smaller than the contribution from mitochondrial respiration, because 5 mM oligomycin, a strong inhibitor of oxidative phosphorylation, was able to inhibit the production of ATP by 83%. It is possible that the ATP being produced after incubation with oligomycin was partially from the presence of adenylate kinase. Consequently, we strongly agree that steps should be taken to account for any signal contribution due to competing ADP reactions (i.e., adenylate kinase) such as coinubating with an inhibitor of adenylate kinase. This is something that we will take into account in future studies and appreciate the suggestion.

Last, in regard to your concerns on the validity and interpretation of our ATP concentration measurements due to changing concentrations of adenine nucleotides, I agree there are alternative ways to measure ATP concentration such as HPLC and NMR; however, the method we introduced is a quick and reproducible methodology for measuring both ATP content and rate of production in isolated mitochondria. Analyses on cell or tissue extracts require a membrane permeabilization step to allow hydrophilic substrates (i.e., ADP) to penetrate, which can interfere with ATP production (19), and therefore other methods are not without their own limitations.

We do appreciate the comments by Dr. Short. It was not our intention to leave out previous works, as most were covered in a previous paper (6). It is with great regret that some of the manuscripting manuscripts (3, 4, 10–18, 24, 25) were left out during the introduction and we apologize for this unintentional mistake. For, if not for their work, we would not have had the foundation in which to undertake this research. Additionally, we add that many of Drs. Short and Nair’s results in previous works were quite influential and beneficial in aiding in our analysis of our own data. We look forward to their upcoming manuscripts, as each one contributes to the increased understanding of many of the mechanisms involved in ATP production and muscle function.

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