Application Note

Assessing mitochondrial dysfunction in primary cardiomyocytes

Mitochondrial reserve capacity is a sensitive measure of cellular stress

RESEARCH AREAS

Cardiovascular disease
Neurodegeneration
Metabolic disease

ASSAY TYPES

Mitochondrial function: mitochondrial profile or "stress test"

KEYWORDS

Mitochondria, oxidative phosphorylation, oligomycin, FCCP, antimycin A, HNE

This application note describes a method for profiling mitochondrial function in cells responding to stress. The mitochondrial profile generated in this way provides four parameters of mitochondrial function that can be measured in one experiment: basal respiration rate, ATP-linked respiration, proton leak, and reserve capacity. The results described here suggest that the development of cardiomyocyte injury, in this case caused by an oxidized lipid, increases ATP-linked oxygen consumption, diminishes respiratory efficiency, and depletes the bioenergetic reserve capacity.

Bradford Hill *et al* [1] measured this 'bioenergetic capacity' in primary cardiomyocytes and determine whether it is utilized in response to stress induced by the pathologically relevant reactive lipid species HNE (4-hydroxynonenal), a product formed during acute and chronic cardiac dysfunction. They found that intact neonatal rat ventricular myocytes (NRVMs) have a substantial capacity to respond to increasing energy demand under basal conditions. However, upon exposure to increasing concentrations of HNE cellular respiration, as measured by oxygen consumption rate (OCR), was increased until the cells' bioenergetic capacity was depleted. These findings led to the concept of 'reserve capacity,' a measure of the mitochondrial energy reserve. Once this energetic reserve was depleted by the HNE treatment, respiration failed, and cell death occurred. Thus, measuring the bioenergetic reserve capacity may be an effective way to assess or predict the ability of cells to manage and overcome stress, such as that encountered during acute oxidative insults.

In Figure 1A, the bioenergetic impact of HNE-mediated damage is illustrated. NVRMs were exposed to various concentrations of HNE while monitoring oxygen consumption in an Seahorse XF Analyzer. Interestingly, HNE stimulated both glycolysis and mitochondrial respiration in a concentration-

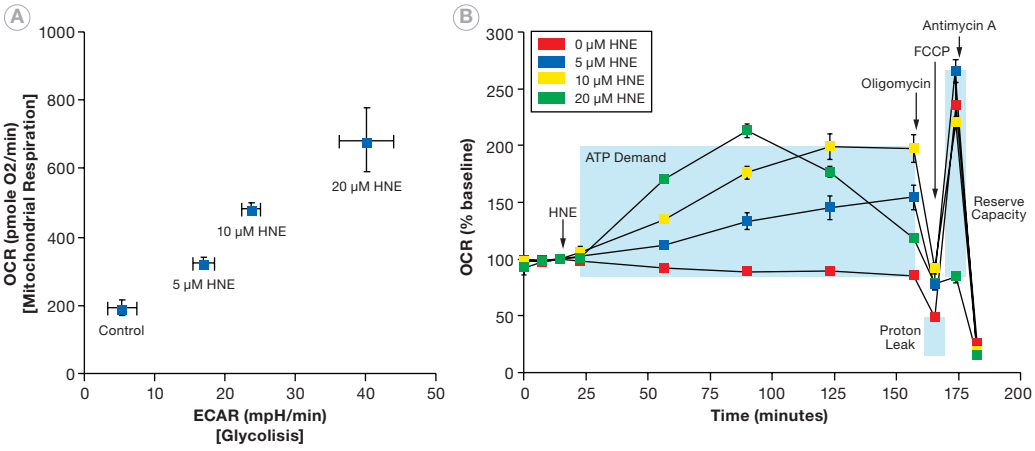

Figure 1 | Bioenergetic response of cardiomyocytes to 4-hydroxynonenal (HNE)

A. HNE stimulates both glycolysis and mitochondrial respiration in a concentration-dependent manner, consistent with an increased energy demand in response to oxidative stress.

B. HNE at concentrations up to 10 µM show no effect on maximal respiratory rate, as determined by addition of FCCP, while HNE at concentrations > 10 µM inhibit the maximal respiratory rate, suggesting the mitochondrial respiratory capacity has been exhausted.

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dependent manner, consistent with an increased energy demand in response to oxidative stress. This is shown by plotting the oxygen consumption rate [OCR], a measure of mitochondrial respiration, against the extracellular acidification rate [ECAR], a marker for glycolysis, giving a 'metabolic image' that allows one to compare and contrast the relative changes in the two major energy-producing pathways of the cell, aerobic and glycolytic metabolism.

To determine the specific mitochondrial perturbations that occur in response to HNE, the authors performed a single experiment that allows mitochondrial profiling in any cell type. This assay was performed by exposing the HNE-treated myocytes to oligomycin, FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) and antimycin A in succession as shown in Figure 1B. After three basal measurements, HNE at various concentrations was injected automatically into the wells containing the myocytes. The OCR increased in a concentration-dependent manner in an effort to confront the increased energy demand caused by the oxidative damage. Oligomycin, a complex V inhibitor, was injected to differentiate the ATP-linked respiration (oligomycin-sensitive fraction) from the proton leak. As shown in Figure 1B, proton leak (measurement 9, oligomycin-insensitive fraction) was increased by HNE in a concentration-dependent manner. These results suggest that HNE increases OCR both by increasing proton leak (thereby decreasing mitochondrial efficiency) and by increasing energy demand.

Following oligomycin addition, maximal respiratory rate was determined by subsequently injecting the wells with FCCP, an uncoupler that raises OCR to an extremely high if not its theoretical maximal rate when titrated correctly. It is clear in Figure 1B that HNE at 0–10 µM concentrations had no effect on this maximal respiratory rate. However, concentrations of HNE in excess of 10 µM resulted in an inhibition of oxygen consumption even after FCCP addition, suggesting the mitochondrial respiratory capacity had been exhausted. Therefore, HNE induced an ATP demand that utilized mitochondrial reserve capacity in a concentration-dependent manner consistent with damage to the respiratory chain that was associated with the formation of HNE-induced protein modifications. The rate of oxygen consumption due to non-mitochondrial sources was determined in the absence and presence of HNE using antimycin A and was unchanged.

Discussion

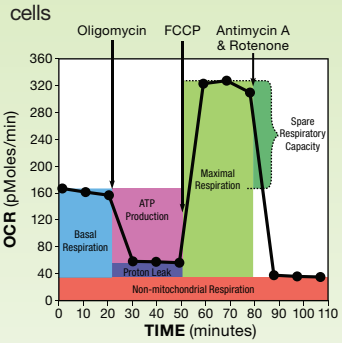
The ability of cells to respond to stress under conditions of increased energy demand is in a large part influenced by the bioenergetic capacity of mitochondria. This bioenergetic capacity is deter-mined by several factors, including the ability of the cell to deliver substrate to mitochondria and the functional capacity of enzymes involved in electron transport.

A rapidly growing number of studies are employing this mitochondrial profile [Figure 3], to assess cellular bioenergetics, identify mitochondrial dysfunction and to predict the ability of cells to respond to stress and/or insults [4-7]. For example, Choi *et al.* [4] was able to correlate a loss of spare respiratory capacity with partial restriction of electron transport through mitochondrial complexes I and II in synaptosomes as a model to mimic possible defects associated with Parkinson's and Huntington's diseases, respectively.

In another study, Atg7, an essential gene required for autophagosome formation, was deleted in mice [5]. Impaired or deficient autophagy is believed to cause or contribute to aging, as well as a number of age-related pathologies. The results suggest that continuous oxidative stress contributes to the decline in mitochondrial function, which may underlie the autophagy-related pathology observed in this model.

Cardiomyocyte injury, in this case caused by an oxidized lipid, increases oxygen consumption, diminishes respiratory efficiency, and depletes the bioenergetic reserve capacity.

Figure 3 | Bioenergetic profile of cells



After measuring the basal respiration rate of cells, compounds modulating mitochondrial function are added sequentially. The effect on oxygen consumption rates (OCR) is measured after each compound addition. This reveals the four fundamental parameters of mitochondrial function: basal respiration, ATP turnover, proton leak, and maximal respiratory capacity.

Materials & Methods:

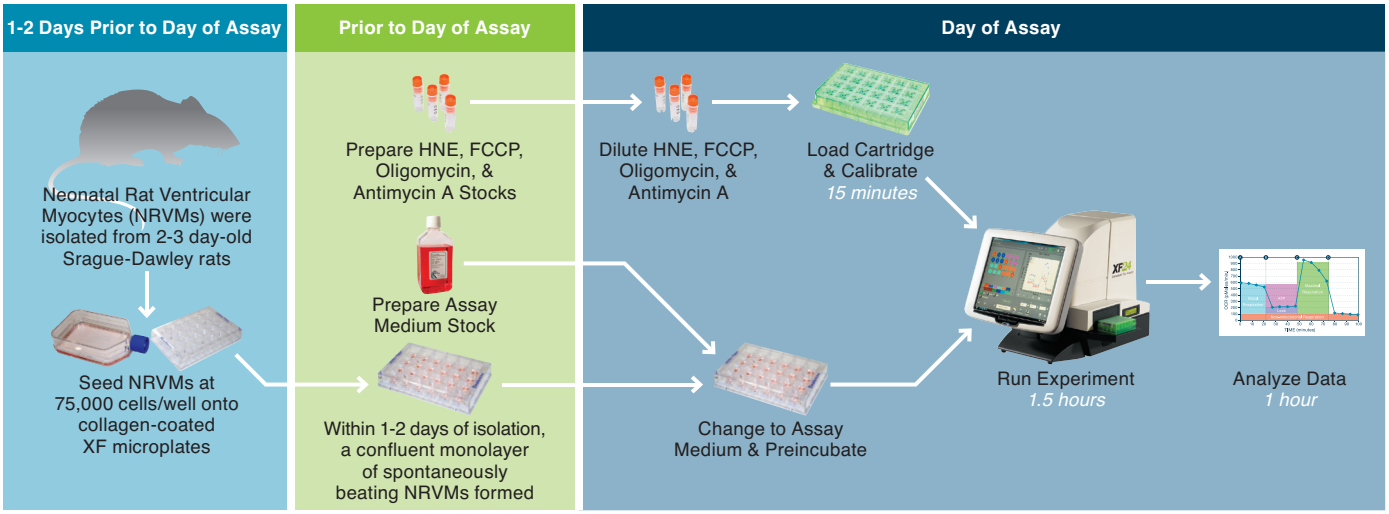
Cells and Compounds: Primary cultures of neonatal rat ventricular myocytes (NRVMs) were obtained from 2–3-day-old neonatal Sprague–Dawley rats and were cultured as described previ-ously [2]. NRVMs were seeded at 75,000 cells/well on to collagen-coated Seahorse XF V7 Cell Culture Microplates in growth medium containing 15% FBS (fetal bovine serum). The next day, medium was replaced, and cells were grown in the growth medium without FBS. Within 1-2 days of isolation, a confluent monolayer of spontaneously beating NRVMs formed. For all bioenergetic measurements, the culture medium was changed 1 hour prior to the assay to unbuffered DMEM (Dulbecco's modified Eagle's medium, pH 7.4) supplemented with 4 mM L-glutamine (Gibco). To determine the optimum number of cells needed, NRVMs were seeded to a density of 25,000, 50,000 or 75,000 cells/well. Oxygen consumption in these cells was linear with respect to cell number within this range and a seeding density of 75,000 cells/well was chosen for the remainder of the experiments.

4-hydroxynonenal (HNE) was obtained from Calbiochem. Oligomycin, FCCP and antimycin A were from Sigma. [Now available as a kit from Seahorse Bioscience.]

XF Analysis

Bioenergetic analyses of intact NRVMs were performed in the Seahorse XF Analyzer. The Seahorse XF Analyzer creates a transient micro-chamber of only a few µl in specialized cell culture microplates. This enables OCR (oxygen consumption rate) and ECAR (extracellular acidification rate) to be monitored in real time [3]. Indices of mitochondrial function were measured as follows: Oligomycin, FCCP and antimycin A were injected sequentially through ports in the Seahorse XF Assay cartridges to final concentrations of 1 µg/ml, 1 µM and 10 µM respectively. This allowed determination of the basal level of oxygen consumption, the amount of oxygen consumption linked to ATP production, the level of non-ATP-linked oxygen consumption (proton leak), the maximal respiration capacity and the non-mitochondrial oxygen consumption. Three basal OCR measurements were recorded prior to injection of HNE and then 5 subsequent measurements were made before oligomycin addition [Figure 1B]. After mixing and recording the oligomycin-insensitive OCR, FCCP was injected and another OCR measurement was recorded. The OCR measured after FCCP injection represents the maximal capacity that cells have to reduce oxygen under the experimental conditions. Finally, antimycin A was injected to inhibit the flux of electrons through complex III, and thus no oxygen was further consumed at cytochrome c oxidase. The remaining OCR determined after this treatment is primarily non-mitochondrial and could have been due to cytosolic oxidase enzymes.

Figure 2 | Flow Chart of XF Assay



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