

Advances around technologies investigating mitochondrial function and insights gained by their applications

Diabetes mellitus is defined as a state of persistently high levels of blood glucose and insulin deficiency, and resistance to it is considered as the cause(s). However, the metabolic abnormalities of diabetes are not limited to glucose, but encompass the entire metabolism. Insulin controls not only glucose, but fat and protein metabolism as well. Furthermore, body structures of patients with this disease become abnormal, termed complications, which eventually lead to the death of patients.

The mitochondrion is an intracellular organelle that plays a central role in the metabolism. Here are the Krebs cycle enzymes and the electron transfer chain (ETC) enzymes, which transduce energy substrates into a usable form of energy, adenosine triphosphate (ATP) and heat. Mitochondrion literally burns glucose, fatty acids and amino acids into CO_2 and water, consuming oxygen. Free energy in those molecules is released in the form of hydrogen, and transferred to the ETC through hydrogenated nicotine amide dinucleotide and flavin adenine dinucleotide, which are then pumped into the intermembranous space by ETC complexes 1, 3 and 4. The huge electrochemical gradient (reaching approximately -170 mV; proton motive force $[\Delta\Psi]$) thus generated is used for ATP synthesis (ATP synthase or complex 5), which compresses inorganic phosphate (Pi) into adenosine diphosphate (ADP) in making ATP. Excessive build-up of $\Delta\Psi$ is prevented by the action of uncoupling proteins (UCP), which dissipates $\Delta\Psi$ (Figure 1).

One might imagine this energy transforming system as being made of four modules: a turbine engine (tri-acetic acid (TCA) cycle or hydrogen generator); an electricity grid connecting the engine to the plant (ETC complexes 1, 2 and 3, all feeding into complex 4); a plant, making energy currency (complex 5); and controllers. It is now appreciated that these five complexes form one supercomplex, which exists in a dynamic state; its function of this supercomplex change according to the need of extracting energy depending on the relative abundance of the nutrients to burn, which in turn reflects the composition of foods consumed.

The mitochondrion is a highly sophisticated machine, but rather fragile, as it has to handle electrons. Electrons are

transferred easily to reactive molecules. As electron leak is inevitable, it forms superoxide (a reactive oxygen species [ROS]) by reacting with oxygen, which could damage the mitochondrial genome, the mitochondrial membrane (its proteins and fats). Hydrogen peroxide (H_2O_2) is another potential toxic product, which is monitored by the nucleus. Animals have evolved delicate protective and repair systems for this reason, and it is under very delicate control. If the mitochondrion is damaged, however, it is either entirely removed or the cell carrying the damaged mitochondrion dies and is removed (termed mitophagy and apoptosis, respectively).

The mitochondrion is under delicate control, and easily changes its morphology

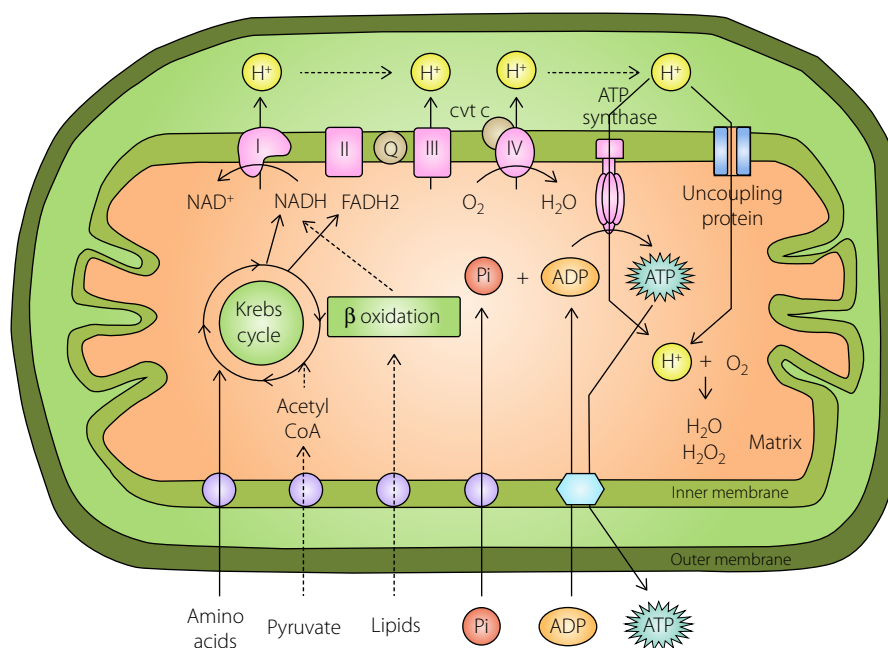


Figure 1 | A simplified view of the mitochondrial electron transfer system and adenosine triphosphate (ATP) production mechanisms, and reactive oxygen species production in the mitochondrion. I–V, electron transfer system complex molecules 1–5; ADP, adenosine diphosphate; CoA, coenzyme A; cytc, cytochrome c; Pi, inorganic phosphate; Q, coenzyme Q.

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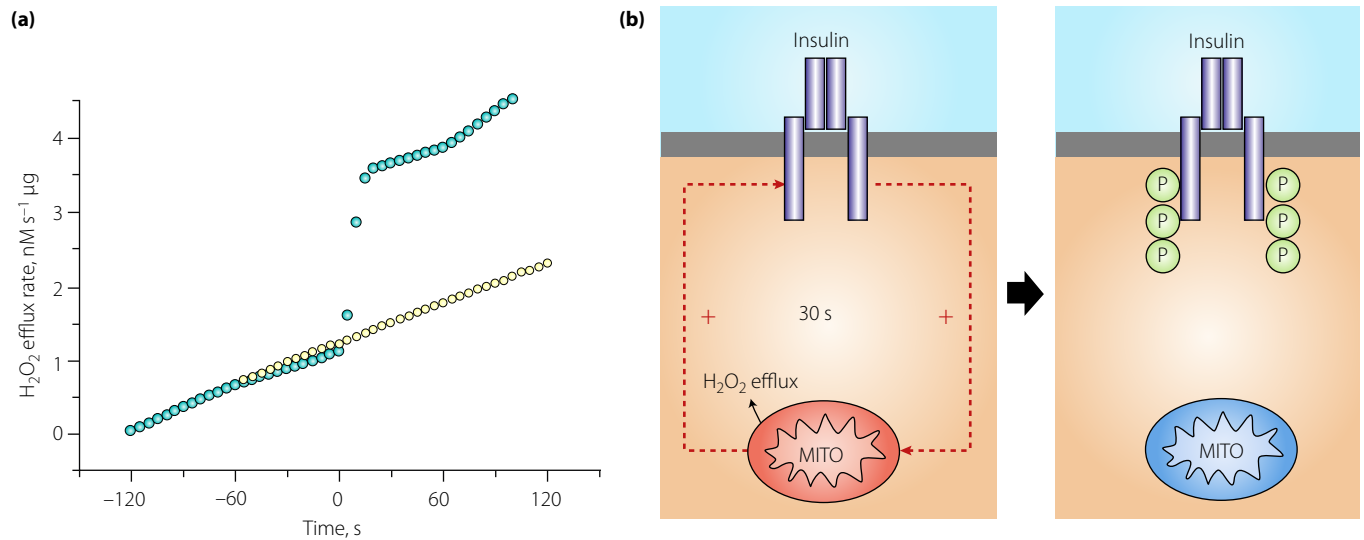


Figure 2 | (a) Very rapid rise in H₂O₂ signal from the mitochondrion after insulin treatment, measured by applying Amplex Red in cultured cerebellar granule neurons. (b) Control of insulin receptor phosphorylation by H₂O₂ (from Pomytkin³ with permission). MITO, mitochondrion; P, phosphate.

and functions. Sometimes it divides into two, sometimes several mitochondria fuse and form networks. It is transported wherever it is required (to provide energy, ATP). It increases its mass per cell according to the need for energy, as happens in the muscle during training.

Insulin is one of the key hormones controlling mitochondrial function. Another key hormone is catecholamine, such as epinephrine. They stimulate general physiological processes and prepare the body for physical activity (fight-or-flight response), which requires energy. Increases in heart rate, blood pressure and blood glucose levels are mediated through adrenergic receptors, and then activate a master regulator of mitochondrion, PPAR- γ (peroxisome proliferator activated receptor gamma) coactivator-1 (PGC1). Insulin complements (after binding to its own receptor) the actions of catecholamine by making glucose enter the cell, allowing it to burn or store the forms of glycogen or fat (if nutrient is excessive). When insulin is lacking, the entire metabolism is reversed; glucose is released from the stored source or newly made using substrates released from muscle. ATP is produced through the glycolytic process, which is much less efficient, as acetyl-coenzyme A (made by

beta oxidation of fatty acids) cannot enter the TCA cycle, but is transformed into ketone bodies. Like an engine emitting smoke. We now know a great deal about these altered intermediary metabolisms of the diabetic state, a state of insulin deficiency or its resistance. However, attention is scarcely paid to the mitochondrion itself.

Scientific advances are made when new technologies of observation are developed. Mitochondriology is no exception. There has been huge development in this area, which is beyond the scope of this short Commentary. For details of newly developed technologies, readers are referred to a recent review published in *Diabetes*¹. Among them, two machines and two techniques stand out.

First, the XF Extracellular Flux analyzer from Seahorse Bioscience (North Billerica, Massachusetts, USA) makes monitoring the mitochondrial function of cells on a large scale easier.

Second, the O2k system from OROBOROS Instruments (Innsbruck, Austria) measures mitochondrial respiration (oxygen consumption and some related parameters) of isolated mitochondria, cells and tissues with precision.

Development of several fluorescent dyes (fluorophores) and chemical probes

has allowed researchers to see the mitochondrion, and monitor its function directly using fluorescent microscopes and detectors. For example, Amplex Red allows us to easily monitor H₂O₂ levels, and the triphenylmethyl phosphonium cation allows researchers to easily probe the mitochondrial membrane potential.

Finally, I would like to remind readers that nuclear magnetic resonance spectroscopy has been used trace high-energy phosphates (i.e., ATP), as well as inorganic phosphate *in vivo* (³¹P-magnetic resonance spectroscopy).

Therefore, one can easily evaluate mitochondrial function either after its isolation or using permeabilized cell or tissues; feed it energy substrates, and monitor oxygen concentration, $\Delta\Psi$, H₂O₂ and ATP levels over time. Because ATP is made from ADP, enough ADP should be present in the media. If all the conditions are fine for the mitochondrion, it is in so-called state 3, happily making ATP. You can treat the body, tissue or cell with various agents, including hormones, drugs, inhibitors and whatever you are interested in, and see how the mitochondrion is responding to the treatments.

Recently, Yu *et al.*² reported in *Diabetes* the results of all those parameters before

and after insulin treatment using isolated mitochondria from diabetic rat gastrocnemius muscle. What they reported was not surprising; mitochondrion of diabetic rats was abnormal, and returned to normal with insulin treatment. Most significant was that the mitochondrion showed the high cost of ROS per unit ATP production in the diabetic state; the mitochondrion was less effective and emitted a large amount ROS, like a defective engine.

What is surprising is the sophistication of the analytical method. They invented a nuclear magnetic resonance spectroscopy method measuring ATP that is almost 40-fold more sensitive, thus creating a way to monitor ATP in isolated mitochondria. Combined with another innovation, the ADP clamp (a certain amount of ADP allows a fixed amount of changes in $\Delta\Psi$), they improved the mitochondrial function test to an unprecedented precision. If this method is applied on a large scale, as they claim, we can further improve our capability to evaluate mitochondrial function in various states.

Amplex red was used by Pomytkin³ to show insulin-treated neuronal cell release H_2O_2 from the mitochondrion. The response was surprisingly rapid, and occurred immediately after insulin treatment, suggesting it is not mediated through activation of nuclear genes (Figure 2). Pomytkin suggested that H_2O_2 goes out to the cytoplasm and induces insulin receptor autophosphorylation, a critical post-translational modification step leading to the activation of the receptor, tyrosine kinase. Although the insulin-induced H_2O_2 spike is short-term, and a set of requirements must be met to trigger this action, Pomytkin pointed out that

fully functional mitochondrion is required as the insulin-sensitive source of H_2O_2 . This phenomenon has huge implications, as it is linked to insulin resistance, the key abnormality of type 2 diabetes or metabolic syndrome. The brain is a target of insulin, and insulin has been known to be an important regulator of feeding behavior, bodyweight and cognitive function. Insulin resistance in the brain is a relatively novel concept⁴, and now it is also linked to mitochondrial function.

Abnormal mitochondrial function has long been known to occur in diabetes. Sir Krebs studied it in 1960s. Thereafter, many scientists considered the possibility of mitochondrial dysfunction as a cause of insulin resistance. What happens to insulin action if there is an inhibitor of mitochondrial function? Could it be a cause of insulin resistance?

Recently, Park *et al.*⁵ reported that diabetic sera contains substances that inhibit the mitochondrial function of a cell (ATP generating capacity), and their inhibitory activities correlated with their stimulation activity of ROS production and their reactivity with the aryl hydrocarbon receptor (AhR) using novel cell-based assay systems. They used several fluorophores including Amplex red, various inhibitors of mitochondrial ETS and the XF-24 analyzer in those analyses, and myoblast cells are used to test sera. Sera from impaired glucose tolerance or diabetes contained more AhR ligands, and made mitochondrion dysfunctional, produced less ATP and produced more ROS in an AhR ligand concentration-dependent manner⁵. Furthermore, the AhR ligands level was positively associated with body size, blood pressure, serum triglyceride and fasting glucose, suggesting that pollutants in our

blood is an important determinant of insulin resistance.

We should know more about the mitochondrion, its functional states and controls. These newly introduced technologies will greatly enhance our ability to understand diabetes mellitus, and pave a way to the prevention and cure of diabetes in the near future.

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