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# Mitochondrial Metabolism and the Age-Associated Cardiovascular Diseases

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## Abstract

Perfusion experiments with isolated hearts have long ago established that heart mitochondria utilize fatty acids as the main source of energy and that there are competitive reciprocal interactions between fatty acids and glucose, known as Glucose-Fatty Acid Cycle. However little was known about heart metabolism at the mitochondrial level. Recent studies on metabolism of rat heart mitochondria (RHM) established that RHM oxidize efficiently and synergistically long-chain acyl carnitine only in the presence of other mitochondrial metabolites derived from catabolism of glucose or proteins. Based on the data presented by Brand., *et al*. [50-53], we suggest that during simultaneous oxidation of palmitoyl-carnitine and supportive substrates (succinate, pyruvate or glutamate), electrons enter the respiratory chain both through the NADH/NAD+ and the $QH_2$/Q pathways, allowing fast production of ATP at high workloads. However, under these conditions, production of superoxide radicals by RHM increases many times. These data substantiate the fact that increased fatty acids oxidation in individuals with metabolic syndrome or during chronic stress with allostatic overload, result in an oversupply of substrates for mitochondria, relative to cellular energy demand. Under conditions of limited physical activity and increased caloric intake, this results in increased oxidative stress, accelerated aging, mitochondrial dysfunctions, and cardiovascular diseases. Mechanisms of ROS-associated dysfunctions of heart mitochondria are discussed.

***Keywords:*** *Mitochondria; Substrate Metabolism; Respiration; ROS; Fatty Acids; Oxidative Stress; Metabolic Syndrome; Allostasis*

## Abbreviations

IF: Flavin Site of Complex I; IQ: Quinone-Binding Site of Complex I; IIF: Flavin Site of Complex II; IIIQo: Quinol Oxidation Site of Complex III; ETF: Electron Transferring Flavoprotein; ETF:QOR: ETF-ubiquinone Oxidoreductase; IPLP: Isoprostane Pathway of Lipid Peroxidation; mGPDH: Mitochondrial Glycerol 3-Phosphate Dehydrogenase; α-KG: α-Ketoglutarate Dehydrogenase; OAA: Oxaloacetate; P-C: Palmitoyl-Carnitine; PDH: Pyruvate Dehydrogenase; PUFA: Polyunsaturated Fatty Acids; Q: Ubiquinone; QH2: Ubiquinol; RHM: Rat Heart Mitochondria; ROS: Reactive Oxygen Species; SDH: Succinate Dehydrogenase (Complex II); TCA: Tricarboxylic Acids, Cycle; T2D: Type 2 Diabetes

## Introduction

Age-associated cardiovascular diseases (CVDs) represent a variety of heart dysfunctions, including heart failures and infarction, and thus represent a serious public health problem, associated with significant mortality, morbidity, and healthcare expenditures, particularly among subjects aged over 65 [1-3]. Age-related cardiovascular pathologies tend to cluster or occur together with other pathologies typical for metabolic syndrome, which also include insulin resistance - type 2 diabetes (T2D), obesity, atherogenic dyslipidemia and hypertension [4]. The clustering of several risk factors and their shared responsiveness to lifestyle modifications suggests that they are not independent of one another and share underlying causes, mechanisms and features [4,5]. There is a large body of evidence that these age dependent common causes and mechanisms are associated with mitochondrial dysfunctions [6-11]. Clinically, cardiovascular diseases are usually represented as a mixture of pathologies of the heart muscle, heart vessels and the vascular system as a whole, as well as some extra cardio-

vascular systems, such as renin-angiotensin-aldosterone system [12]. Therefore, clinical pictures of CVDs and their causes are complex. There has been no clear understanding of the nature of age-associated metabolic syndrome yet. Currently, it is mostly the enumeration of related diseases and symptoms. In this review we discuss the latest developments in understanding of the roles mitochondria play in aging, oxidative stress, and organ-specific metabolic features of heart mitochondria. We also present a new vision on development of metabolic syndrome and provide a strong substantiation for the conception of allostasis and the mitochondrial allostatic overload.

## Heart metabolism as studied by perfusion of the isolated heart

In a recent comprehensive review on heart mitochondria, Murphy., *et al*. [13] cited Opie's vivid notion that "the incessant energy requirements of the heart are sustained by the consumption of a mass of ATP daily that surpasses cardiac weight itself by approximately 5 to 10 fold" [14]. Energy for the constant pumping of blood through the vascular system is provided by a large amount of mitochondria in cardiomyocytes, which constitute between 23% and 32% of the myocellular volume [15]. The distribution of mitochondria in cardiomyocytes is illustrated in figure 1.


***Figure 1:*** *Distribution of mitochondria in cardiomyocytes. Cardiac muscle structure. Colored scanning electron micrograph (SEM) of cardiac muscle fibrils (green and brown) from a healthy heart. Mitochondria (pink) supply the muscle cells with energy. The muscle fibrils, or myofibrils, are crossed by transverse tubules (vertical lines), which mark the division of the myofibrils into contractile units (sarcomeres). Credit: Steve Gschmeissner/Science photo library.*

The density of cardiac mitochondria varies between species and increases from human to mouse in parallel with increased heart rate and oxygen consumption [15]. Similar to other hard working organs (kidneys, brain), heart mitochondria have a very large number of respiratory chain complexes, and the matrix has a relatively small osmotically active volume [16]. This limits diffusion of small molecules and promotes enzymes in the matrix and inner mitochondrial membrane (IMM) to function as multienzyme complexes [16,17].

In the middle of the last century the first important information on heart metabolism was obtained in experiments with perfusion of the isolated heart, which established competition for respiration between glucose and fatty acids [18-22]. In 1963 Randle., *et al*. [23] proposed the so called Glucose-Fatty Acid Cycle, which depicts the reciprocal and independent metabolic relationship between glucose and fatty acids (Figure 2). Briefly, the essence of the glucose fatty acid cycle is: "provision of glucose promotes glucose oxidation and glucose and lipid storage, and inhibits fatty acid oxidation; provision of free fatty acids promotes fatty acid oxidation and storage, inhibits glucose oxidation and may promote glucose storage if glycogen reserves are incomplete" [24]. The experimental details and discussions of reciprocal influences of glucose and fatty acids on their metabolism in the heart were discussed in numerous papers [18-22,25,26] and reviews [8,23,24,27-29], of which we cite only few.

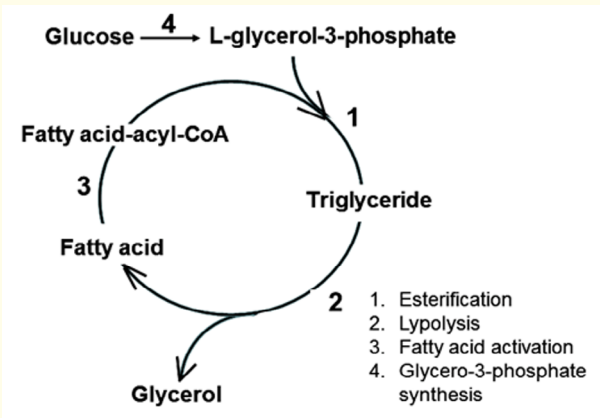


***Figure 2:*** *The glucose fatty-acid cycle. The concept of a glucose fatty-acid cycle is shown as a simplified diagram. The essential features are (1) the restrictions imposed on glucose metabolism in muscle by the release for oxidation of more fatty acids derived from muscle or adipose-tissue glycerides, and (2) those imposed on release of fatty acids from glycerides by uptake of glucose. In the tissue phase, fatty acids and glycerol are released from glycerides in both muscle and adipose tissue (lipolysis): they may be reincorporated into glycerides by esterification with glycerol phosphate formed from glucose, but not by reaction with glycerol, which is released into extracellular fluid. Fatty acids may also be oxidized (both tissues), or transferred to plasma albumin (adipose tissue). The figure was adapted from [26].*

A number of important observations have been made during studies of metabolic responses of the heart experiencing increased workloads. In a human at rest, the oxygen consumption index (OCI) (OCI is the ratio: % of $O_2$ consumption by the organ/ % of the total body weight) of the heart is the highest among all organs: OCI = 27.5 (30, 31). However, at high workload the rate of oxygen consumption may increase nine fold [32]. When working with small animals, it should be kept in mind that in a rat, for example, the OCI for the heart (OCI = 6) is second only to the kidney (OCI = 7.8) [30,31].

Neely., *et al*. [22,33] observed significant changes in heart metabolism of glucose and fatty acids at high workloads. He made the fundamental conclusion that energy production in glucose-perfused hearts appeared to be carbon substrate-limited, and increased mechanical performance could be maintained only by further reductions in energy levels [33]. In hearts receiving fatty acids as substrate, in addition to glucose + insulin, energy production was not substrate-limited, and the levels of acetyl-CoA, citric acid cycle intermediates, the mitochondrial NADH/NAD ratio, and the phosphate potential were all high. In this case, hearts were able to respond to increased mechanical activity with smaller changes of phosphate potential. This is an important notion, not only for understanding the energy supply in the heart at different workloads, but also for understanding the mechanisms of reactive oxygen species (ROS) production by heart mitochondria.

In these pioneering and fundamental publications on heart metabolism under various workloads and substrates conditions [22,33], the authors discussed changes in the TCA cycle intermediary metabolites, NADH/NAD+ ratio in mitochondria, and the phosphate potential in cardiomyocytes in the frames of the classical thermodynamics and classical single ring-like functioning of the TCA cycle.

In 1994 Yudkoff., *et al*. [34] suggested that in the presence of glutamate + pyruvate, the TCA cycle in brain mitochondria operates as two coupled cycles (see figure 3). Cycle A is leading from α-ketoglutarate (α-KG) to oxaloacetate (OAA), and cycle B - from OAA to α-KG that includes the citrate synthase reaction. According to Yudkoff., *et al*. [34], the flux of substrates through cycle A is 3-5 times faster than through cycle B. This agrees with the fact that in brain mitochondria oxidizing glutamate + pyruvate + malate, the concentration of α-KG was increased 30-fold [35]. In 2014, the two-circles functioning of the TCA cycle was suggested for the glial mitochondria, which for the expense of fatty acids oxidation provides both carbons and reducing equivalents for the anaplerotic synthesis of glutamate and glutamine through functioning of the B cycle and maintains the aerobic glycolysis due to functioning of the A cycle [36]. The ability of TCA cycle to function as two coupled cycles makes the cycle metabolically much more flexible and becomes particularly important when mitochondria

must utilize simultaneously different substrates, support various metabolic pathways and swiftly accommodate to changes in the organ's workload [37]. We suggest that the two-circles functioning of the TCA cycle takes place in the heart also.

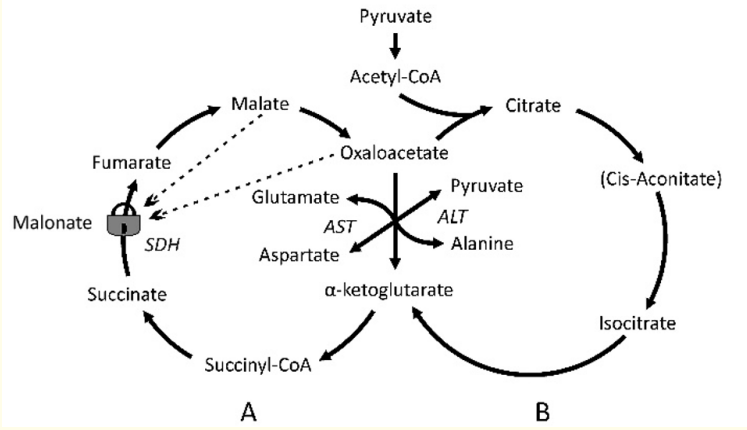


***Figure 3:*** *A schematic presentation of operation of the tricarboxylic acid cycle in brain mitochondria oxidizing glutamate and pyruvate. The figure was adapted from [95]. Abbreviations: AST: Aspartate Aminotransferase; ALT: Alanine Aminotransferase, SDH: Succinate Dehydrogenase. The symbol of the closed lock means the step catalyzed by SDH is inhibited. The dashed arrows indicate inhibitory influences of malate and oxaloacetate on SDH. The figure was taken from [39].*

Unlike substrate interactions in the whole heart [8] or body [24,29], substrate interactions in heart mitochondria have remained almost unstudied. Therefore in the next sections we will focus on metabolic properties of isolated rat heart mitochondria (RHM).

## Metabolic properties of heart mitochondria

### Intrinsic inhibition of succinate dehydrogenase

During the first 70 years of Mitochondriology, scientists learned very much about the structure and functions of isolated mitochondria, including heart mitochondria. During this period, scientists required a steadily high supply of electrons to the respiratory chain, which would provide high energization of mitochondria. For several reasons, discussed in [38], the best substrate choice was succinate in the presence of rotenone - a powerful inhibitor of the respiratory chain Complex I. Succinate + rotenone became an almost universal substrate for all types of mitochondria, including heart mitochondria. Oxidation of succinate in the presence of rotenone provided high rates of respiration with high respiratory control ratios and was very useful for studies of many mitochondrial functions. However, the practice of usage of single substrates, often in the presence of malate, such as glutamate + malate, pyruvate + malate, and succinate + rotenone, turned out to be unacceptable in studies of the mitochondrial physiology *in situ*.

Needless to say, there is normally no rotenone present *in vivo*. Brand and colleagues stressed that usage of inhibitors for mitochondrial research, particularly production of ROS, "is fundamentally flawed, because blocking a site of electron transport will invariably interrupt normal electron flow and alter the redox states of other sites in the electron flow pathway, which can dramatically alter their rates of ROS production, leading to unreliable or incorrect conclusions" [39]. Thus, it would be logical to study succinate as a substrate for heart mitochondrial energization without the inhibitor. Figure 4A illustrates how rat heart mitochondria oxidized succinate in the absence of rotenone. From figure 4A we see that in the absence of rotenone, heart mitochondria perfectly well oxidized succinate during resting respiration (State 4), but upon activation of oxidative phosphorylation by addition of ADP (State 3), respiration quickly became inhibited and inhibition was increased further by the addition of uncoupler (State 3U). The inhibition of respiration was instantly released by the addition of glutamate or pyruvate (Figure 4A) [31]. This indicates that the spontaneous inhibition of succinate oxidation was caused by

oxaloacetate (OAA) formed from succinate in the TCA cycle. Properties of intrinsic inhibition of SDH in heart and brain mitochondria were described in detail [38,40].

### Oxidation of palmitoyl-carnitine, pyruvate and glutamate by rat heart mitochondria

Figure 4B illustrates oxidation of palmitoyl-carnitine (P-C), a natural fatty acid substrate for the heart mitochondria. From figure 4B, we see that P-C effectively energized mitochondria in the absence of ADP (resting respiration), although the degree of energization was lower than with RHM oxidizing succinate (Figure 4A), pyruvate (Figure 5A), or glutamate (Figure 5B). The degree of mitochondrial energization during resting respiration with various substrates can be qualitatively evaluated by comparing positions of steady-state $TPP^+$ concentration after addition of mitochondria to the incubation medium containing 2 μM $TPP^+$. The larger shift of $TPP^+$ trace upward indicates that more hydrophobic cation was consumed by energized mitochondria. Addition of ADP to RHM oxidizing P-C caused a significant drop in membrane potential (Figure 4B), which then was slowly restored, but to a lower steady-state level than before. Addition of an uncoupler prevented stimulation of P-C oxidation, likely because transport of P-C and formation of palmitoyl-CoA in the matrix are energy-dependent functions. In comparison with rates of oxidative phosphorylation (State 3) in heart mitochondria oxidizing pyruvate (5A) or glutamate (5B), State 3 respiration rate with P-C alone was lower correspondingly, 1.5 and 2 fold. Thus, figure 4B shows that acyl-carnitines, when used alone, are poor substrates for oxidative phosphorylation in RHM.

Unlike succinate (with no rotenone) (Figure 4A) and P-C (Figure 4B), heart mitochondria oxidize pyruvate and glutamate perfectly well (Figure 5A and 5B). Pyruvate, the product of glycolysis, and amino acid glutamate are popular substrates to study mitochondria from different organs. Malate is usually added to increase transport of glutamate into mitochondria, providing oxaloacetate for transamination of glutamate [41] and activation of malate-aspartate shuttle. By itself, malate is a very poor substrate for respiration. Both pyruvate (Figure 5A) and glutamate (Figure 5B) are efficient substrates for energization of mitochondria *in vitro.* However, from the physiological and metabolic points of view, in the incessantly working heart glucose and aminoacids, including glutamate, they are not used as main substrates for prolonged provision of energy. This is because the body's stores of glucose and aminoacids are very limited, and the metabolites have other important functions beyond just being fuels for energy [31].

The results presented in figures 4A, 4B, 5A and 5B clearly illustrate that the old methodology to study respiratory activities of heart mitochondria using single substrates glutamate, pyruvate, succinate + rotenone, or acyl-carnitines contradicted the results obtained by many scientists in perfusion experiments, which showed that heart mitochondria oxidize fatty acids at a high rate and effectively accommodate energy metabolism to high workloads without significant changes in phosphate potential [8,18-27,33]. According to some estimates, fatty acids make up to 90% of the heart's energy demands [42]. The details of the fatty acids activation, oxidation and regulation have been described in numerous publications [8,22,43-46].

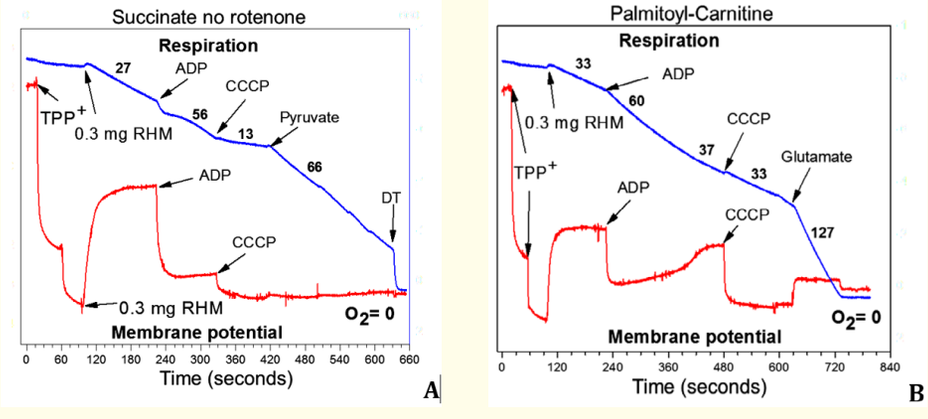


***Figure 4:*** *Respiratory rates and membrane potential of the rat heart mitochondria oxidizing succinate. Heart mitochondria were isolated from male Sprague Dawley rat by the method described in [33]. The incubation medium contained: 125 mM KCl, 10 mM MOPS, pH 7.2, 2 mM $MgCl_2$, 2 mM $KH_2PO_4$, 10 mM NaCl, 1 mM EGTA, 0.7 mM $CaCl_2$. Substrates: (A) succinate 5 mM, (B) palmitoyl-carnitine 0.05 mM in 50% ethanol. The incubation chamber volume 0.65 ml. Additions: TPP+ 1 μM (final [$TPP^+$] 2 μM), mitochondria 0.3 mg, ADP 150 μM, CCCP 0.1 μM. Membrane potential was measured with a TPP+-sensitive electrode as described in [33]. Numbers at the respiration trace indicate the rates of $O_2$ consumption in nmol $O_2$/min/mg mitochondrial protein. The figures are taken from [34].*

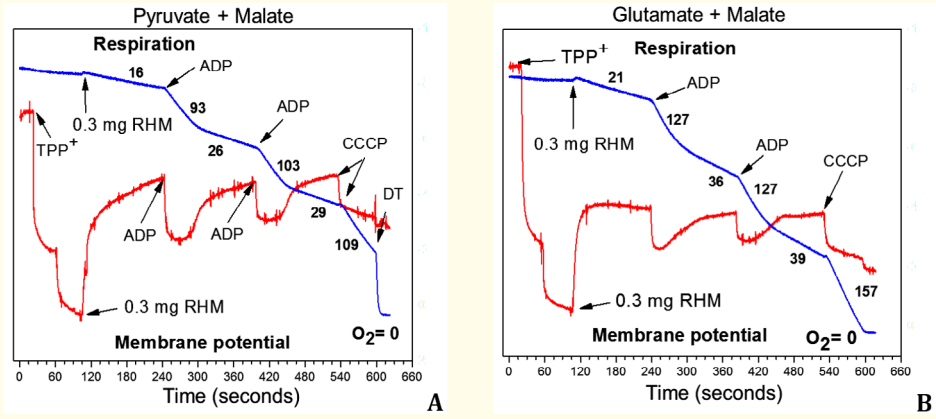


***Figure 5:*** *Respiratory rates and membrane potential of the rat heart mitochondria oxidizing pyruvate + malate or glutamate + malate in different metabolic states. Incubation conditions and additions as in figure 4. Substrates: pyruvate 2.5 mM (A), glutamate 5 mM (B), malate 2 mM. The figures were taken from [34].*

## Oxidation by isolated rat heart mitochondria of substrates mixtures

Recently we have shown that with the isolated rat heart mitochondria, the highest rates of oxidative phosphorylation were obtained when mitochondria oxidized mixtures of substrates [31]. Figure 6A illustrates that succinate in the presence of glutamate and pyruvate showed no sign of inhibition of respiration, even in the presence of an uncoupler, which increases 10 fold the affinity of SDH for inhibition by OAA [47].

It is of importance that P-C also released intrinsic inhibition of SDH (Figure 6B). Thus, the two "bad" substrates, when mixed together, supported high rates of respiration not only during oxidative phosphorylation, but, importantly, during uncoupling as well. Pyruvate (Figure 6C) and glutamate (Figure 6D) also significantly increased rates of oxidative phosphorylation when oxidized together with P-C. Figure 7 summarizes results of several experiments.

In general, oxidation of P-C in the presence of supportive substrates increased the resting respiration several fold, although there was a large scattering of the results (Figure 7, State 4). Oxidative phosphorylation during oxidation of P-C with pyruvate or glutamate increased by 30%, and increased almost 50% when P-C was oxidized with succinate, when compared correspondingly with pyruvate and glutamate. In comparison with P-C alone, the rate of ADP phosphorylation during oxidation of P-C with pyruvate, glutamate, or succinate increased correspondingly 2.7, 2.8 and 3.2 fold. In all cases of P-C oxidation in the presence of other metabolites, the rates of uncoupled respiration were high. This indicates that simultaneous oxidation of several substrates abolished all restrictions for the transport of electrons down the respiratory chain.

The most important features of oxidation by RHM of succinate and P-C in the presence of other substrates, which for brevity we denote as supporting substrates, is a significant increase, sometimes several fold, in the rate of State 4 respiration (compare figures 5 and 6, see also figure 7). Earlier, in our studies of brain and spinal cord mitochondria from the SOD1 mutant rats, we found that increased rates of resting respiration were accompanied by increased production of ROS [48,49]. Figure 7 shows that with the rat heart mitochondria oxidation of P-C in the presence of pyruvate, glutamate or succinate also increased 7 - 10 fold the rate of ROS production, as compared with RHM oxidizing pyruvate, which was taken as 100%. In the next section we will discuss in more detail production of ROS in RHM oxidizing various substrates.

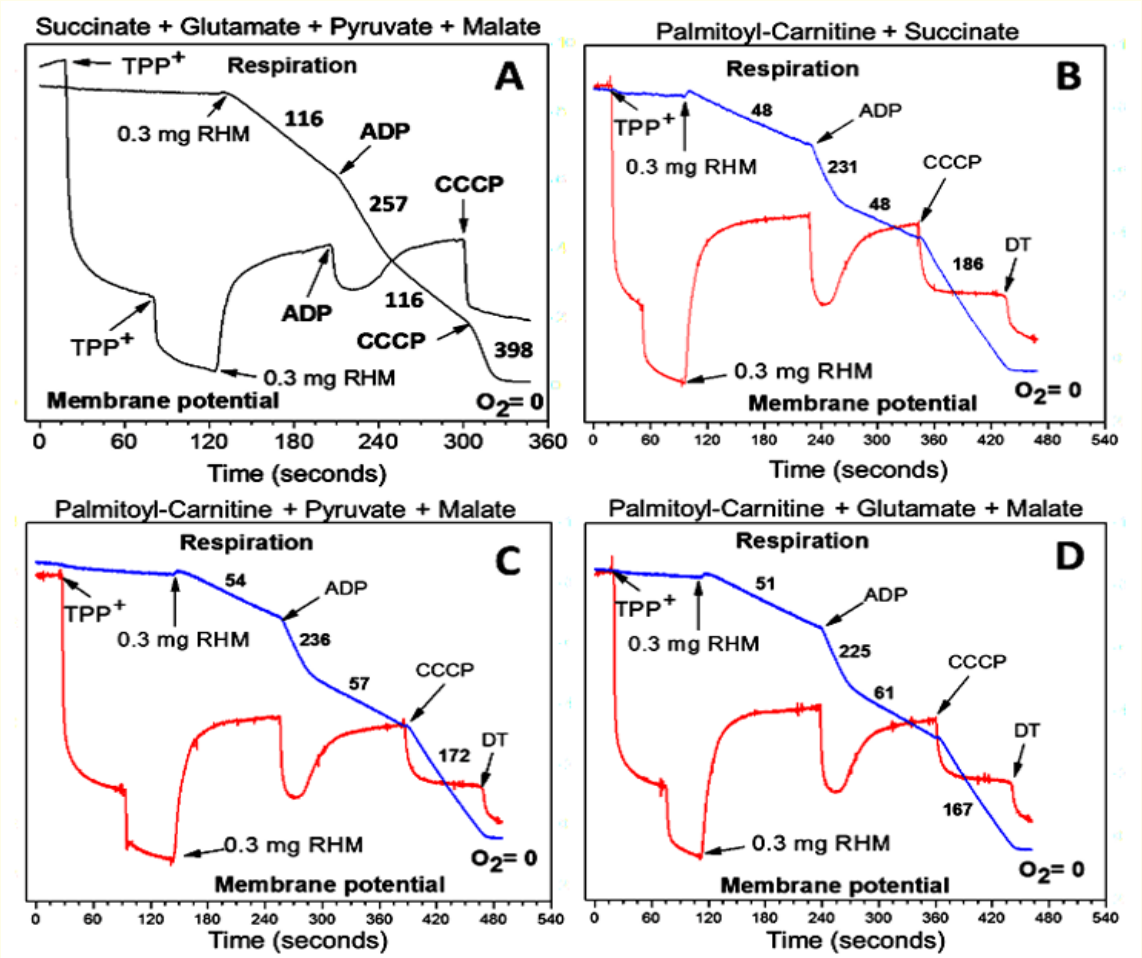


***Figure 6:*** *Respiratory rates and membrane potential of rat heart mitochondria oxidizing succinate and palmitoyl-carnitine in the presence of other metabolites. Incubation conditions and additions as in figure 4 and figure 5.*

### Activation of succinate and palmitoyl-carnitine oxidation by supporting metabolites increases the production of ROS

Reactive oxygen species (ROS) is a collective name for radicals originating from the superoxide radical ($O_2^{\bullet}$), a molecule of $O_2$ with an extra electron. In experiments presented in figure 8, the term ROS is used for brevity because the rates represent predominantly the $O_2^{\bullet}$ formation by mitochondria, but also reflect release of $H_2O_2$ by mitochondria also [50]. Brain and heart mitochondria possess very high activities of superoxide dismutases 2 and 1, which rapidly dismutate $O_2^{\bullet}$ to $H_2O_2$ and thus allow the determination of rates of superoxide radical formation using the Amplex Red method.

Figures 8A-8C show rates of ROS production for P-C, pyruvate (8A), glutamate (8B) and succinate (8C), as well as the mixtures of P-C with each of the supporting substrate. Oxidation of P-C in the presence of any supporting substrate led to an increase 5 - 10 fold in production of ROS, whereas with all substrates used alone, the rates of ROS production were low. However, when succinate and P-C were used alone, variations in the rates of ROS production were relatively high (see figure 7 in comparison with figures 8A-8C). Earlier, we showed [40] that with brain mitochondria oxidizing succinate the intrinsic inhibition of SDH caused almost complete loss of resting respiration and generation of membrane potential, which could be prevented by isolation of brain mitochondria in the presence of 0.1% BSA. With heart mitochondria oxidizing succinate, the resting (State 4) respiration was normal, but ADP did not stimulate oxidative phosphorylation (State 3), and both State 4 respiration and State 3 inhibition were insensitive to BSA. The intrinsic inhibition of SDH in heart mitochondria could be prevented or removed only metabolically by addition of glutamate or pyruvate [38], or palmitoyl-carnitine [31] (See also figure 6 in this paper).

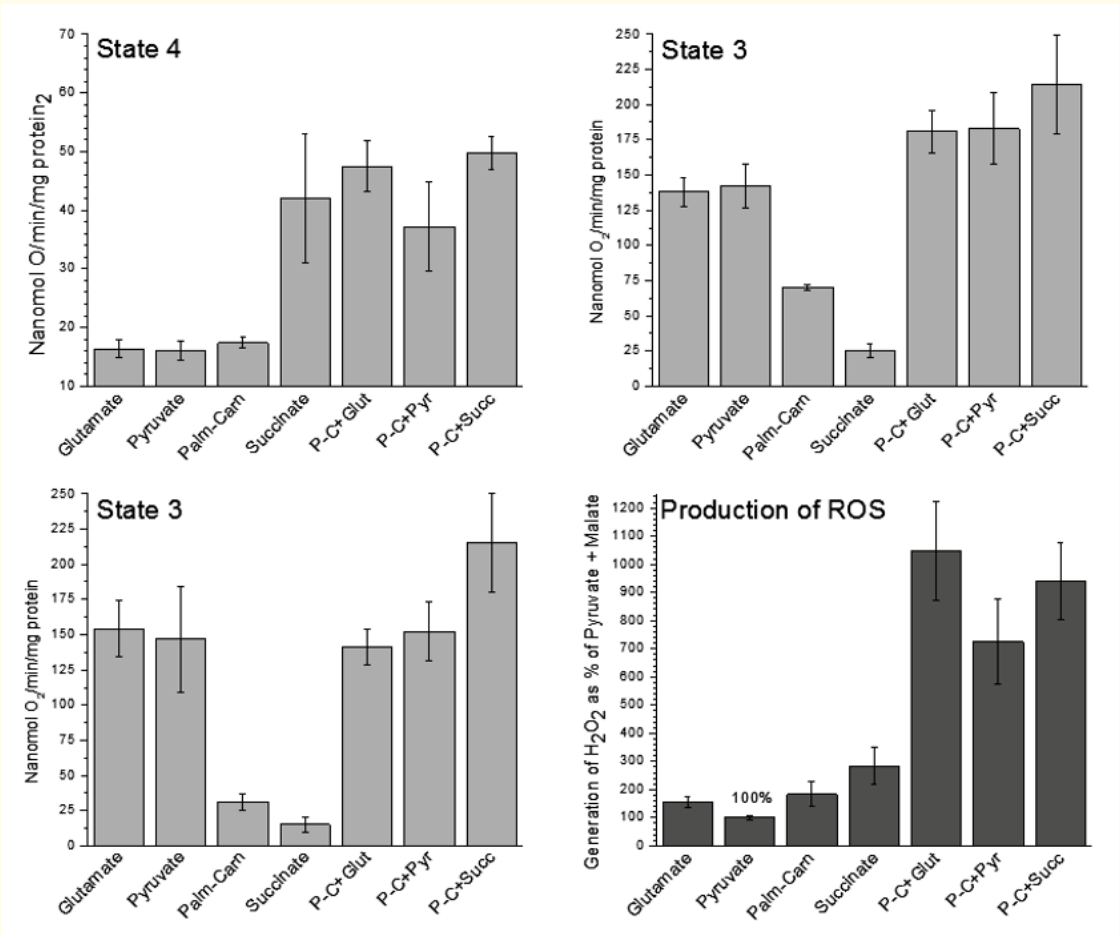


***Figure 7:*** *Respiratory activities of RHM and ROS production during synergistic oxidations of palmitoyl-carnitine and other mitochondrial metabolites. Respiratory data are presented as M ± SE of 4 - 8 separate isolations. ROS productions are presented as percent of H2O2 produced by RHM oxidizing pyruvate + malate, which was taken as 100%, as M ± SE of 3 - 5 experiments. The figure was taken from [34].*

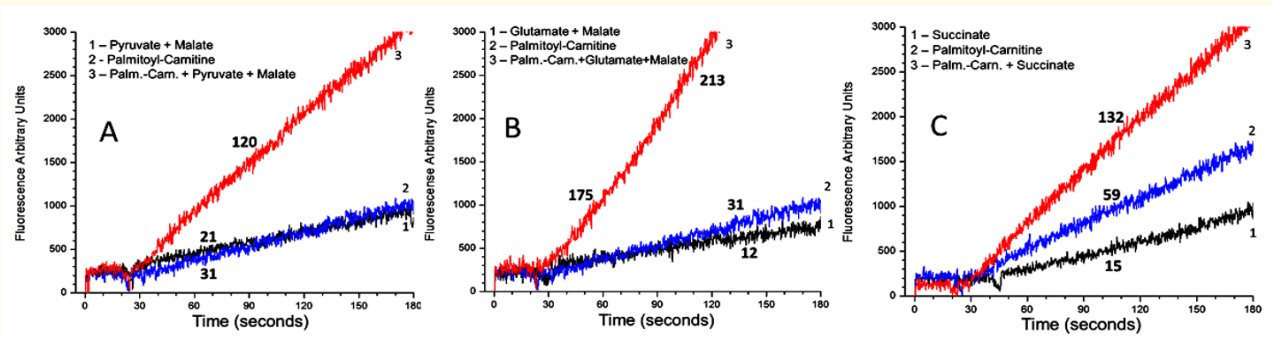


***Figure 8:*** *Production of superoxide radicals by RHM oxidizing palmitoyl-carnitine. 1. supporting substrate only; 2. palmitoyl-carnitine only, and 3. palmitoyl-carnitine + substrate. Substrates: Figure 4A - pyruvate + malate, Figure 4B - glutamate + malate, and Figure 4C - succinate. Experimental conditions are described in Methods. The incubation medium contained: Amplex Red 2 µM, horse radish peroxidase 2 Units, substrates as indicated in Methods, volume 1 ml. The reaction was initiated by addition of 50 µg of mitochondria. Initial rates were measured for 3 minutes. Numbers at the traces are the rates of $H_2O_2$ production in picomol $H_2O_2$/min/mg protein RHM. The rates were corrected for the time control rate with RHM incubated without added substrates. The figures were taken from [34].*

Another important distinction between brain and spinal cord mitochondria from the mitochondria of other organs is the complete absence of endogenous substrates in neuronal mitochondria. When isolated neuronal mitochondria were placed into an incubation medium without added substrates, there was zero oxygen consumption, whereas mitochondria from other organs supported respiration for several minutes. We suggest that the differences between the content of endogenous substrates explain the differences in the intrinsic inhibition of SDH between brain and heart mitochondria, and also variations in the rates of resting respiration of heart mitochondria oxidizing palmitoyl-carnitine.

### Role of endogenous substrates in supporting resting respiration and ROS production

Figure 9 shows an experiment with RHM incubated in a medium without externally added substrates. Unlike brain mitochondria, which consume no oxygen, the heart mitochondria not only supported the State 4 respiration, but upon addition of ADP, supported a relatively high rate of oxidative phosphorylation. After 8 minutes of respiration on endogenous substrates, the addition of palmitoyl-carnitine was able to activate State 3 respiration at a low rate, which was further stimulated by the addition of glutamate.

The experiment shown in figure 9 suggests that responses of isolated RHM to palmitoyl-carnitine or succinate strongly depend on the amount of endogenous substrates. During storage of mitochondrial suspension on ice, mitochondria gradually lose endogenous substrates. We usually followed changes in the quality of mitochondria during a working day by comparing the rates of respiration on glutamate + malate obtained in the first and the last experiments, and we found that normally there was no deterioration in the quality of RHM or RBM during the 5 hours of storage of isolated mitochondria on ice, in spite of losing much of endogenous substrates.

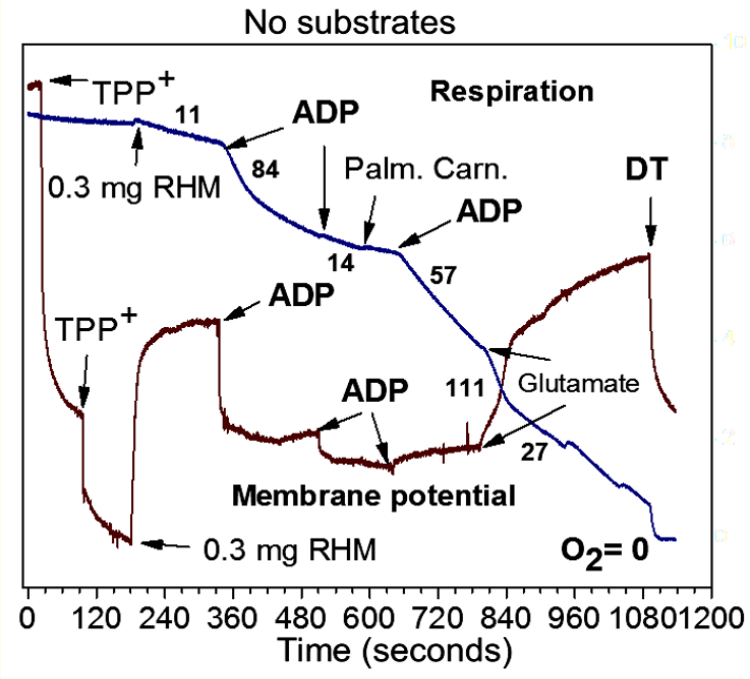


***Figure 9:*** *Respiratory activity and membrane potential of RHM incubated in the absence of externally added substrates. Incubation conditions as described in Methods, except that initially no substrate was added to the incubation medium. Additions: ADP 150 µM, palmitoyl-carnitine 0.05 mM, glutamate 5 mM. dithiothreitol (DT) 50 µL was added to eliminate remaining $O_2$ to save time.*

We have paid so much attention to the problems of metabolic interactions and endogenous substrates of heart mitochondria because these issues are of great practical importance. For example, it is known that patients suffering from heart infarction often have signs characteristic of metabolic syndrome, which is known for lower efficiency of glucose metabolism. However, long time ago it was established that glucose is a key metabolite for survival of cardiomyocytes during ischemia [13,28,51]. Our results presented in this paper suggest that metabolic interactions in heart mitochondria may affect not only production of energy, but also a change in the rate of ROS production. Figure 7 provides examples on how different metabolic patterns in mitochondria affect production of ROS. Figure 8 shows that simultaneous oxidation of P-C and succinate increased production of ROS more than 7 fold in comparison with succinate alone. However, when palmitoyl-carnitine was oxidized in the presence of several supporting substrates (see figure 10 trace 4), the ROS production increased even further.

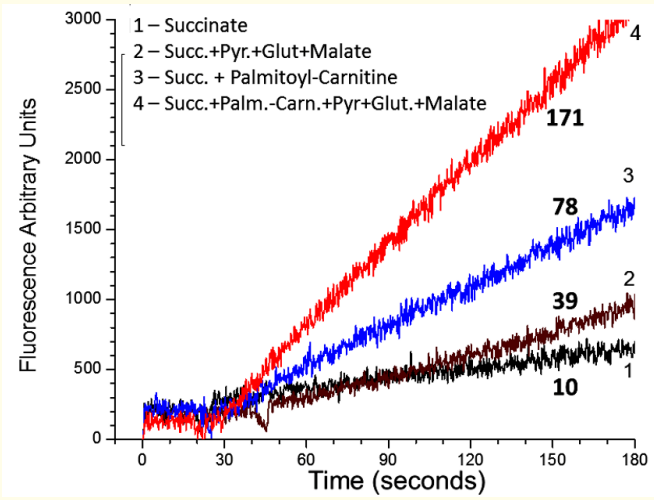


***Figure 10:*** *Effect of pyruvate plus malate and palmitoyl-carnitine on ROS production by RHM oxidizing succinate. Incubation conditions as in figure 8.*

Thus, experiments with the isolated heart mitochondria show that simultaneous oxidation of fatty acids and other mitochondrial metabolites support not only high rates of ATP production, but may increase manifold production of ROS and thus accelerate oxidative damages to mitochondria and other components in cardiomyocytes. This raises a question what is the mechanism that allows heart mitochondria to change rates of ATP production in a very wide range and why do the rates of ROS production increase so dramatically during simultaneous oxidation of fatty acids and other mitochondrial substrates? At least some of the most important answers to these questions can be found in recent fundamental publications by Martin Brand and colleagues [39,50,52,53].

## Different mitochondrial substrates produce ROS at different sites

Brand., *et al*. [39] analyzed rates of superoxide/$H_2O_2$ production at different sites in rat skeletal muscle mitochondria oxidizing a variety of conventional substrates in the absence of added inhibitors: succinate, glycerol 3-phosphate, palmitoylcarnitine plus carnitine, or glutamate plus malate. The authors concluded that the overall rates differed by an order of magnitude between substrates, and that the relative contribution of each site was very different with different substrates [39,50]. In a comprehensive review [50], Brand indicated that mammalian mitochondria can generate superoxide and/or hydrogen peroxide from at least 11 different sites associated with substrates catabolism and the electron transport chain. He concluded that these different sites have very distinct properties [50]. Six sites operate at the redox potential of the NADH/$NAD^+$ isopotential pool, about -280 mV, and five sites operate at the redox potential of the ubiquinol/ubiquinone ($QH_2$/Q) isopotential pool, about +20 mV [39,50].

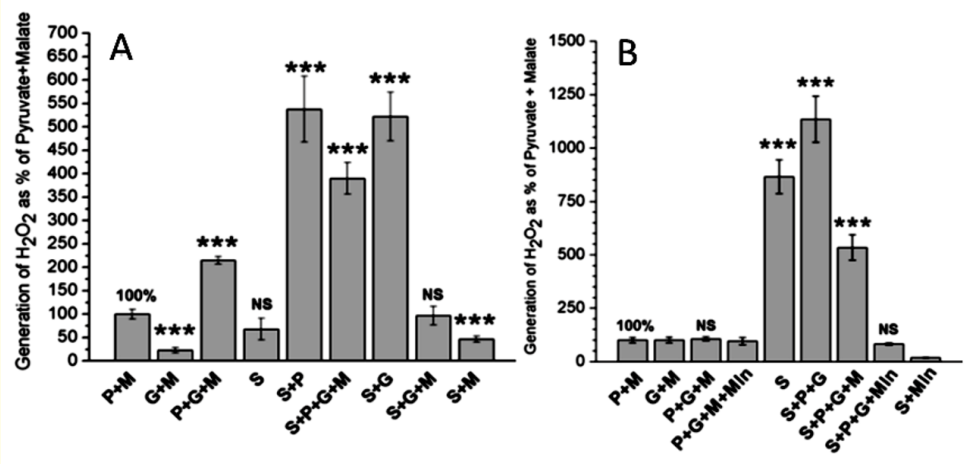


***Figure 11:*** *Effects of malate on ROS production by the rat brain and heart mitochondria oxidizing various substrates and substrate mixtures. Incubation conditions as in figure 4, substrate concentrations as described in the Methods. The data are the average of three different isolations (M ± St. error). ROS production was measured with the Amplex red method as described in [2]. ROS production with pyruvate + malate was taken as 100%. A: Rat Brain Mitochondria. B: Rat Heart Mitochondria. Statistics: *** - p < 0.001; NS: Non-Significant. All differences are comparisons with pyruvate + malate. Abbreviations: P: Pyruvate; G: Glutamate; M: Malate; S: Succinate; Mln: Malonate 5 mM. The figure was taken from [41].*

Increases in respiration rate can cause either an increase in production of superoxide/hydrogen peroxide, if the increase in respiration rate is driven by increased substrate supply (so the electron transport chain becomes more reduced), or a decrease, if respiration is driven by increased ATP demand [50].

In order to satisfy the increased ATP demands under conditions of high workloads of the heart, mitochondria must receive the correspondingly increased supply of electrons into the respiratory chain. The conventional mitochondrial substrates, often regarded as complex I substrates, which provide electrons through complex I (pyruvate and glutamate and some others), have relatively low rates of respiration because the NADH dehydrogenase of complex I is the rate limiting step. Accelerated rates of respiration can be achieved either by transamination of glutamate or with mixed substrates, such as pyruvate + glutamate + malate, when in addition to NADH, the transaminase reactions can produce 2-oxoglutarate, which is then converted to succinate in the TCA cycle [36,37]. But this mechanism works specifically well in neuronal mitochondria and does not work in the heart mitochondria [31]. The importance of fatty acids oxidation for the bioenergetics of skeletal muscle and heart mitochondria was explained by Brand and his colleagues [50,53]. During β-oxidation of fatty acids by the multienzyme complexes [54], the electron-transferring flavoprotein-ETF:Q oxidoreductase system reduces the membrane's pool of ubiquinone. This reverses the flux of electrons through the complex II from the membrane's pool of $QH_2$ into respiratory chain [50,53]. In the presence of P-C and supporting substrates there is no inhibition of SDH (complex II), and thus electrons enter the respiratory chain both through the NADH/$NAD^+$ and $QH_2$/Q pathways allowing fast production of ATP at high workloads. If consumption of ATP is limited, the excess of electrons is redirected to reverse electron transport and this results in increased reduction of several potential sites that produce superoxide and $H_2O_2$, of which the highest rates are, according to Brand [50], at complex I (sites $I_F$ and $I_Q$), at complex II (site $II_F$), and complex III (site $III_{Qo}$). This suggests that aging of the heart may be accelerated in people with metabolic syndrome and who lead a sedentary lifestyle.

## Oxidative stress and mtDNA mutations

MtDNA mutations are one of the hallmarks of ageing and age-related diseases. An aging-dependent accumulation of mtDNA point mutations has been demonstrated in the majority of individuals above a certain age [54], and an exponential increase with age was described for deletions of mtDNA [55]. The mutations occur in the mtDNA main control region at critical sites for mtDNA replication and the extraordinary tissue specificity and nucleotide selectivity of these mutations strongly suggest that they are functionally relevant [54]. Accumulation of mtDNA mutations with increasing age was demonstrated, mainly in heart, muscle and brain, which undergo a high range of workloads and associated metabolic activities. In the liver, which in addition to relatively stable high rate of metabolism has a very high regeneration capacity, there was shown no significant accumulation of mtDNA mutations with age [56]. For decades, it was generally agreed that ROS-associated somatic mutations of mtDNA contribute to human aging and to the decline of energetic capabilities at higher age [55,57].

In recent years, however, new experimental data have discredited to some extent the original theory of direct participation of free radicals in mtDNA mutations [58,59]. The major sources of mitochondrial DNA mutations seem to be replication errors and failure of the repair mechanisms. The accumulation of these mutations observed in aged organisms seems to occur by clonal expansion and not to be caused by a reactive oxygen species-dependent vicious cycle [58-60]. It has been found that most commonly discussed ROS species either are not chemically active enough ($O_{2}^{\bullet-}$, $^{\bullet}NO$) to initiate mutations, or too active ($^{\bullet}OH$) and thus have a too short life span in order to reach mtDNA [61,62]. In addition to endomitochondrial micro-compartmentalization [63], mtDNA appears to be well protected from action of reactive oxygen species (ROS) through robust protein coating of nucleosome [59]. The new hypotheses of how age-associated mitochondrial dysfunction may lead to aging are based on the role of reactive oxygen species as signaling molecules and on their role in mediating stress responses to age-dependent damage [58,59]. However, this formulation sounds too vague.

About thirty years ago, Roberts and Morrow have discovered that polyunsaturated fatty acids (PUFA), which are part of the mitochondrial phospholipids, may undergo autoxidation with formation of extremely wide range of final products with prostaglandin-like activities [64,65]. Therefore this type of lipid peroxidation was named isoprostane pathway of lipid peroxidation (IPLP). Some of the products of IPLP, for example isoketals and isolevuglandins, are highly toxic molecules. Adducts of these molecules with phosphatidylethanolamine and proteins have been found as the most reliable and sensitive early markers of oxidative stress and age-associated diseases [66,67]. However, the mechanism of IPLP initiation remained obscure.

Recently, we have suggested that perhydroxyl radical ($HO_{2}^{\bullet}$), a protonated form of superoxide radical, is responsible for activation of PUFA autoxidation and thus the process of aging [62]. The molecule of $HO_{2}\bullet$ is highly hydrophobic, is a strong oxidant, and has a very high affinity for reacting with PUFA. We suggested that during interaction with a molecule of PUFA, $HO_{2}^{\bullet}$ is converted to two molecules of water, and the PUFA loses two double bonds, and becomes a highly unstable molecule, which undergoes peroxidation and random intramolecular re-arrangements, thus causing a very large isomerism of the final products. The extremely high reactivity of $^{\bullet}HO_{2}$ with polyunsaturated fatty acids is the cause of very subtle and slow accumulation of damages in the membrane and membrane associated proteins, even though the concentration of $^{\bullet}HO_{2}$ relative to superoxide radical may be very low [62].

The type of a final product of IPLP strongly depends on the local conditions and thus contributes to the organ's specificity of mtDNA mutations [62]. By itself, $HO_{2}^{\bullet}$ does not react with mtDNA because they are spatially separated, but some toxic products of IPLP are highly reactive and stable enough to reach mtDNA or proteins responsible for the housekeeping of nucleosomes harboring mtDNA. Because concentration of $HO_{2}^{\bullet}$ in mitochondrial membrane depends on the steady state concentration of $O_{2}^{\bullet}$ [50], the increased production of superoxide radicals during oxidation of FAs in the presence of supportive substrates also increases $HO_{2}^{\bullet}$ and thus affects the rate of mtDNA modifications.

## Oxidative stress and the mitochondrial membrane integrity

Mitochondria, particularly the inner membrane, contain very large amount of proteins, many of them in hundreds and thousands copies. Between the proteins are located phospholipids, which comprise only around 25% of the total mass. Cardiolipin is located almost

exclusively in the inner membrane of mitochondria (IMM). In the IMM of liver mitochondria, phosphatidylcholine (40%), phosphatidylethanolamine (34%) and cardiolipin (18%) together comprise 92% of all phospholipids [68]. However, only phosphatidylcholine, together with few other phospholipids, form a biological membrane. Phosphatidylethanolamine (PEA) and cardiolipin (CL) have a conical form and interact with proteins and thus allow integration of large proteins and multiprotein complexes into numerous curves of the inner membrane [69]. PEA has a conical form because at C2 this phospholipid has a polyunsaturated fatty acid, usually arachidonic acid (C20:4, ω-6) or docosahexaenoic acid (C22:6, ω3), which have a curved shape. The conical shape of CL comes from its structure: two phosphatidic acid moieties connect with a glycerol backbone in the center to form a dimeric structure. The small head, made of glycerol and two phosphates, bears one or two negative charges, and four fatty acids make a wide "skirt". In most animals, the four fatty acids are usually linoleic acid with two unsaturated bonds (C18:2).

During oxidative stress, the final products of IPLP: isoprostanes, isofurans or isoketals remain esterified with phospholipids and can form stable adducts with N atoms of the lysine containing proteins and amino group of PEA. This results in the malfunctions of lipid-protein interactions and dysfunction of proteins. Accumulation of oxidized CL and its depletion are hallmarks of aging [70-72]. The mechanism of cardiolipin peroxidation remains uncertain, but recent data show that during aging and diabetes the fatty acid composition of CL may undergo remodeling when C18:2 fatty acids can be replaced by arachidonic acid (C20:4, ω-6) or docosahexaenoic acid (C22:6, ω3) [73,74], which are substrates for IPLP [62,64,65].

Thus aging and increased oxidative stress in aged individuals with metabolic syndrome cause not only mutations and deletions of mtDNA, but also diminish energy production by heart mitochondria due to accumulation of damaged proteins and dysfunction of the membrane-associated protein complexes. From the evidence presented above, it is conceivable that $HO_2^{\bullet}$, which initiates IPLP of PUFA, is the main species of ROS that is responsible for aging and mitochondrial dysfunctions.

## Metabolic syndrome and cardiovascular diseases

Oxidative stress is regarded as the main cause of aging, mitochondrial dysfunctions, and thus being one of the main pathogenic mechanisms of many diseases [6,7,11,13]. This is particularly true for those diseases, which are associated with the metabolic syndrome that develops at the certain stage of aging in humans [5-7]. We have recently pointed out that available definitions of metabolic syndrome do not reflect the fundamental causes of this body condition [31]. It is clear, that metabolic syndrome as part of aging is not just the result of accumulated errors and dysfunctions in the cause of life for whatever reason. Recently, we have suggested that the main signs of metabolic syndrome - rearrangement of the body to a more bulky structure, accumulation of fat, increased consumption of fatty acids as the source of energy, dysregulation of metabolic relationships between glucose and fatty acids, etc., suggest that aging human organisms gradually become governed by the set of genes that control the final stage of the postembryonic ontogenesis of an individual [31].

From the point of view of the development of an individual after birth, the genetic clock determines switches between the stages of ontogenesis and the longevity of each stage of the organism's progress in its development. It is conceivable that for an individual, each step is determined by internal factors, such as hormonal spectrum, quality of stage-specific genes, intrinsic production of ROS, etc., and external factors determined by the quality of life conditions and the environmental factors. For humans, the stages after the reproductive age were much less subject for natural selection than the earlier stages of the postembriogenesis. Therefore, the metabolic features after age of 50 - 60 resemble those of our ancestors. There are many direct and indirect indices that many characteristics of metabolic syndrome reflect not the results of aging and accumulated damages, but a new type of metabolism which we inherited from our ancestors who were poorly protected from cold weather, often starved, and did not eat food rich in carbohydrates. One of the typical manifestations of metabolic syndrome is a high incidence of the diabetes type 2 (T2D). Therefore research on pathogenesis of T2D may serve as a good example which indirectly supports our hypothesis on the nature of metabolic syndrome.

Intensive studies have shown that the number of identified gene loci conferring an increased risk for T2D now surpasses one hundred. There is currently very strong evidence to support the notion that failure of insulin secretion is the defining event in the pathogenesis

of T2D (Reviewed in 75). This breakthrough has changed the focus of much of the research on the pathogenesis of T2D. It has prompted research on genomic processes, e.g. genetic polymorphisms, epigenetic mechanisms, micro and linker RNAs, and chromatin structure, which are robustly linked to the disease and centering on the pancreatic β-cell [75]. Thus, there is no single mutation or other single course of T2D, and researchers are focused on metabolic diversities of our ancient ancestors from the Northern part of the globe. Metabolic dysregulation in contemporary elderly people with metabolic syndrome more likely results from the fact that our ancestors did not use food rich in carbohydrates. The sedentary life and excessive food consumption rich in carbohydrates are not compatible with the genetic pattern of our ancestor's metabolism, and therefore are probably the main cause of accelerated aging and the diseases accompanying the metabolic syndrome.

## Chronic stress and the hypothesis of mitochondrial allostatic load as the cause of metabolic syndrome, T2D and cardiovascular diseases

In 1988 Sterling and Eyer [76] put forward the concept of Allostasis as an alternative to the concept of Homeostasis, which was suggested in XIX century by French physiologist Claude Bernard [77]. The word "homeostasis" was later coined by Walter Bradford Cannon [78]. A thorough discussion of allostasis versus homeostasis can be found in [79]. Briefly, allostasis is the process whereby an organism maintains physiological stability (homeostasis) through changing parameters of its internal milieu by matching them appropriately to environmental demands [80]. Because allostatic mechanisms are particularly active during chronic stress, organs and tissues experience "biological wear and tear", which predisposes organs to development of diseases. The physiological consequences of chronic stress were named "allostatic load" [81].

Further developments of the allostatic load model of chronic stress were focused on glucocorticoid dysregulation of the metabolic processes, particularly glucose imbalance. It has been proposed that mitochondrial dysfunction constitutes an early, modifiable target of chronic stress and stress-related health behaviors [80]. During stress, particularly chronic ones, glucocorticoids trigger physiological processes that ultimately increase circulating levels of glucose and lipids [80]. This response in turn promotes the development of hypertension, central obesity and glucose intolerance, collectively recapitulating the key features of the metabolic syndrome [82]. Chronically elevated glucose and fatty acids levels damage both mitochondria and mtDNA, generating toxic products that can promote systemic inflammation, alter gene expression, and accelerate cell aging [80]. An extreme form of allostatic load was coined "allostatic overload", in which dysregulation of allostatic responses, coupled with health-damaging behaviors (poor sleep, increased caloric intake, lack of exercise, smoking, and drinking alcohol, often in response to chronic stressors, lead to disease [80].

The allostatic load model proposes a specific three-stage temporal and causal sequence of biological damage linked to health outcomes [83]. First, overactivation or underactivation of primary mediators such as glucocorticoids, induces direct effects and outcomes on cellular processes. Secondary outcomes (metabolic, cardiovascular, neural, and second-order immune biomarkers) become dysregulated as indicated by their abnormal patterns, including the lack of adaptation, prolonged response or blunted response. Third, this process culminates in tertiary outcomes or clinical end points [80]. However, the primary effects or the mechanisms that link primary mediators to secondary and tertiary outcomes remain unclear [80].

Allostatic overload resulting in the oversupply of substrates for mitochondria (metabolic oversupply) [84], relative to cellular energy demand during stress, generates metabolic stress and generally results in weight gain and mitochondrial stress and/or damage [80]. The results, presented in [31] and in this review, substantiate the conclusions of allostatic load hypothesis by offering the evidence that mitochondria increase production of ROS and thus initiate the damaging processes in the heart and brain by activation of IPLP.

As we have shown, during simultaneous oxidation of fatty acids and the products of catabolism of glucose and/or proteins, namely pyruvate, succinate, glutamate, the production of ROS, mostly superoxide radicals, may increase manifold. According to Brand [50,52,53], under conditions of excessive substrates supply, electrons enter the respiratory chain both through the $NADH/NAD^+$ and $QH_2/Q$ pathways allowing fast production of ATP at high workloads. If energy demand is limited, the excess of electrons is redirected to reverse electron

transport and this results in increased reduction of several potential sites that produce superoxide and $H_2O_2$. Increased production of $O_2^{\bullet}$ inevitably increases the level of perhydroxyl radicals ($HO_2^{\bullet}$) that initiate the isoprostane pathway of lipid peroxidation of mitochondrial PUFA. It was suggested recently that $HO_2^{\bullet}$-stimulated IPLP is the main mechanism driving the process of aging [62,85]. There arises a logical question, how does the antioxidant system react and respond to the process of aging?

### Antioxidant functions in aging

The sections above described specific metabolic mechanisms leading to mitochondrial ROS overproduction and increased lipid peroxidation which accelerates aging and cardiac dysfunction. We discussed evidence that although different species of ROS contribute to the overall wear and tear of an organism in the course of postembryonic ontogenesis, it is the perhydroxyl radical that is responsible for the subtle and persistent accumulation of damages that some researchers consider as the process of aging.

Protonation of $O_2^{\bullet}$ occurs at the membrane/water interface, and activation of autoxidation of mitochondrial PUFA by $HO_2^{\bullet}$ occurs inside the membrane's lipid phase [64,65]. Therefore, the slow process of aging is not sensitive to water soluble antioxidants. Barja summarized the results of experiments on various species of living organisms and selected those features, which affected the life span in all species tested. He concluded that only two known factors correlate with animal longevity in vertebrates, including mammals and birds. Long life span is associated with (a) low rates of mitochondrial ROS production and (b) low degree of fatty acid polyunsaturation of cellular membranes, including the mitochondrial ones [11]. Because formation of $HO_2^{\bullet}$ is proportional to the level of $O_2^{\bullet}$, which at any moment is determined by the rates of its production and elimination [50], the activities of Mn-SOD2 and Cu, Zn-SOD1 are of most importance for the heart and the central nerve system, where alternative antioxidant systems are relatively week, whereas the contents of AA and DHA are at the highest [68].

During aging, however, the increased ROS production occurs in parallel with diminished activities of key mitochondrial antioxidant enzymes such as superoxide dismutase (SOD2) [86], and reduced thiol-dependent scavengers of $H_2O_2$ and lipid peroxides by glutathione peroxidases [87]. Mitochondrial deacetylase Sirtuin 3 is the key node in regulation of mitochondrial metabolism and antioxidant activity, and we have recently shown that Sirtuin 3 S-glutathionylation contributes to Sirtuin 3 inactivation in hypertension [88]. The expression and activity of Sirtuin 3 declines with age [89] and this contributes to SOD2 inactivation due to SOD2 hyperacetylation leading to increased superoxide levels and lipid peroxidation. This results in the development of mitochondrial oxidative stress due to imbalance between the ROS overproduction and diminished antioxidant activity. The age-associated Sirtuin 3 impairment may contribute to the development of mitochondrial oxidative stress and development of CVD.

## Conclusions

In this review we provided evidence that heart mitochondria under normal conditions oxidize mixtures of substrates where fatty acids serve as the main source of hydrogen for production of energy. Synergistic interactions between fatty acids and the catabolic metabolites of glucose and amino acids allow energy supply by mitochondria in a wide range of functional loads. However, during insufficient physical activity, the excessive supply of substrates due to chronic stress and the stress-associated behavioral deviations (overeating, alcohol consumption, etc.) may result in excessive production of ROS by mitochondria, which leads to diseases typical of the metabolic syndrome. We have also suggested that when an individual enters the last stage of postembryonic ontogenesis, the development of metabolic syndrome may result from the inconsistencies between contemporary life style and the genetically predetermined metabolic phenotype inherited from our ancestors.

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