ISSN 0006-2979, Biochemistry (Moscow), 2019, Vol. 84, No. 11, pp. 1411-1423. © Pleiades Publishing, Ltd., 2019.
Russian Text © The Author(s), 2019, published in Biokhimiya, 2019, Vol. 84, No. 11, pp. 1743-1758.

REVIEW

Brain Ischemia/Reperfusion Injury and Mitochondrial Complex I Damage

A. Galkin

Division of Neonatology, Department of Pediatrics, Columbia University William Black Building, NY 10032, New York, USA
e-mail: ag4003@cumc.columbia.edu

Received June 7, 2019
Revised July 8, 2019
Accepted July 9, 2019

Abstract—Ischemic stroke and neonatal hypoxic-ischemic encephalopathy are two of the leading causes of disability in adults and infants. The energy demands of the brain are provided by mitochondrial oxidative phosphorylation. Ischemia/reperfusion (I/R) affects the production of ATP in brain mitochondria, leading to energy failure and death of the affected tissue. Among the enzymes of the mitochondrial respiratory chain, mitochondrial complex I is the most sensitive to I/R; however, the mechanisms of its inhibition are poorly understood. This article reviews some of the existing data on the mitochondria impairment during I/R and proposes two distinct mechanisms of complex I damage emerging from recent studies. One mechanism is a reversible dissociation of natural flavin mononucleotide cofactor from the enzyme I after ischemia. Another mechanism is a modification of critical cysteine residue of complex I involved into the active/deactive conformational transition of the enzyme. I describe potential effects of these two processes in the development of mitochondrial I/R injury and briefly discuss possible neuroprotective strategies to ameliorate I/R brain injury.

DOI: 10.1134/S0006297919110154

Keywords: stroke, ischemia-reperfusion injury, mitochondria, complex I, flavin, thiols, nitrosation

Brain tissue ischemia/reperfusion (I/R) injury occurs under pathological conditions, such as ischemic stroke, cardiac arrest, head trauma or perinatal hypoxic-ischemic injury. Stroke is the fifth leading cause of death in the world, and one in six people worldwide will have a stroke in their lifetime. Due to the increase in the average population age, the frequency of stroke is predicted to rise rapidly in the near future. Another clinical manifestation of I/R is a perinatal hypoxic-ischemic (HI) injury and the worldwide mortality in infants had reached 1.15 million in 2010 [1]. In survivors, the estimated cost for clinical and lifetime care is projected to be trillions of dollars [2]. Despite the prevalence and devastating costs of brain ischemia, the major effective treatment in the clinic for stroke cases is still mechanical or pharmacological reinstatement of blood supply or reperfusion. The intracellular mechanisms of acute brain injury in I/R are far from being completely understood. Development of therapeutic interventions ameliorating the tissue damage is an area of active research. The present review summarizes recent advances in the studies of impairment of mitochondrial complex I during acute primary and secondary energy failure in the brain I/R. I have limited this review to only acute changes that take place within the first hours of tissue injury, as various aspects of the I/R effect on mitochondrial metabolism have been covered elsewhere [3-6].

Abbreviations: A/D transition, active/deactive transition; FAD, flavin adenine dinucleotide; FMN/$FMNH_2$, oxidized/reduced flavin mononucleotide; HI, hypoxia-ischemia; I/R, ischemia/reperfusion; MCAO, middle cerebral artery occlusion; Q, ubiquinone; RET, reverse electron transfer; ROS, reactive oxygen species; TCA, tricarboxylic acid (cycle).

MITOCHONDRIAL RESPIRATORY CHAIN AND MITOCHONDRIAL COMPLEX I

Our brain relies mostly on mitochondria to produce energy via the combined activity of metabolic enzymes, respiratory chain, and ATP synthase (Fig. 1). Carbohydrates, fats, and amino acids are eventually degraded to simpler, universal intermediates that can be oxidized releasing energy to be captured. The oxidation process begins with the transfer of electrons derived from substrates through a system of carriers of the respiratory chain located in the inner mitochondrial membrane.

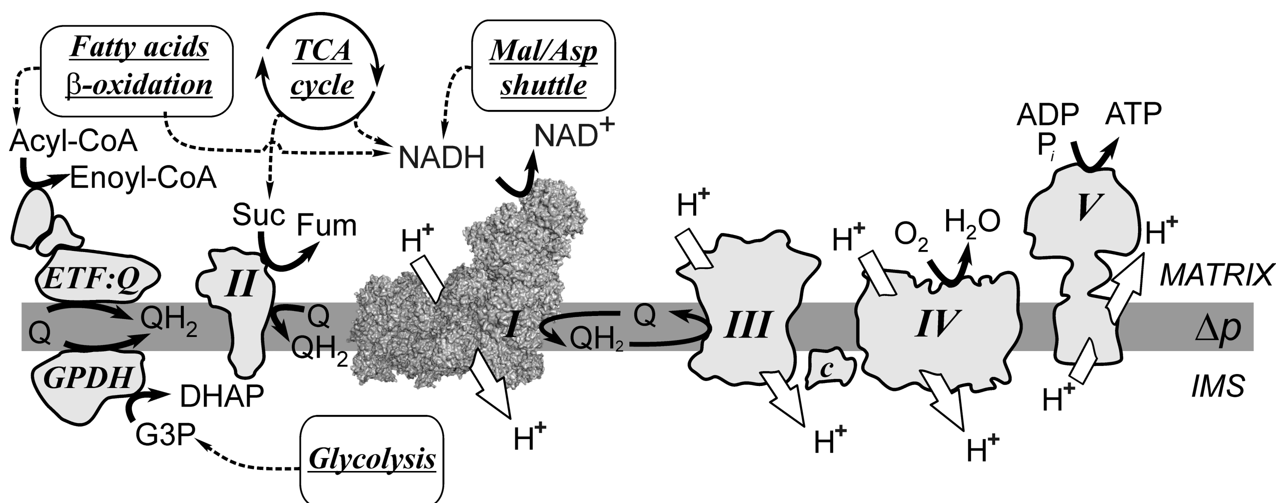

Fig. 1. Mitochondrial oxidative phosphorylation in the inner mitochondria membrane. Electrons from substrates are supplied to at the ubiquinone (Q) level from various dehydrogenases. ETF:ubiquinone oxidoreductase (ETF:Q) transfers electrons from the dehydrogenation step of various acyl-CoA to enoyl-CoA in fatty acids oxidation in the matrix. Glycerol 3-phosphate dehydrogenase (GPDH) at the outer side of the inner membrane oxidizes glycolytic glycerol 3-phosphate (G3P) to dihydroxyacetone phosphate (DHAP). Complex II or succinate dehydrogenase of the tricarboxylic (TCA) cycle oxidizes succinate (Suc) to fumarate (Fum). NADH dehydrogenase (NADH:ubiquinone oxidoreductase) or complex I is depicted as structural model (PDB 6G2J) with atoms shown as spheres. Complex I oxidizes NADH generated in fatty acids oxidation, TCA cycle, and malate/aspartate shuttle (Mal/Asp shuttle). Respiratory chain complexes I, III, and IV pump protons (H^+) across the membrane (white arrows) to create proton motive force (Δp) to drive ATP synthesis at complex V.

Respiratory chain complexes transfer electrons in a series of steps to oxygen, the terminal electron acceptor (Fig. 1). The free energy of the redox reactions is used by complexes I, III, and IV to translocate protons and form the proton motive force (Δp) across the membrane (Fig. 1). The back-flow of protons from the intermembrane space to the matrix drives the synthesis of ATP from ADP and inorganic phosphate at complex V. The energy demand of the brain is provided by the process above and most of this energy in form of ATP is required for ion transport during neuronal action potential propagation.

Contrary to the biochemical textbook scheme depicting complexes I and II as the only points of electron entry into the respiratory chain, there are other dehydrogenases involved in this process. In the brain, two other major dehydrogenases are located at the inner mitochondrial membrane and transfer electrons from their substrates to ubiquinone. These are glycerol 3-phosphate dehydrogenase (GPDH) on the outer side [7] and electron transfer flavoprotein (ETF) of fatty acids oxidation (ETF:ubiquinone oxidoreductase system) at the inner side of the mitochondrial membrane [8] (Fig. 1).

Complex I, or proton-pumping NADH dehydrogenase (NADH:ubiquinone oxidoreductase), is solely responsible for the oxidation of matrix NADH and regeneration of NAD^+ required for the steady-state operation of catabolism (e.g., TCA cycle, β-oxidation of fatty acids, and glycolysis). Complex I also pumps 4 protons per 2 electrons passed from NADH to ubiquinone and, therefore, has the highest efficiency output in the respiratory chain [9] (Fig. 1).

Mitochondrial complex I is a membrane-bound L-shaped enzyme with a membrane arm and a hydrophilic peripheral arm that extends into the matrix. The mammalian enzyme is composed of 45 subunits and the function of many of them is still not clear. Among 14 conserved core subunits that are sufficient for catalysis in the bacterial enzyme, seven membrane ND subunits are encoded by the mitochondrial DNA [10]. The enzyme molecule can be divided into three functional modules: peripheral N module where NADH binds, central Q module that reduces ubiquinone, and membrane P module which pumps protons across the membrane [11]. All redox centers of the enzyme are located in the N and Q modules. Complex I contains one molecule of tightly, but non-covalently bound flavin mononucleotide (FMN) and eight iron-sulfur clusters. Flavin directly accepts electrons from NADH and transfers them towards a series of iron-sulfur clusters that provide an electron pathway to the membrane ubiquinone. The final step of ubiquinone reduction most likely involves the formation of semiquinone intermediate(s) involved in energy transformation [12, 13]. The energy released at this final step drives long-range conformational changes, driving proton pumping by ND subunits in the membrane P module [14].

Complex I oxidizes NADH by membrane ubiquinone during physiological forward electron transfer, which involves probably all redox centers of complex I, but the enzyme is also able to catalyze other reactions (see [15] for the review). NADH:ubiquinone reductase reaction is sensitive to classical quinone-like inhibitors of

the enzyme (rotenone, piericidin A). NADH can be also oxidized by so-called artificial acceptors (ferricyanide, hexaammineruthenium), which react with the enzyme at the level of FMN or first iron-sulfur clusters. This reaction occurs in the N module and it is not sensitive to specific hydrophobic inhibitors or genetic modifications in the subunits of the Q or P modules. Complex I can also reduce molecular oxygen and generate reactive oxygen species (ROS) in the form of superoxide or H_2O_2. Under physiological conditions, complex I is probably a major contributor to ROS production by the mitochondrial respiratory chain.

Importantly, the physiological forward reaction of NADH oxidation by ubiquinone is reversible. Complex I can oxidize membrane ubiquinol and reduce NAD^+ during in the process called reverse electron transfer (RET). During energy-consuming RET reaction, electrons are pushed "uphill" against the difference of redox potential, and this process requires a high QH_2/Q ratio and the presence of a proton motive force across the membrane. These conditions are fulfilled during oxidation of succinate, glycerol 3-phosphate, or fatty acids. Corresponding dehydrogenases generate ubiquinol, while the proton motive force is created through combined operation of complexes III and IV downstream. Succinate-supported RET reaction was first characterized *in vitro* more than 60 years ago [16, 17], but recently gained interest in *in vivo* studies, since the level of succinate can be significantly elevated under some pathological conditions including tissue ischemia [18-22]. The highest rate of ROS production in intact mitochondria or submitochondrial particles was found during RET [23-29]. It should be noted that after reduction of all available matrix NAD^+, mitochondria come into the steady-state conditions of RET, in which all redox centres of complex I are reduced and the rate of the transfer via complex I probably represents a one-electron leak to oxygen. Most likely, reduced or semireduced flavin of complex I is the direct reductant for oxygen in ROS generation under these conditions [24, 30-34].

Complex I from mammals can undergo slow conformational active/deactive (A/D) transition, which was initially characterized by the Vinogradov's group [35-38]. Two distinct catalytic forms of the enzyme were described in mitochondrial preparations *in vitro* [39-45] and in various tissues *ex vivo* [46-52], indicating the significance of A/D transition in the regulation of the enzyme *in situ*. The difference in the kinetic and structural properties between the A and the D forms, as well as diagnostic tests for both forms, are covered in several comprehensive reviews [53-55] and I only briefly describe them here.

In situ or *in vitro* complex I is represented by an equilibrating mixture of the A and the D forms of the enzyme [53, 55]. In the absence of substrates at physiological temperatures, the equilibrium is shifted towards the catalytically dormant D-form. If both substrates (NADH and ubiquinone) are available, the D-form can perform a very slow (1 min^{-1}) turnover resulting in the conformational D to A transition and formation of the catalytically active (10^4 min^{-1}) A-form [53, 55]. Interestingly, turnover-dependent activation can take place only during forward reaction and D to A conversion cannot take place during RET, since the transfer of electrons from ubiquinol to the last iron-sulfur cluster is blocked in the D-form [31, 56]. Physiologically, deactivation of the enzyme occurs during tissue ischemia or metabolic hypoxia, when electron transfer is halted at the level of complex IV, mitochondrial respiration suspended so that the entire quinone pool is reduced [46-51]. In the absence of substrate quinone, complex I is idle and A to D conversion takes place. Intriguingly, following arrest of blood circulation, deactivation occurs at a faster rate in brain than in cardiac tissue [57]. Upon reoxygenation and increase in the level of oxidized quinone, the dormant D-form of complex I is converted to the A-form within minutes, but the time course of activation is not completely defined yet. Natural effectors, such as free fatty acids [58] and calcium [56], can significantly decrease the rate of D to A conversion, while sodium slightly increases this rate [59].

Structural differences between the A- and D-form identified in our labelling and crosslinking studies were found to all be located close to the Q-binding site near the junction of Q and P modules [40, 44, 60]. Critical thiol residue (cysteine 39) of the flexible hydrophilic loop in the membrane ND3 subunit was found to be exposed to the outside only in the D-form, but not in the A-form [37, 40]. We also found that deactivation led to a higher exposure of several other subunits, such as membrane-bound ND1 and NADPH-binding subunit NDUFA9 [44, 60]. Surprisingly, no subunits were found to be more exposed in the A-form [55], suggesting that the D-form corresponds to a "relaxed" conformation, while the A-form corresponds to a "tense" conformation of the enzyme. This was further confirmed by cryoelectron microscopy studies of the mammalian [61] and yeast [62] enzymes. Both catalytic forms of complex I were described for the enzyme from mouse heart [63], where it was revealed that significant changes occur in the transmembrane helical structures of mitochondrial-encoded ND subunits. Several catalytically important amino acids rotate upon deactivation around the α-helical axis within the lipid bilayer. Structures of both catalytic forms are available in PDB format (PDB 6G2J and 6G72) [63].

Although complex I has been extensively studied for over half a century, its structure has been resolved only recently [64-66], and the regulation of the enzyme *in situ* is not completely understood. Impairment of this enzyme is the most frequently found cause of inherited mitochondrial disorders [67], affected in aging [68], and neurodegeneration [69]. Complex I is also a major source, as well as a target, of deleterious ROS [55].

PHYSIOLOGICAL CHANGES IN RESPONSE TO BRAIN ISCHEMIA/REPERFUSION

Before discussing the details of the I/R effect on mitochondrial complex I, it would be best to briefly describe changes in the energy metabolism after arrest of blood circulation in the brain. The amounts of glucose and alternative substrates, such as glycogen, lactate, and fatty acids, are significantly higher than tissue oxygen available for their oxidation [5]. At any given moment, the amount of oxygen present in a human brain in the hemoglobin-bound and dissolved forms is estimated around 250 μmol per brain [70]. This would be enough to support metabolism at the average oxygen consumption rate during resting wakefulness of 1.88 mmol/min per brain [71] for around 10 s, which is about the time of consciousness after the complete arrest of brain circulation. In mice, brain oxygen is completely depleted within only a few seconds following cardiac arrest [72]. Mitochondria of neurons and astrocytes consume more than 75% of oxygen delivered to the brain [5] and therefore these cells are the most sensitive to oxygen deprivation. Oxygen deficit strongly affects mitochondrial metabolism which provides most intracellular ATP by oxidation of substrates generated during glycolysis, Krebs cycle, and fatty acids degradation (Fig. 1). In mice and rats, a rapid fall in the ATP level occurs within 2-5 s of brain ischemia [3, 73]. Ischemia-induced arrest of mitochondrial respiration and ATP generation is defined as a primary energy failure. Without oxygen, electron transfer at complex IV is stopped, leading to a complete reduction of mobile electron carriers (NAD(P)H and quinone) and all components of the respiratory chain. Since metabolic substrates are still available, rapid accumulation of catabolic intermediates (lactate, succinate, free fatty acids, acyl-CoA, and glycerol) takes place. Energy crisis due to the low ATP production, in turn, has a deleterious effect on ion homeostasis. Under physiological conditions, ion transport machinery maintains a 30-fold higher concentration of intracellular potassium ions, 7-fold lower concentration of intracellular sodium, and a five orders-of-magnitude lower intracellular calcium concentration in comparison to the extracellular fluid. The lack of ATP leads to the arrest of energy-dependent ion pumps and therefore, ion imbalance. Eventually, this results in a drop of plasma membrane potential and dramatic increase in the intracellular sodium concentration. The activity of the Na^+/Ca^{2+} exchanger in the plasma membrane leads to a significant rise of cytoplasmic as well as intramitochondrial calcium concentrations and development of tissue edema [74, 75]. At the same time, residual carbohydrates are metabolized to lactate in the process of anaerobic glycolysis, which is linked to the acidification of the cytoplasm. The magnitude of acidification depends on the initial brain glucose and glycogen content prior to ischemia and further influences the ionic imbalance of excitable brain cells. Neurons are also extremely sensitive to the so-called glutamate excitotoxicity. Due to the rise of the intracellular calcium level, the neurotransmitter glutamate is released from synaptic vesicles; glutamate activation of ionotropic receptors results in increased energy-consuming electrical activity, especially under conditions of reduced glutamate uptake by astrocytes in ischemia. This causes more calcium imbalance and further decreases energy supplies of the cell, augmenting the neuronal damage [5].

Permanent ischemia results in the eventual death of cerebral tissue, specifically damaging neurons and oligodendrocytes in the area supplied by the occluded artery [76]. Restoration of oxygen is a prerequisite for cell survival after ischemia; therefore, adequate reestablishment of blood flow (reperfusion) is required within 15-30 min. Ischemia-affected tissue can be formally divided into the "core" with a severe drop in the oxygen level and surrounding "penumbra", where collateral circulation provides partial oxygen delivery. The time course of ATP decline, lactate rise, acidification, and changes in the intracellular calcium concentration in the core and penumbra areas are different, reflecting different levels of metabolism. When the blood supply is restored, the dynamics of oxygen delivery and consumption is also different in the core and the penumbra, indicating differences in the cell damage and microvasculature state. Irreversibly damaged cells in the core area eventually undergo degradation and within weeks, this area becomes a lesion. Cells in the penumbra with partial energy metabolism may survive, depending on the duration of the occlusion and regiment of reoxygenation [77]. Redelivery of oxygen initiates mitochondria respiration leading to the recovery of ATP to satisfy cellular energy demands. The early stage of reperfusion is also associated with a transient rise of the level of ROS probably produced via several enzymatic systems, including mitochondria. This leads to the development of oxidative stress and further tissue injury. In several *in vivo* models of brain I/R, ATP levels and intensity of mitochondrial oxidative phosphorylation decline again a few hours later, manifesting the so-called secondary energy failure mediated by still unknown mechanism [3-5].

EFFECT OF BRAIN ISCHEMIA/REPERFUSION ON MITOCHONDRIAL COMPLEX I

Brain I/R and mitochondrial respiration. Swelling of mitochondria and disruption of the inner membrane after prolonged ischemia were observed by electron microscopy and after histochemical analysis in early studies [78, 79]. The impairment of NADH-dependent dehydrogenases activity after ischemia takes place earlier than the decline of succinate-dependent activity, detected by staining with cell viability tetrazolium-based dyes [78,

80]. Today, this can be interpreted as direct evidence for a higher vulnerability of complex I to ischemia in comparison with the rest of the respiratory chain. The inhibitory effect of the absence of oxygen on mitochondrial respiration was first demonstrated in a model of global ischemia in exsanguinated rats [81, 82]. With glutamate as a substrate, respiration of intact mitochondria isolated following as short as 2 min of ischemia was only 25% when compared to the naпve animals. Using a model of compressive brain ischemia in rabbits, a significant decrease in ADP-stimulated respiration on malate and glutamate was found after 20-40 min of increased intracranial pressure, but no effect of ischemia on the ATPase activity was observed [83, 84]. Similar results were obtained using bilateral brain ischemia in gerbils, where a twofold decline in respiration following 60 min of oxygen deprivation was demonstrated [85]. It can be concluded, that ischemia only inhibits mitochondrial respiration when substrates of NAD^+-dependent dehydrogenases of the TCA cycle are used to provide NADH for complex I.

The pathophysiology of the primary energy failure due to the absence of oxygen demonstrated in early studies is complicated by the fact that the severity of initial tissue injury is further affected by the recirculation. Redelivery of oxygen is essential for tissue survival, but at the same time, reperfusion leads to multiple indirect or secondary consequences for cell recovery in this area. The results obtained using complete brain ischemia model in rats showed almost full recovery of respiration with malate and glutamate or succinate after 30 min of recirculation, even when a significant inhibition of respiratory function occurred during the ischemic event [86]. In this work, respiration on malate and glutamate was affected more than succinate-supported oxygen consumption in complete as well as incomplete models of ischemia. However, 30 min reperfusion after incomplete ischemia led to a further decrease of respiration, indicating that the severity of ischemia and intensity of reperfusion define I/R damage to the tissue [86, 87]. The possible scenario of I/R injury development is further complicated by a phenomenon of secondary energy failure. Thus, transient focal ischemia in rats resulted in a twofold decline of ADP-stimulated oxygen uptake in brain homogenates supplied with malate and pyruvate, which was followed by a complete recovery after 1 h of recirculation and then a secondary gradual decrease (3-24 h) [88]. This effect was also dependent on a brain subregion taken for sampling. The assessment of isolated intact mitochondria in a similar model [89] confirmed changes of respiration after I/R, describing ischemia-induced decline first, then recovery shortly after reoxygenation, and a secondary decline of complex I-supported oxygen consumption.

Practical aspects. There are several practical considerations that should be taken into account when discussing the available data on the measurements of mitochondrial respiration in brain samples following I/R. A problem in interpreting the results produced in earlier works is that few comparable studies used the same *in vivo* models of ischemia, applied the same anesthetics, or even used the same brain subregions for sampling. These parameters affect the outcome of these models, as well as biochemical characteristics of the obtained samples. The measurements of oxygen consumption by a preparation of intact mitochondria isolated from the affected brain area after I/R can indeed provide a convenient and physiologically relevant marker of mitochondrial function. However, respiration measurements must be interpreted with caution. Brain tissue is composed of different cell types, e.g., there is almost an equal number of neurons and glial cells in the mouse cortex, while in the cerebellum, the glia/neurons ratio is 1 : 6 [90]. Therefore, preparations of intact brain mitochondria obtained by standard homogenization/differential centrifugation techniques of cerebral tissue are most likely represented by fractions of mitochondria from various cell types, and contribution of each fraction depends on the brain area taken for isolation. Since the absolute content of mitochondria and intensity of oxidative phosphorylation in neurons and glial astrocytes are not the same and these cell types are affected differently in I/R, the resulting respiration data is hard to attribute to mitochondria of "neurons only".

In practice, for characterization of oxygen consumption by isolated mitochondria, several combinations of substrates are used to initiate respiration at various entry points. For example, palmitoyl carnitine can be used to reduce quinone, which is a substrate for the membrane ETF:ubiquinone oxidoreductase system of fatty acids oxidation [91]. Glycerol 3-phosphate is a substrate of mitochondrial glycerol 3-phosphate dehydrogenase that passes electrons to the quinone pool which then feeds them to complexes III-IV [92]. The succinate/glutamate combination provides respiration via complexes II-III-IV, while malate and pyruvate or malate and glutamate are used to reduce matrix NAD^+ to support respiration from complex I to complexes III-IV. The effect of I/R on mitochondria, detected as a change in respiration when mitochondria oxidize various substrates, may not solely reflect the effect on respiratory chain enzymes (i.e., complexes I-IV). Mitochondrial respiration is a product of several factors, including membrane integrity and composition, transport of substrates, and activities of NAD^+-dependent dehydrogenases of the TCA cycle, ATP synthase, and eventually respiratory chain complexes. Therefore, the assessment of activity of individual complexes in permeabilized mitochondria or mitochondrial fragments is essential for pinpointing impairments in individual complexes. It can be assumed that at the early stages of reperfusion (up to 3-4 h), the mitochondrial content in the sample does not change significantly [27, 74, 93]. At longer time intervals, however, necrotic or apoptotic degradation of tissue could lead to a decrease in the number of live cells and therefore, the apparent decline of respiratory complexes activ-

ity is due to a decrease in mitochondrial content. Mitochondrial content is usually assessed as specific citrate synthase activity or by the quantification of cytochrome content per milligram of protein in the preparation. Most likely, in some early studies, a drop in respiration after reperfusion-induced recovery could be attributed to the tissue lysis-induced decrease in mitochondrial content, i.e., decrease in the number of live cells in the affected area. It is very likely, that a decline in mitochondrial respiration within 12-56 h of recirculation in the model of complete cerebral ischemia in cats can be explained by tissue degradation as well as the relatively harsh proteolysis used for isolating mitochondrial fractions [94].

Brain I/R and mitochondrial complex I. Early finding showing the direct effect of I/R on mitochondrial respiration supported by the substrates of NAD^+-dependent dehydrogenases (malate/pyruvate or malate/glutamate) can now be interpreted as clear indications of complex I involvement. To the author's knowledge, the first demonstrations of the I/R-induced injury of mitochondrial complex I was carried out using a model of brain ischemia in gerbils who possess the circle of Willis. This is a better model of human global or focal brain ischemia than commonly used rats and mice. Cerebral ischemia was applied via carotid artery occlusion for 30 min and then mitochondria isolated from the fresh tissue at different time intervals following reperfusion were analyzed [95, 96]. Ischemia resulted in the decline of mitochondrial complex I-mediated respiration (malate and pyruvate or malate and glutamate) by ~ 25%, but it recovered gradually during the first 30 min of reperfusion and declined again at 2 h. Analysis of individual respiratory chain components showed that ischemic inhibition of respiration was due to the decline of complex I NADH:ubiquinone reductase activity. Succinate oxidation or citrate synthase activity were not significantly affected. Initial recovery of malate and glutamate mitochondrial respiration upon reperfusion followed by a decline after 4 h was demonstrated later using the model of transient focal ischemia in rats [97]. Results obtained on the models of I/R injury in adult animals are comparable to the data acquired using the neonatal model of hypoxia-ischemia (HI) [27]. Oxygen deprivation decreased malate and glutamate-supported respiration by twofold, however reoxygenation recovered respiration almost fully until 4 h when respiration declined again. Oxidation of succinate was not affected by the I/R [27]. Suppression of mitochondrial activity through the ischemic inhibition of complex I was recently shown by positron emission tomography *in situ* after 3 h of middle cerebral artery occlusion (MCAO) in nonhuman primate brain [98].

While a decrease in the mitochondrial complex I activity has been shown in several brain I/R-related studies, the mechanism of enzyme impairment has not been identified. Therefore, we reexamined the temporal dynamics of enzyme impairment due to I/R in our recent studies using the MCAO model in mice [93] and neonatal model of brain HI [49, 50, 99]. We found that brain I/R caused inhibition of mitochondrial respiration via inactivation of complex I. The time course of complex I dysfunction was either multiphasic in the model of MCAO in adult mice [93] but was biphasic in the neonatal HI model of I/R [99]. In both models, we observed strong inhibition of complex I immediately after ischemia and before reperfusion. This finding is in agreement with previous studies [27, 95, 97]. Rapid and almost full recovery of complex I activity after 10-15 min of reoxygenation was followed by a respiratory decline at times longer than 4 h. We identified at least two novel distinct mechanisms of complex I impairment at different time points. One is the dissociation of FMN [34, 93, 99] and another is A/D conformational change of the enzyme resulting in the exposure of critical cysteine residue [49, 50, 53, 55]. I will discuss them in more detail below.

Flavin release. Using *in vivo* models of neonatal HI [99] and the MCAO model of ischemic stroke in adult mice [93] we found that I/R induced inactivation of complex I. Enzyme activity decline was associated with a loss of complex I flavin mononucleotide (FMN). One molecule of non-covalently bound FMN is attached at the 51 kDa subunit (NDUFV1) of complex I N-module and directly interacts with the substrates NADH or NAD^+ [64, 65, 100]. We identified the mechanism of FMN loss from complex I following brain I/R injury. Accordingly, in our studies [34, 93, 99], tissue ischemia-induced FMN dissociation due to the stimulation of RET through the enzyme. It has been shown in various *in vivo* models that brain ischemia leads to a significant increase in the succinate content in the tissue [18, 20-22]. In our neonatal model of I/R [22], we found a 30-fold increase in the succinate level and it took at least 30 min of reperfusion to bring it back to the basal level. β-Oxidation of fatty acids is strongly inhibited with the onset of ischemia, which leads to rapid accumulation of total unesterified fatty acids and acyl-CoA in the brain [101, 102]. In addition, another RET-supporting substrate, glycerol 3-phosphate, is increased after brain ischemia [103]. Along with succinate, these substrates will be oxidized by ubiquinone during ischemia when tissue oxygen level is very low (but not zero) or after reperfusion, supporting RET-like conditions when electrons from ubiquinol are pushed uphill towards complex I nucleotide-binding site. In the steady-state, this would maintain high reduction of complex I FMN, which induces dissociation of the reduced cofactor [29, 34]. The FMN-deficient enzyme is not able to catalyze physiological NADH oxidation, restricting the efficiency of the respiratory chain and ATP production.

The detrimental effects of FMN dissociation may not be limited to complex I dysfunction only. The release of $FMNH_2$ into the mitochondrial matrix would be strongly prooxidative. Reduced flavin rapidly reacts with

oxygen in the aqueous phase (rate constant, 250 $M^{-1}·s^{-1}$), leading to the generation of superoxide and hydrogen peroxide [104, 105]. The content of complex I in the preparation of mouse brain mitochondria is ~20-50 pmol/mg protein (Stepanova and Galkin, unpublished results) and the degree of complex I inactivation is 25-35% [93, 99]. Therefore, after ischemia-induced release, the concentration of the free flavin in the matrix could reach 15-30 μM. At such a high level, the $FMN/FMNH_2$ couple could be involved in a dismutation reaction producing semi-reduced species [105], as well as serve as a redox mediator between electron donors, such as NADH, and available electron acceptors in the matrix. Production of superoxide and hydrogen peroxide during the fast non-enzymatic reaction of reduced flavin with oxygen immediately after reoxygenation may contribute to the transient burst of ROS generation during the initial phases of reperfusion. The metabolic fate of oxidized free flavin is hard to predict. Some fraction of free FMN is going to bind to the apo-form of complex I, and another fraction might be dephosphorylated to riboflavin or converted to FAD by the ATP-dependent FAD pyrophosphatase [106].

Since the first attempts of complex I purification, it has been recognized that FMN can be easily lost during purification or dialysis of the mitochondrial NADH dehydrogenase preparations [100]. Reduction-induced reversible dissociation of FMN has been shown previously for the fragmented [107] and membrane-bound [108] preparations of mammalian complex I *in vitro* and recently for the bacterial enzyme [109]. Enzyme reduced by NADH quickly loses FMN; exogenously added flavin prevents the dissociation and also reactivates the FMN-deficient enzyme. In our *in vitro* studies, FMN added during incubation of intact brain mitochondria under the RET-like conditions partially prevented complex I inactivation [99]. More importantly, administration of the FMN precursor riboflavin (vitamin B2) before *in vivo* I/R of the neonatal brain decreased the infarct size by half, lessened the neurological deficit, and preserved complex I activity when compared to vehicle-treated mice [99]. Interestingly, a significant fraction of acute stroke patients was deficient of riboflavin immediately post-infarct, but the clinical significance of these findings is not clear [110]. Our results [34, 93, 99] provide a mechanistic explanation behind clinical neuroprotective action of riboflavin supplement for patients with ischemic stroke [111]. Prophylactic administration of riboflavin to pregnant women may be potentially used as a supplement to therapeutic interventions against ischemia-induced injury under pathological conditions of fetal distress.

Thiol oxidation. Multiple studies showed reoxygenation-induced oxidative stress after reperfusion as one of the main contributors to tissue damage in brain I/R injury [112, 113] and that endogenous glutathione is a major component in the mitochondrial maintenance of redox balance. Reduced glutathione, as well as other thiol reductases, prevent or repair oxidation injury induced by oxidative stress in I/R [6, 114]. Both glutathione content and its redox state are strongly affected following I/R, therefore making cysteine residues of mitochondrial proteins a major target of oxidative stress [114, 115]. Various antioxidant-based intervention strategies were proven to be neuroprotective in *in vivo* models [116-118], but the mechanism of their action is not completely understood.

Using the MCAO model of stroke [93], we found that after reoxygenation-induced recovery, however, an additional 30 min of recirculation led to a secondary decrease in the mitochondrial complex I activity. *Ex vivo* treatment of mitochondrial membranes obtained from these samples with reduced glutathione partially recovered complex I activity. Consequently, we hypothesized that the enzyme impairment was likely to occur via oxidative modification of the critical thiol(s) of complex I [93]. We found that 20 min of focal ischemia led to a decrease in the total glutathione content in the affected brain area [93]. The administration of cell-permeable glutathione ethyl ester at the beginning of reoxygenation phase restored glutathione content, prevented the decline of complex I activity, and was associated with smaller infarct size and improved neurological outcome [93]. This is in agreement with previous studies using a rat model of stroke, but the mechanism of neuroprotection was not identified [118].

What residues are involved in the enzyme sensitivity towards ROS? Based on our early studies [41, 47] and the work of Murphy's group [48, 119], it was reasonable to expect that modification of the critical thiol of complex I involved in the A/D conformational change may contribute to the oxidative damage in brain I/R. The lack of oxygen induced the change in the conformation of complex I converting it to the dormant D-form in various *in vivo* studies [45-48, 51]. It should be emphasized, that the D-form is a dormant state of complex I, not inactive or covalently modified enzyme; the D-form can be quickly "reactivated" given access to the substrates [41, 54]. Historically, the term "deactivation" was used to indicate the difference in kinetic behavior between the two forms *in vitro*, but the D-form of the enzyme can be considered *in situ* as "resting" or "dormant". Unless specifically stated, when assessing the complex I activity *ex vivo*, the enzyme was activated in the assay and both forms contributed to the measured enzymatic rate [54].

The A to D conformational change leads to the exposure of Cys39 residue of the ND3 subunit to the outside [40, 55]. Modification of this critical thiol arrests reactivation of the enzyme. Oxidizing agents, such as ROS and peroxynitrite, induced an irreversible modification of the cysteine, while modification by low molecular weight nitrosothiols was reversible [41, 47]. The A-form is not sensitive to the same treatment. Therefore, the sensitivity of mitochondrial complex I to the reperfusion-

induced oxidative stress can be determined by the exposure of the thiol residue involved in the ischemia-induced A/D transition.

Recently, we confirmed the presence of reversible ischemia-induced A/D conformational change of complex I in the brain mitochondria. Combining ischemia and hypoxia in the Rice–Vannucci neonatal brain I/R model resulted in the increase in the D-form content from the basal level of 10 to 35% in rats [49] and from 5 to 40% in mice [50]. Only partial deactivation of the enzyme was found, which is not surprising, due to the inaccuracy in the excision of the ischemic area in the focal ischemia models before the demarcation of infarction in the brain was developed. This is why 15-min global brain ischemia after cardiac arrest in mice increased the fraction of the D-form almost to 90% [120].

Therefore, prior to reperfusion, a significant fraction of the enzyme is in the D-form with the cysteine residue of the ND3 subunit exposed and susceptible to modification. Reperfusion reactivates the D-form into the A-form, but also induces oxidative stress resulting in the irreversible inhibition of the enzyme. We found that the reversible protective modification of the critical cysteine of the D-form via administration of mitochondria-targeted nitrosating agent MitoSNO [48, 119] before reperfusion attenuated oxidative stress and significantly improved neurological recovery in I/R of the neonatal brain [50]. The beneficial action of MitoSNO can be explained via two mechanisms: one is the above-mentioned protection of the critical thiol of the enzyme preventing its irreversible oxidative modification and inactivation of complex I. Another mechanism is a delay of reperfusion-induced D to A transition confining complex I-originated ROS release by the A-form [49, 50].

Based on our recent studies, we propose the following scenario to describe the nature of complex I damage and its role in neuroprotection in I/R (Fig. 2). During normoxia, the enzyme is mostly in the A-form and catalyzes physiological oxidation of NADH by ubiquinone, contributing to the formation of the potential across the membrane. The critical Cys39 of the ND3 subunit is not exposed, and the reduction of the enzyme is not maximal

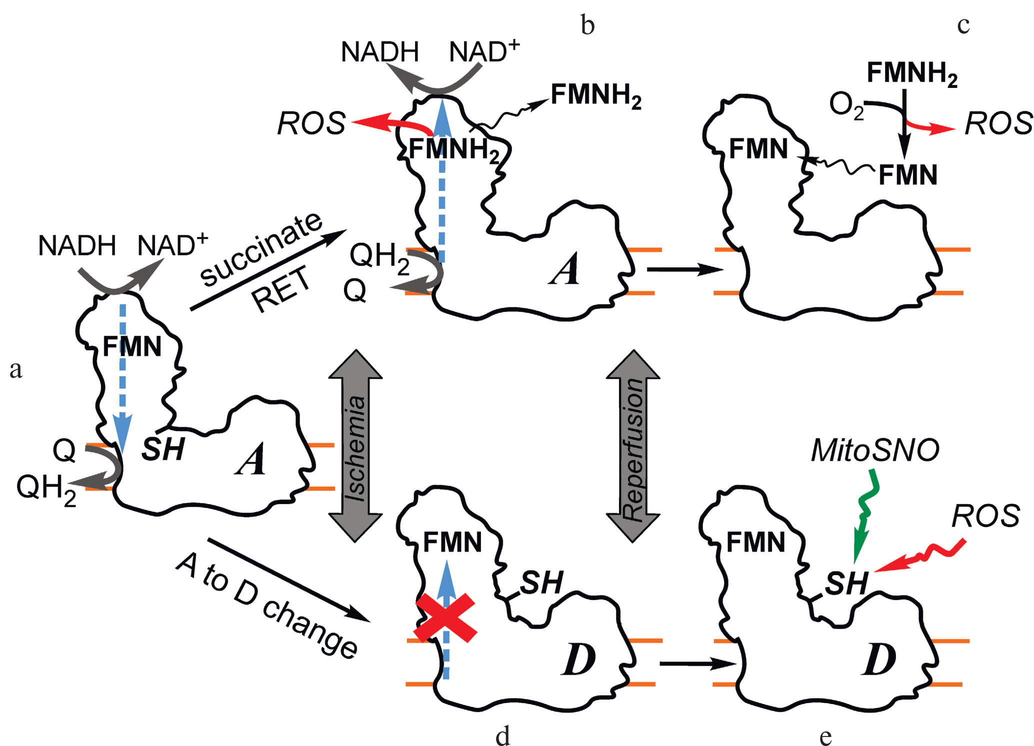

Fig. 2. Schematic diagram illustrating proposed effects of I/R on the mitochondrial complex I. a) When oxygen is present, the enzyme catalyzes a physiological forward reaction of NADH oxidation by ubiquinone (Q) supplying electrons to the rest of the respiratory chain. A large fraction of complex I is in the catalytically active A-form with the critical Cys39 of the ND3 inaccessible. b) Succinate level is increased when oxygen is lacking, and therefore, the active fraction of the active enzyme is able to catalyze reversed reaction (RET), when electrons are transferred from ubiquinol towards flavin (FMN) at the nucleotide-binding site of complex I. RET supports the highest rate of enzymatic ROS generation by complex I. Reduction of flavin ($FMNH_2$) under RET conditions causes its dissociation and inactivation of complex I. c) $FMNH_2$ can nonenzymatically react with molecular oxygen producing ROS and oxidized flavin, which can bind back to complex I. d) Prolonged ischemia results in the conformational change of complex I to the D-form and exposure of the ND3 Cys39 residue to the outside. e) Cys39 is irreversibly modified by ROS during reoxygenation phase but can be protected by transient nitrosation by the mitochondria-targeted S-nitrosothiol MitoSNO.

(Fig. 2a). The lack of oxygen induces accumulation of succinate and glycerol 3-phosphate that can potentially diffuse from the anoxic core and become available for the mitochondria in the more oxygenated penumbra area. Succinate can be oxidized by mitochondria supporting RET-like conditions, which dramatically increases the reduction state of complex I. This may also stimulate the production of ROS from the enzyme; however, the maintenance of the enzyme's flavin in the reduced state induces dissociation of $FMNH_2$ (Fig. 2b). Free cofactor can be auto-oxidized upon reperfusion, generating non-enzymatic ROS, and probably can bind back to the apoenzyme (Fig. 2c). At the same time, the low flux of electrons via the enzyme during tissue ischemia may induce the A to D conformational change of complex I, exposing the critical Cys39 residue of the ND3 subunit (Fig. 2d). Deactivation of complex I blocks the process of RET via the enzyme [35], preventing ROS generation and FMN dissociation. Most likely, the intensity of the reduction-induced FMN-dissociation and the A/D conversion depends on the time and depth of ischemia (i.e., core and penumbra) and cell enzymatic status.

Reperfusion of the ischemic tissue reactivates complex I (D→A transition) within seconds to minutes time interval. Such delayed activation of the enzyme may hinder the burst of respiration from the pool of accumulated NADH or succinate at the early reperfusion stage and may serve as an intrinsic protective mechanism (Fig. 2d). At the same time, reperfusion-induced oxidative stress may cause thiol modification of the D-form of complex I, leading to the enzyme impairment. If MitoSNO is present during reperfusion, some fraction of the D-form becomes nitrosated at the exposed critical Cys39 of the ND3 subunit (Fig. 2e). This temporarily locks complex I in the D-form, decreasing ROS production during early reoxygenation. Moreover, nitrosation protects this critical thiol from irreversible oxidative modification during reperfusion-driven oxidative stress. The S-nitrosated complex I is subsequently reduced by matrix thiol reductases [48], gradually recovering the D-form and eventually being converted to the catalytically competent A-form of the enzyme.

Ultimately, determining whether A/D transition and FMN loss protect brain tissue *in vivo* or contribute to the damage will require further direct experimental testing. Depending on the oxygen availability and length of ischemia, flavin release and A/D transition can be either detrimental or beneficial for the tissue survival after I/R. On one hand, FMN release from complex I and reversible A to D transition lowers production of enzymatic ROS during early stages of reoxygenation. On the other hand, free reduced flavin may be non-enzymatically auto-oxidized, generating a transient burst of ROS while leaving complex I impaired. The same stands for the A/D transition: reversible deactivation delays ROS generation during reoxygenation but makes the enzyme vulnerable to the oxidative damage and may result in the modification of Cys39 residue by ROS or peroxynitrite, leaving the enzyme in the inactive state.

SUMMARY AND PERSPECTIVES

The impairment of complex I during brain ischemia and the following reoxygenation is well-established, although mitochondrial damage in I/R is clearly not caused by a single mechanism. We proposed two distinct mechanisms of complex I impairment: RET-induced dissociation of flavin cofactor and specific modifications of the critical thiols of the enzyme. It is therefore of great importance to characterize cell type specificity of these mechanisms and identify to what extent neuronal and astrocytic mitochondria are affected using established animal models of the brain I/R. The proteomics assessment of post-translational modification of mitochondrial complex I amino acids in the brain after I/R [121] is required in order to identify specific residues involved into the oxidative functional damage of the enzyme. From a more global perspective, examining whether these processes occur in humans during ischemic stroke or neonatal HI encephalopathies is needed to warrant a sustained effort for translational research.

Funding. The work in the author's laboratory was supported by the NIH grant NS-100850 and MRC UK grants G1100051 and MR/L007339/1.

Acknowledgements. I am grateful to Dr. Anna Stepanova and Dr. Vadim Ten for critical reading of the manuscript. I also wish to thank Dr. Vera Grivennikova for the help with the Russian version of this review and valuable discussions. Thanks are due to Nicole Sayles for her help in the preparation of the review.

Conflict of interest. The author declares no conflict of interest in financial or in any other area.

Compliance with ethical norms. This article does not contain any studies with human participants or animals.

REFERENCES

1. Lee, A. C., Kozuki, N., Blencowe, H., Vos, T., Bahalim, A., Darmstadt, G. L., Niermeyer, S., Ellis, M., Robertson, N. J., Cousens, S., and Lawn, J. E. (2013) Intrapartum-related neonatal encephalopathy incidence and impairment at regional and global levels for 2010 with trends from 1990, *Pediatr. Res.*, **74**, 50-72, doi: 10.1038/pr.2013.1206.
2. Roger, V. L., Go, A. S., Lloyd-Jones, D. M., Benjamin, E. J., Berry, J. D., Borden, W. B., Bravata, D. M., Dai, S., Ford, E. S., Fox, C. S., Fullerton, H. J., Gillespie, C., Hailpern, S. M., Heit, J. A., Howard, V. J., Kissela, B. M., Kittner, S. J., Lackland, D. T., Lichtman, J. H., Lisabeth, L. D., Makuc, D. M., Marcus, G. M., Marelli, A., Matchar, D. B., Moy, C. S., Mozaffarian, D., Mussolino,

M. E., Nichol, G., Paynter, N. P., Soliman, E. Z., Sorlie, P. D., Sotoodehnia, N., Turan, T. N., Virani, S. S., Wong, N. D., Woo, D., Turner, M. B., and American Heart Association Statistics Committee and Stroke Statistics Subcommittee (2012) Heart disease and stroke statistics – 2012 update: a report from the American Heart Association, *Circulation*, **125**, e2-e220.

3. Siesjo, B. K., Elmer, E., Janelidze, S., Keep, M., Kristian, T., Ouyang, Y. B., and Uchino, H. (1999) Role and mechanisms of secondary mitochondrial failure, *Acta Neurochir. Suppl.*, **73**, 7-13.
4. Vannucci, R. C., Towfighi, J., and Vannucci, S. J. (2004) Secondary energy failure after cerebral hypoxia-ischemia in the immature rat, *J. Cereb. Blood Flow Metab.*, **24**, 1090-1097.
5. Hertz, L. (2008) Bioenergetics of cerebral ischemia: a cellular perspective, *Neuropharmacology*, **55**, 289-309, doi: 10.1016/j.neuropharm.2008.05.023.
6. Sims, N. R., and Muyderman, H. (2010) Mitochondria, oxidative metabolism and cell death in stroke, *Biochim. Biophys. Acta*, **1802**, 80-91, doi: 10.1016/j.bbadis.2009.09.003.
7. Mracek, T., Drahota, Z., and Houstek, J. (2013) The function and the role of the mitochondrial glycerol-3-phosphate dehydrogenase in mammalian tissues, *Biochim. Biophys. Acta*, **1827**, 401-410, doi: 10.1016/j.bbabio.2012.11.014.
8. Watmough, N. J., and Frerman, F. E. (2010) The electron transfer flavoprotein:ubiquinone oxidoreductases, *Biochim. Biophys. Acta*, **1797**, 1910-1916, doi: 10.1016/j.bbabio.2010.10.007.
9. Galkin, A. S., Grivennikova, V. G., and Vinogradov, A. D. (1999) $H^+/2e^-$ stoichiometry in NADH-quinone reductase reactions catalyzed by bovine heart submitochondrial particles, *FEBS Lett.*, **451**, 157-161.
10. Hirst, J. (2013) Mitochondrial complex I, *Annu. Rev. Biochem.*, **82**, 551-575.
11. Brandt, U. (2006) Energy converting NADH:quinone oxidoreductases, *Annu. Rev. Biochem.*, **75**, 69-92.
12. Burbaev, D. S., Moroz, I. A., Kotlyar, A. B., Sled, V. D., and Vinogradov, A. D. (1989) Ubisemiquinone in the NADH-ubiquinone reductase region of the mitochondrial respiratory chain, *FEBS Lett.*, **254**, 47-51.
13. De Jong, A. M., and Albracht, S. P. (1994) Ubisemiquinones as obligatory intermediates in the electron transfer from NADH to ubiquinone, *Eur. J. Biochem.*, **222**, 975-982.
14. Cabrera-Orefice, A., Yoga, E. G., Wirth, C., Siegmund, K., Zwicker, K., Guerrero-Castillo, S., Zickermann, V., Hunte, C., and Brandt, U. (2018) Locking loop movement in the ubiquinone pocket of complex I disengages the proton pumps, *Nat. Commun.*, **9**, 4500, doi: 10.1038/s41467-018-06955-y.
15. Vinogradov, A. D., Gavrikova, E. V., Grivennikova, V. G., Zharova, T. V., and Zakharova, N. V. (1999) Catalytic properties of mitochondrial NADH-ubiquinone reductase (Complex I), *Biochemistry (Moscow)*, **64**, 136-152.
16. Chance, B., and Hollunger, G. (1960) Energy-linked reduction of mitochondrial pyridine nucleotide, *Nature*, **185**, 666-672.
17. Klingenberg, M., and Slenczka, W. (1959) Pyridine nucleotide in liver mitochondria. An analysis of their redox relationships, *Biochemische Zeitschrift*, **331**, 486-517.
18. Folbergrova, J., Ljunggren, B., Norberg, K., and Siesjo, B. K. (1974) Influence of complete ischemia on glycolytic metabolites, citric acid cycle intermediates, and associated amino acids in the rat cerebral cortex, *Brain Res.*, **80**, 265-279, doi: 0006-8993(74)90690-8.
19. Solberg, R., Enot, D., Deigner, H. P., Koal, T., Scholl-Burgi, S., Saugstad, O. D., and Keller, M. (2010) Metabolomic analyses of plasma reveals new insights into asphyxia and resuscitation in pigs, *PLoS One*, **5**, e9606, doi: 10.1371/journal.pone.0009606.
20. Benzi, G., Arrigoni, E., Marzatico, F., and Villa, R. F. (1979) Influence of some biological pyrimidines on the succinate cycle during and after cerebral ischemia, *Biochem. Pharmacol.*, **28**, 2545-2550.
21. Chouchani, E. T., Pell, V. R., Gaude, E., Aksentijevic, D., Sundier, S. Y., Robb, E. L., Logan, A., Nadtochiy, S. M., Ord, E. N., Smith, A. C., Eyassu, F., Shirley, R., Hu, C. H., Dare, A. J., James, A. M., Rogatti, S., Hartley, R. C., Eaton, S., Costa, A. S., Brookes, P. S., Davidson, S. M., Duchen, M. R., Saeb-Parsy, K., Shattock, M. J., Robinson, A. J., Work, L. M., Frezza, C., Krieg, T., and Murphy, M. P. (2014) Ischemic accumulation of succinate controls reperfusion injury through mitochondrial ROS, *Nature*, **515**, 431-435.
22. Sahni, P. V., Zhang, J., Sosunov, S., Galkin, A., Niatsetskaya, Z., Starkov, A., Brookes, P. S., and Ten, V. S. (2017) Krebs cycle metabolites and preferential succinate oxidation following neonatal hypoxic-ischemic brain injury in mice, *Pediatr. Res.*, **83**, 491-497, doi: 10.1038/pr.2017.277.
23. Hinkle, P. C., Butow, R. A., Racker, E., and Chance, B. (1967) Partial resolution of the enzymes catalyzing oxidative phosphorylation. XV. Reverse electron transfer in the flavin-cytochrome beta region of the respiratory chain of beef heart submitochondrial particles, *J. Biol. Chem.*, **242**, 5169-5173.
24. Turrens, J. F., and Boveris, A. (1980) Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria, *Biochem. J.*, **191**, 421-427.
25. Grivennikova, V. G., and Vinogradov, A. D. (2006) Generation of superoxide by the mitochondrial complex I, *Biochim. Biophys. Acta*, **1757**, 553-561.
26. Pryde, K. R., and Hirst, J. (2011) Superoxide is produced by the reduced flavin in mitochondrial complex I: a single, unified mechanism that applies during both forward and reverse electron transfer, *J. Biol. Chem.*, **286**, 18056-18065.
27. Niatsetskaya, Z. V., Sosunov, S. A., Matsiukevich, D., Utkina-Sosunova, I. V., Ratner, V. I., Starkov, A. A., and Ten, V. S. (2012) The oxygen free radicals originating from mitochondrial complex I contribute to oxidative brain injury following hypoxia-ischemia in neonatal mice, *J. Neurosci.*, **32**, 3235-3244.
28. Quinlan, C. L., Perevoshchikova, I. V., Hey-Mogensen, M., Orr, A. L., and Brand, M. D. (2013) Sites of reactive oxygen species generation by mitochondria oxidizing different substrates, *Redox. Biol.*, **1**, 304-312.
29. Stepanova, A., Konrad, C., Manfredi, G., Springett, R., Ten, V., and Galkin, A. (2018) The dependence of brain mitochondria reactive oxygen species production on oxygen level is linear, except when inhibited by antimycin A, *J. Neurochem.*, **148**, 731-745, doi: 10.1111/jnc.14654.
30. Kudin, A. P., Bimpong-Buta, N. Y., Vielhaber, S., Elger, C. E., and Kunz, W. S. (2004) Characterization of superoxide-producing sites in isolated brain mitochondria, *J. Biol. Chem.*, **279**, 4127-4135.
31. Vinogradov, A. D., and Grivennikova, V. G. (2005) Generation of superoxide-radical by the NADH:ubiquinone

oxidoreductase of heart mitochondria, *Biochemistry (Moscow)*, **70**, 120-127.
32. Galkin, A., and Brandt, U. (2005) Superoxide radical formation by pure complex I (NADH:ubiquinone oxidoreductase) from *Yarrowia lipolytica*, *J. Biol. Chem.*, **280**, 30129-30135.
33. Kussmaul, L., and Hirst, J. (2006) The mechanism of superoxide production by NADH:ubiquinone oxidoreductase (complex I) from bovine heart mitochondria, *Proc. Natl. Acad. Sci. USA*, **103**, 7607-7612.
34. Stepanova, A., Kahl, A., Konrad, C., Ten, V., Starkov, A. S., and Galkin, A. (2017) Reverse electron transfer results in a loss of flavin from mitochondrial complex I: potential mechanism for brain ischemia reperfusion injury, *J. Cereb. Blood Flow Metab.*, **37**, 3649-3658, doi: 10.1177/0271678X17730242.
35. Kotlyar, A. B., and Vinogradov, A. D. (1990) Slow active/inactive transition of the mitochondrial NADH-ubiquinone reductase, *Biochim. Biophys. Acta*, **1019**, 151-158.
36. Maklashina, E. O., Sled, V. D., and Vinogradov, A. D. (1994) Hysteresis behavior of complex I from bovine heart mitochondria: kinetic and thermodynamic parameters of retarded reverse transition from the inactive to active state, *Biochemistry (Moscow)*, **59**, 946-957.
37. Gavrikova, E. V., and Vinogradov, A. D. (1999) Active/deactive state transition of the mitochondrial complex I as revealed by specific sulfhydryl group labeling, *FEBS Lett.*, **455**, 36-40.
38. Grivennikova, V. G., Kapustin, A. N., and Vinogradov, A. D. (2001) Catalytic activity of NADH-ubiquinone oxidoreductase (complex I) in intact mitochondria. evidence for the slow active/inactive transition, *J. Biol. Chem.*, **276**, 9038-9044.
39. Roberts, P. G., and Hirst, J. (2012) The deactive form of respiratory complex I from mammalian mitochondria is a Na^+/H^+ antiporter, *J. Biol. Chem.*, **287**, 34743-34751.
40. Galkin, A., Meyer, B., Wittig, I., Karas, M., Schagger, H., Vinogradov, A., and Brandt, U. (2008) Identification of the mitochondrial ND3 subunit as a structural component involved in the active/deactive enzyme transition of respiratory complex I, *J. Biol. Chem.*, **283**, 20907-20913.
41. Galkin, A., and Moncada, S. (2007) S-nitrosation of mitochondrial complex I depends on its structural conformation, *J. Biol. Chem.*, **282**, 37448-37453.
42. Maklashina, E., Kotlyar, A. B., and Cecchini, G. (2003) Active/de-active transition of respiratory complex I in bacteria, fungi, and animals, *Biochim. Biophys. Acta*, **1606**, 95-103.
43. Matsuzaki, S., and Humphries, K. M. (2015) Selective inhibition of deactivated mitochondrial complex I by biguanides, *Biochemistry*, **54**, 2011-2021.
44. Babot, M., Labarbuta, P., Birch, A., Kee, S., Fuszard, M., Botting, C. H., Wittig, I., Heide, H., and Galkin, A. (2014) ND3, ND1 and 39 kDa subunits are more exposed in the de-active form of bovine mitochondrial complex I, *Biochim. Biophys. Acta*, **1837**, 929-939.
45. Galkin, A., Abramov, A. Y., Frakich, N., Duchen, M. R., and Moncada, S. (2009) Lack of oxygen deactivates mitochondrial complex I: implications for ischemic injury? *J. Biol. Chem.*, **284**, 36055-36061.
46. Maklashina, E., Sher, Y., Zhou, H. Z., Gray, M. O., Karliner, J. S., and Cecchini, G. (2002) Effect of anoxia/reperfusion on the reversible active/de-active transition of NADH-ubiquinone oxidoreductase (complex I) in rat heart, *Biochim. Biophys. Acta*, **1556**, 6-12.
47. Gorenkova, N., Robinson, E., Grieve, D., and Galkin, A. (2013) Conformational change of mitochondrial complex I increases ROS sensitivity during ischaemia, *Antioxid. Redox. Signal.*, **19**, 1459-1468.
48. Chouchani, E. T., Methner, C., Nadtochiy, S. M., Logan, A., Pell, V. R., Ding, S., James, A. M., Cocheme, H. M., Reinhold, J., Lilley, K. S., Partridge, L., Fearnley, I. M., Robinson, A. J., Hartley, R. C., Smith, R. A., Krieg, T., Brookes, P. S., and Murphy, M. P. (2013) Cardioprotection by S-nitrosation of a cysteine switch on mitochondrial complex I, *Nat. Med.*, **19**, 753-759.
49. Stepanova, A., Konrad, C., Guerrero-Castillo, S., Manfredi, G., Vannucci, S., Arnold, S., and Galkin, A. (2019) Deactivation of mitochondrial complex I after hypoxia-ischemia in the immature brain, *J. Cereb. Blood Flow Metab.*, **39**, 1790-1802, doi: 10.1177/0271678X18770331, Epub 2018, Apr 9.
50. Kim, M., Stepanova, A., Niatsetskaya, Z., Sosunov, S., Arndt, S., Murphy, M. P., Galkin, A., and Ten, V. S. (2018) Attenuation of oxidative damage by targeting mitochondrial complex I in neonatal hypoxic-ischemic brain injury, *Free Radic. Biol. Med.*, **124**, 517-524, doi: 10.1016/j.freeradbiomed.2018.06.040.
51. Hernansanz-Agustin, P., Ramos, E., Navarro, E., Parada, E., Sanchez-Lopez, N., Pelaez-Aguado, L., Cabrera-Garcia, J. D., Tello, D., Buendia, I., Marina, A., Egea, J., Lopez, M. G., Bogdanova, A., and Martinez-Ruiz, A. (2017) Mitochondrial complex I deactivation is related to superoxide production in acute hypoxia, *Redox Biol.*, **12**, 1040-1051, doi: 10.1016/j.redox.2017.04.025.
52. Lopez-Fabuel, I., Le Douce, J., Logan, A., James, A. M., Bonvento, G., Murphy, M. P., Almeida, A., and Bolanos, J. P. (2016) Complex I assembly into supercomplexes determines differential mitochondrial ROS production in neurons and astrocytes, *Proc. Natl. Acad. Sci. USA*, **113**, 13063-13068, doi: 10.1073/pnas.1613701113.
53. Vinogradov, A. D., and Grivennikova, V. G. (2001) The mitochondrial complex I: progress in understanding of catalytic properties, *IUBMB Life*, **52**, 129-134.
54. Babot, M., Birch, A., Labarbuta, P., and Galkin, A. (2014) Characterisation of the active/de-active transition of mitochondrial complex I, *Biochim. Biophys. Acta*, **1837**, 1083-1092, doi: 10.1016/j.bbabio.2014.02.018.
55. Drose, S., Stepanova, A., and Galkin, A. (2016) Ischemic A/D transition of mitochondrial complex I and its role in ROS generation, *Biochim. Biophys. Acta*, **1857**, 946-957, doi: 10.1016/j.bbabio.2015.12.013.
56. Kotlyar, A. B., Sled, V. D., and Vinogradov, A. D. (1992) Effect of Ca^{2+} ions on the slow active/inactive transition of the mitochondrial NADH-ubiquinone reductase, *Biochim. Biophys. Acta*, **1098**, 144-150.
57. Babot, M., and Galkin, A. (2013) Molecular mechanism and physiological role of active-deactive transition of mitochondrial complex I, *Biochem. Soc. Trans.*, **41**, 1325-1330.
58. Loskovich, M. V., Grivennikova, V. G., Cecchini, G., and Vinogradov, A. D. (2005) Inhibitory effect of palmitate on the mitochondrial NADH:ubiquinone oxidoreductase (complex I) as related to the active-de-active enzyme transition, *Biochem. J.*, **387**, 677-683.
59. Stepanova, A., Valls, A., and Galkin, A. (2015) Effect of monovalent cations on the kinetics of hypoxic conformational change of mitochondrial complex I, *Biochim.*

Biophys. Acta, **1847**, 1085-1092, doi: 10.1016/j.bbabio. 2015.05.012.

60. Ciano, M., Fuszard, M., Heide, H., Botting, C. H., and Galkin, A. (2013) Conformation-specific crosslinking of mitochondrial complex I, *FEBS Lett.*, **587**, 867-872.
61. Blaza, J. N., Vinothkumar, K. R., and Hirst, J. (2018) Structure of the deactive state of mammalian respiratory complex I, *Structure*, **26**, 312-319e3, doi: 10.1016/j.str.2017.12.014.
62. Parey, K., Brandt, U., Xie, H., Mills, D. J., Siegmund, K., Vonck, J., Kuhlbrandt, W., and Zickermann, V. (2018) Cryo-EM structure of respiratory complex I at work, *Elife*, **7**, doi: 10.7554/eLife.39213.
63. Agip, A. A., Blaza, J. N., Bridges, H. R., Viscomi, C., Rawson, S., Muench, S. P., and Hirst, J. (2018) Cryo-EM structures of complex I from mouse heart mitochondria in two biochemically defined states, *Nat. Struct. Mol. Biol.*, **25**, 548-556, doi: 10.1038/s41594-018-0073-1.
64. Fiedorczuk, K., Letts, J. A., Degliesposti, G., Kaszuba, K., Skehel, M., and Sazanov, L. A. (2016) Atomic structure of the entire mammalian mitochondrial complex I, *Nature*, **537**, 644-648, doi: 10.1038/nature19794.
65. Zhu, J., Vinothkumar, K. R., and Hirst, J. (2016) Structure of mammalian respiratory complex I, *Nature*, **536**, 354-358, doi: 10.1038/nature19095.
66. Zickermann, V., Wirth, C., Nasiri, H., Siegmund, K., Schwalbe, H., Hunte, C., and Brandt, U. (2015) Mechanistic insight from the crystal structure of mitochondrial complex I, *Science*, **347**, 44-49.
67. Koopman, W. J., Willems, P. H., and Smeitink, J. A. (2012) Monogenic mitochondrial disorders, *N. Engl. J. Med.*, **366**, 1132-1141, doi: 10.1056/NEJMra1012478.
68. Stefanatos, R., and Sanz, A. (2011) Mitochondrial complex I: a central regulator of the aging process, *Cell Cycle*, **10**, 1528-1532.
69. Breuer, M. E., Koopman, W. J., Koene, S., Nooteboom, M., Rodenburg, R. J., Willems, P. H., and Smeitink, J. A. (2013) The role of mitochondrial OXPHOS dysfunction in the development of neurologic diseases, *Neurobiol. Dis.*, **51**, 27-34, doi: 10.1016/j.nbd.2012.03.007.
70. Ndubuizu, O., and LaManna, J. C. (2007) Brain tissue oxygen concentration measurements, *Antioxid. Redox. Signal.*, **9**, 1207-1219.
71. Madsen, P. L., Holm, S., Herning, M., and Lassen, N. A. (1993) Average blood flow and oxygen uptake in the human brain during resting wakefulness: a critical appraisal of the Kety-Schmidt technique, *J. Cereb. Blood Flow Metab.*, **13**, 646-655, doi: 10.1038/jcbfm.1993.83.
72. Reneau, D. D., Guilbeau, E. J., and Null, R. E. (1977) Oxygen dynamics in brain, *Microvasc. Res.*, **13**, 337-344.
73. Lowry, O. H., Passonneau, J. V., Hasselberger, F. X., and Schulz, D. W. (1964) Effect of ischemia on known substrates and cofactors of the glycolytic pathway in brain, *J. Biol. Chem.*, **239**, 18-30.
74. Hillered, L., Siesjo, B. K., and Arfors, K. E. (1984) Mitochondrial response to transient forebrain ischemia and recirculation in the rat, *J. Cereb. Blood Flow Metab.*, **4**, 438-446, doi: 10.1038/jcbfm.1984.63.
75. Kristian, T. (2004) Metabolic stages, mitochondria and calcium in hypoxic/ischemic brain damage, *Cell Calcium*, **36**, 221-233, doi: 10.1016/j.ceca.2004.02.016.
76. Sugawara, T., Lewen, A., Noshita, N., Gasche, Y., and Chan, P. H. (2002) Effects of global ischemia duration on neuronal, astroglial, oligodendroglial, and microglial reactions in the vulnerable hippocampal CA1 subregion in rats, *J. Neurotrauma*, **19**, 85-98, doi: 10.1089/089771502753460268.
77. Astrup, J., Siesjo, B. K., and Symon, L. (1981) Thresholds in cerebral ischemia – the ischemic penumbra, *Stroke*, **12**, 723-725.
78. Becker, N. H. (1961) The cytochemistry of anoxic and anoxioischemic encephalopathy in rats. II. Alterations in neuronal mitochondria indentified by diphosphopyridine and triphosphopyridine nucleotide diaphorases, *Am. J. Pathol.*, **38**, 587-597.
79. Def Webster, H., and Ames, A. (1965) Reversible and irreversible changes in the fine structure of nervous tissue during oxygen and glucose deprivation, *J. Cell Biol.*, **26**, 885-909.
80. Zeman, W. (1963) Histochemical and metabolic changes in brain tissue after hypoxaemia, in *Selective Vulnerability of the Brain in Hypoxemia* (Schade, J. P., and McMenemey, W. H., eds.) Blackwell Publishing Co, London, pp. 327-348.
81. Ozawa, K., Seta, K., Araki, H., and Handa, H. (1967) The effect of ischemia on mitochondrial metabolism, *J. Biochem.*, **61**, 512-514.
82. Ozawa, K., Itada, N., Kuno, S., Seta, K., and Handa, H. (1966) Biochemical studies on brain swelling. II. Influence of brain swelling and ischemia on the formation of an endogenous inhibitor in mitochondria, *Folia Psychiatr. Neurol. Jpn.*, **20**, 73-84.
83. Schutz, H., Silverstein, P. R., Vapalahti, M., Bruce, D. A., Mela, L., and Langfitt, T. W. (1973) Brain mitochondrial function after ischemia and hypoxia. I. Ischemia induced by increased intracranial pressure, *Arch. Neurol.*, **29**, 408-416.
84. Ljunggren, B., Schutz, H., and Siesjo, B. K. (1974) Changes in energy state and acid-base parameters of the rat brain during complete compression ischemia, *Brain Res.*, **73**, 277-289, doi: 0006-8993(74)91049-X.
85. Ginsberg, M. D., Mela, L., Wrobel-Kuhl, K., and Reivich, M. (1977) Mitochondrial metabolism following bilateral cerebral ischemia in the gerbil, *Ann. Neurol.*, **1**, 519-527, doi: 10.1002/ana.410010603.
86. Rehncrona, S., Mela, L., and Siesjo, B. K. (1979) Recovery of brain mitochondrial function in the rat after complete and incomplete cerebral ischemia, *Stroke*, **10**, 437-446.
87. Nordstrom, C. H., Rehncrona, S., and Siesjo, B. K. (1978) Effects of phenobarbital in cerebral ischemia. Part II: Restitution of cerebral energy state, as well as of glycolytic metabolites, citric acid cycle intermediates and associated amino acids after pronounced incomplete ischemia, *Stroke*, **9**, 335-343.
88. Sims, N. R., and Pulsinelli, W. A. (1987) Altered mitochondrial respiration in selectively vulnerable brain subregions following transient forebrain ischemia in the rat, *J. Neurochem.*, **49**, 1367-1374.
89. Sims, N. R. (1991) Selective impairment of respiration in mitochondria isolated from brain subregions following transient forebrain ischemia in the rat, *J. Neurochem.*, **56**, 1836-1844.
90. Herculano-Houzel, S., Ribeiro, P., Campos, L., Valotta da Silva, A., Torres, L. B., Catania, K. C., and Kaas, J. H. (2011) Updated neuronal scaling rules for the brains of Glires (rodents/lagomorphs), *Brain Behav. Evol.*, **78**, 302-314, doi: 10.1159/000330825.
91. Panov, A., Orynbayeva, Z., Vavilin, V., and Lyakhovich, V. (2014) Fatty acids in energy metabolism of the central nervous system, *Biomed. Res. Int.*, **2014**, 472459, doi: 10.1155/2014/472459.

92. Tretter, L., Takacs, K., Hegedus, V., and Adam-Vizi, V. (2007) Characteristics of alpha-glycerophosphate-evoked H_2O_2 generation in brain mitochondria, *J. Neurochem.*, **100**, 650-663.
93. Kahl, A., Stepanova, A., Konrad, C., Anderson, C., Manfredi, G., Zhou, P., Iadecola, C., and Galkin, A. (2018) Critical role of flavin and glutathione in complex I-mediated bioenergetic failure in brain ischemia/reperfusion injury, *Stroke*, **49**, 1223-1231, doi: 10.1161/STROKEAHA.117.019687.
94. Linn, F., Paschen, W., Ophoff, B. G., and Hossmann, K. A. (1987) Mitochondrial respiration during recirculation after prolonged ischemia in cat brain, *Exp. Neurol.*, **96**, 321-333.
95. Almeida, A., Allen, K. L., Bates, T. E., and Clark, J. B. (1995) Effect of reperfusion following cerebral ischaemia on the activity of the mitochondrial respiratory chain in the gerbil brain, *J. Neurochem.*, **65**, 1698-1703.
96. Allen, K. L., Almeida, A., Bates, T. E., and Clark, J. B. (1995) Changes of respiratory chain activity in mitochondrial and synaptosomal fractions isolated from the gerbil brain after graded ischaemia, *J. Neurochem.*, **64**, 2222-2229.
97. Yoshimoto, T., Kristian, T., Hu, B., Ouyang, Y. B., and Siesjo, B. K. (2002) Effect of NXY-059 on secondary mitochondrial dysfunction after transient focal ischemia; comparison with cyclosporin A, *Brain Res.*, **932**, 99-109.
98. Tsukada, H., Ohba, H., Nishiyama, S., Kanazawa, M., Kakiuchi, T., and Harada, N. (2014) PET imaging of ischemia-induced impairment of mitochondrial complex I function in monkey brain, *J. Cereb. Blood Flow Metab.*, **34**, 708-714, doi: 10.1038/jcbfm.2014.5.
99. Stepanova, A., Sosunov, S., Niatsetskaya, Z., Konrad, C., Starkov, A. A., Manfredi, G., Wittig, I., Ten, V., and Galkin, A. (2019) Redox-dependent loss of flavin by mitochondrial complex I in brain ischemia/reperfusion injury, *Antioxid. Redox Signal.*, doi: 10.1089/ars.2018.7693.
100. Rao, N. A., Felton, S. P., Huennekens, F. M., and Mackler, B. (1963) Flavin mononucleotide: the coenzyme of reduced diphosphopyridine nucleotide dehydrogenase, *J. Biol. Chem.*, **238**, 449-455.
101. Yoshida, S., Abe, K., Busto, R., Watson, B. D., Kogure, K., and Ginsberg, M. D. (1982) Influence of transient ischemia on lipid-soluble antioxidants, free fatty acids and energy metabolites in rat brain, *Brain Res.*, **245**, 307-316.
102. Deutsch, J., Kalderon, B., Purdon, A. D., and Rapoport, S. I. (2000) Evaluation of brain long-chain acylcarnitines during cerebral ischemia, *Lipids*, **35**, 693-696.
103. Nguyen, N. H., Gonzalez, S. V., and Hassel, B. (2007) Formation of glycerol from glucose in rat brain and cultured brain cells. Augmentation with kainate or ischemia, *J. Neurochem.*, **101**, 1694-1700, doi: 10.1111/j.1471-4159.2006.04433.x.
104. Massey, V. (1994) Activation of molecular oxygen by flavins and flavoproteins, *J. Biol. Chem.*, **269**, 22459-22462.
105. Gibson, Q. H., Massey, V., and Atherton, N. M. (1962) The nature of compounds present in mixtures of oxidized and reduced flavin mononucleotides, *Biochem. J.*, **85**, 369-383.
106. Barile, M., Brizio, C., Valenti, D., De Virgilio, C., and Passarella, S. (2000) The riboflavin/FAD cycle in rat liver mitochondria, *Eur. J. Biochem.*, **267**, 4888-4900.
107. Sled, V. D., and Vinogradov, A. D. (1993) Reductive inactivation of the mitochondrial three subunit NADH dehydrogenase, *Biochim. Biophys. Acta*, **1143**, 199-203.
108. Gostimskaya, I. S., Grivennikova, V. G., Cecchini, G., and Vinogradov, A. D. (2007) Reversible dissociation of flavin mononucleotide from the mammalian membrane-bound NADH:ubiquinone oxidoreductase (complex I), *FEBS Lett.*, **581**, 5803-5806.
109. Holt, P. J., Efremov, R. G., Nakamaru-Ogiso, E., and Sazanov, L. A. (2016) Reversible FMN dissociation from *Escherichia coli* respiratory complex I, *Biochim. Biophys. Acta*, **1857**, 1777-1785, doi: 10.1016/j.bbabio.2016.08.008.
110. Gariballa, S., and Ullegaddi, R. (2007) Riboflavin status in acute ischaemic stroke, *Eur. J. Clin. Nutr.*, **61**, 1237-1240, doi: 10.1038/sj.ejcn.1602666.
111. Da Silva-Candal, A., Perez-Diaz, A., Santamaria, M., Correa-Paz, C., Rodriguez-Yanez, M., Arda, A., Perez-Mato, M., Iglesias-Rey, R., Brea, J., Azuaje, J., Sotelo, E., Sobrino, T., Loza, M. I., Castillo, J., and Campos, F. (2018) Clinical validation of blood/brain glutamate grabbing in acute ischemic stroke, *Ann. Neurol.*, **84**, 260-273, doi: 10.1002/ana.25286.
112. Kalogeris, T., Bao, Y., and Korthuis, R. J. (2014) Mitochondrial reactive oxygen species: a double edged sword in ischemia/reperfusion vs preconditioning, *Redox Biol.*, **2**, 702-714, doi: 10.1016/j.redox.2014.05.006.
113. Cao, W., Carney, J. M., Duchon, A., Floyd, R. A., and Chevion, M. (1988) Oxygen free radical involvement in ischemia and reperfusion injury to brain, *Neurosci. Lett.*, **88**, 233-238.
114. Anderson, M. F., and Sims, N. R. (2002) The effects of focal ischemia and reperfusion on the glutathione content of mitochondria from rat brain subregions, *J. Neurochem.*, **81**, 541-549.
115. Mizui, T., Kinouchi, H., and Chan, P. H. (1992) Depletion of brain glutathione by buthionine sulfoximine enhances cerebral ischemic injury in rats, *Am. J. Physiol.*, **262**, H313-317.
116. Folbergrova, J., Zhao, Q., Katsura, K., and Siesjo, B. K. (1995) N-tert-Butyl-alpha-phenylnitrone improves recovery of brain energy state in rats following transient focal ischemia, *Proc. Natl. Acad. Sci. USA*, **92**, 5057-5061.
117. Khan, M., Sekhon, B., Jatana, M., Giri, S., Gilg, A. G., Sekhon, C., Singh, I., and Singh, A. K. (2004) Administration of N-acetylcysteine after focal cerebral ischemia protects brain and reduces inflammation in a rat model of experimental stroke, *J. Neurosci. Res.*, **76**, 519-527, doi: 10.1002/jnr.20087.
118. Anderson, M. F., Nilsson, M., Eriksson, P. S., and Sims, N. R. (2004) Glutathione monoethyl ester provides neuroprotection in a rat model of stroke, *Neurosci. Lett.*, **354**, 163-165.
119. Prime, T. A., Blaikie, F. H., Evans, C., Nadtochiy, S. M., James, A. M., Dahm, C. C., Vitturi, D. A., Patel, R. P., Hiley, C. R., Abakumova, I., Requejo, R., Chouchani, E. T., Hurd, T. R., Garvey, J. F., Taylor, C. T., Brookes, P. S., Smith, R. A., and Murphy, M. P. (2009) A mitochondria-targeted S-nitrosothiol modulates respiration, nitrosates thiols, and protects against ischemia-reperfusion injury, *Proc. Natl. Acad. Sci. USA*, **106**, 10764-10769.
120. Stepanova, A., Shurubor, Y., Valsecchi, F., Manfredi, G., and Galkin, A. (2016) Differential susceptibility of mitochondrial complex II to inhibition by oxaloacetate in brain and heart, *Biochim. Biophys. Acta*, **1857**, 1561-1568, doi: 10.1016/j.bbabio.2016.06.002.
121. Kang, P. T., Chen, C. L., Lin, P., Zhang, L., Zweier, J. L., and Chen, Y. R. (2018) Mitochondrial complex I in the post-ischemic heart: reperfusion-mediated oxidative injury and protein cysteine sulfonation, *J. Mol. Cell. Cardiol.*, **121**, 190-204, doi: 10.1016/j.yjmcc.2018.07.244.