Reverse electron transfer results in a loss of flavin from mitochondrial Complex I. Potential mechanism for brain ischemia reperfusion injury

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Abstract

Ischemic stroke is one of the most prevalent sources of disability in the world. The major brain tissue damage takes place upon the reperfusion of ischemic tissue. Energy failure due to alterations in mitochondrial metabolism and elevated production of reactive oxygen species (ROS) is one of the main causes of brain ischemia-reperfusion (IR) damage. Ischemia resulted in the accumulation of succinate in tissues, which favors the process of reverse electron transfer (RET) when a fraction of electrons derived from succinate is directed to mitochondrial complex I for the reduction of matrix NAD^+.

We demonstrate that in intact brain mitochondria oxidizing succinate, complex I became damaged and was not able to contribute to the physiological respiration. This process is associated with a decline in ROS release and a dissociation of the enzyme's flavin. This previously undescribed phenomenon represents the major molecular mechanism of injury in stroke and induction of oxidative stress after reperfusion. We also demonstrate that the origin of ROS during RET is flavin of mitochondrial complex I. Our study highlights a novel target for neuroprotection against IR brain injury and provides a sensitive biochemical marker for this process.

Key words: flavin, ischemia; mitochondria; stroke; succinate

Introduction

Ischemic stroke occurs as a result of a blockage of blood flow to the brain. It is one of the most prevalent sources of disability in modern times, almost pandemics in developed countries, with the number of people affected ~1,184 per 100,000 population [1]. It is a consensus that brain tissue damage occurs upon reperfusion of ischemic tissue. Mitochondrial bioenergetics failure and elevated production of reactive oxygen species (ROS) are considered among the major causes of brain ischemia-reperfusion (IR) tissue damage. The mitochondrial IR-induced damage is usually delayed; after seemingly complete recovery of aerobic ATP production in the first phase of reperfusion, mitochondria function fails later on, a process is termed "secondary energy failure". The molecular mechanisms of this secondary failure are not understood (reviewed in [2]). Several putative mechanisms had been suggested for IR-induced brain mitochondria damage [3-5], including a decline in mitochondrial Complex I function caused by IR [6, 7]. However, the reasons for Complex I failure are also not known.

Mitochondrial Complex I oxidizes tricarboxylic acid cycle (TCA)-produced NADH by ubiquinone in the inner mitochondrial membrane. Electrons from NADH are accepted by non-covalently bound flavin (FMN) and further transferred to ubiquinone in the inner mitochondrial membrane [8, 9], generating the protonmotive force [10] which fuels the production of ATP by mitochondrial ATPase. The electron transfer in Complex I can be reversed so the enzyme can catalyze the reduction of matrix NAD^+ by the ubiquinol at the expense of mitochondrial membrane potential [11, 12]. This reaction is termed "reverse electron transfer" (RET). Despite the fact of that more than 55 years since the first observation of RET in the intact mitochondria have passed [11, 13] not much is known about regulation and significance of this process *in situ*. However, it is known that the minimal requirements for this reaction to occur are the presence of the membrane potential and a high level of ubiquinone reduction, which in the brain can be provided by succinate oxidation. The accumulation of succinate well above its physiological levels is a common marker of brain ischemia. In rat brain, 5 min of ischemia results in ~300% increase in succinate concentration, to the millimolar range [14, 15]. Upon reperfusion, succinate in post-ischemic brain returns to the control levels only after 30-40 min [16]. Succinate had also been shown to inhibit the oxidation of pyruvate and other NAD-linked respiratory substrates and cause an over-reduction of mitochondrial pyridine nucleotides [17]. Succinate had been proposed lately to be the primary oxidized substrate in brain homogenates of post-ischemic neonatal mouse brains (*Dr. Vadim Ten's lab, submitted*).

Recently, succinate-driven RET emerged as a potential area of great physiological importance [18-21]. In this manuscript, we demonstrate that in intact brain mitochondria under conditions of RET, prolonged succinate oxidation damages Complex I by inducing a dissociation of the enzyme's flavin. This previously undescribed phenomenon may represent the major molecular mechanism of IR-induced secondary energy failure in stroke. Our data also demonstrate that the origin of ROS during RET is flavin of mitochondrial Complex I.

Materials and Methods.

Intact mitochondria isolation for in vitro studies

The animal protocol was approved by the Institutional Animal Care and Use Committee of Weill Cornell Medicine. All experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health and the ARRIVE guidelines. To isolate intact mitochondria a modified protocol combining differential centrifugation and digitonin treatment was used [22]. The forebrain hemispheres of C57BL/6 8-10 weeks male mice were excised and immediately immersed into ice-cold isolation medium (225 mM mannitol, 75 mM sucrose, 20 mM HEPES-Tris, 1mM EGTA, pH 7.4) supplemented with 1 mg/ml BSA. Tissue was homogenized with 40 strokes by pestle "B" (tight) of a Dounce homogenizer in 10 ml of the isolation medium, diluted two-fold and transferred into centrifuge tubes. The homogenate was centrifuged at 5,900 g for 4 min in a refrigerated (+4°C) Beckman centrifuge. The supernatant was centrifuged at 12,000g for 10 min, and the pellets were resuspended in the same buffer and 0.02% digitonin was added. The suspension was homogenized briefly with five strokes in a loosely fitted Potter homogenizer and centrifuged again at 12,000g for 10 min, then gently resuspended in the isolation buffer without BSA and washed once by centrifuging at 12,000g for 10 min. The final mitochondrial pellet was resuspended in 0.1 ml of washing buffer and stored on ice.

Accessing respiration and ROS production in intact mitochondria.

Mitochondrial respiration and extramitochondrial release of H_2O_2 were measured using a high-resolution respirometer (Oroboros) equipped with two-channel fluorescence optical setup to monitor simultaneously oxygen level and fluorescence in 2 ml of mitochondrial suspension. For H_2O_2 measurements fluorescence was recorded using a green 525 nm LED as an excitation light source, a photodiode (S2386-8K, Hamamatsu Photonics, Japan) as a sensor, and an emission filter with cut off 580 nm (#34, Rosco Laboratories, USA), fixed in a 3D printed mount (Form2, Formlabs, USA). The photodiode was connected to the amperometric port of the Oxygraph and the fluorescence was recorded using the Datlab software. The calibration of Amplex UltraRed response was performed at the end of the run by adding several 93 pmol/ml aliquots of freshly made H_2O_2 to the chamber containing all the components of the assay.

Mitochondria (0.14-0.18 mg of protein) were added to 2 ml respiration buffer composed of 125 mM KCl, 0.2 mM EGTA, 20 mM HEPES-Tris, 4 mM KH_2PO_4, pH 7.4, 2 mM $MgCl_2$, 2 mg/ml BSA, 10μM Amplex UltraRed (Invitrogen) and 4 U/ml horseradish peroxidase at 37°C. The following substrates were used: 2 mM malate and 5 mM pyruvate or 5 mM succinate and 1 mM glutamate. To initiate State III respiration 200 μM ADP was added to the mitochondrial suspension. Respiration was fully sensitive (inhibited) by 1 mM cyanide or 1 μM antimycin A.

To enable us to rapidly change oxygen concentration in the mitochondrial suspension we have included a gas headspace (1 ml) above the measurement chamber. The headspace could be continuously purged with humidified gaseous nitrogen through the stopper at a rate of 10-60 ml/min. By varying the partial pressure of gaseous nitrogen in the headspace we are able to control its concentration in the liquid sample via N_2 exchange between the two phases. When required, the gas flow was turned off, and the stoppers moved to seal the oxygen chamber so that no headspace remained.

Complex I activity measurements in disrupted mitochondria

The NADH:HAR oxidoreductase activity of Complex I [23] was determined spectrophotometrically using a plate reader (SpectraMax M5, Molecular Devices, USA). Oxidation of 150 μM NADH (ε_{340nm} = 6.22 $mM^{-1}\times cm^{-1}$) by 1mM HAR was followed at 340 nm in 0.2 ml of standard respiration buffer (pH=7.5) supplemented with 25 μg/ml alamethicin (to disrupt mitochondria), 2 mM $MgCl_2$, 1mM cyanide and 0.01-0.025 mg protein/ml mitochondria.

Flavin fluorescence measurements

The flavin fluorescence in mitochondria oxidizing succinate was measured by Hitachi 7000 spectrofluorimeter set at 450 nm excitation and 525 nm emission. The reaction was carried out under exactly the same conditions as the respiratory assays, except that Amplex UltraRed and horseradish peroxidase were omitted from the medium. Special controls for photobleaching of the preparation during prolonged measurements were performed.

Mitochonfrial membrane potential measurements

Mitochondrial membrane potential was asessed indirectly using membrane-permeable fluorescent safranin O probe (2 μM), excitation 525 nm, emission 580 nm essentially as described previously [24].

All chemicals were purchased from Sigma unless stated otherwise. Protein content was determined by BCA assay with deoxycholate (0.1%) for solubilizing the mitochondrial membranes (ThermoFisher, USA). All activities were measured at 37°C except NADH:HAR (25°C). All results are expressed as means ± SEM. Six different preparations of mitochondria were tested. When comparing different groups, a two-sample t-test was used. Other experimental details are described in the legends to the figures.

Results.

In order to assess the oxygen dependence of the hydrogen peroxide emission from intact brain mitochondria first we carried out experiments in the closed respirometer chamber. Fig. 1A shows representative traces of oxygen consumption and hydrogen peroxide release by intact brain mitochondria oxidizing NAD-dependent substrates, or succinate. Table 1 shows quantitative characteristics of the respiration and H_2O_2 emission. The rate of H_2O_2 emission upon coupled succinate oxidation was around tenfold higher than in the presence of NAD-dependent substrates. As expected, the succinate-supported H_2O_2 production was suppressed by either adding ADP or an uncoupler, as well as by rotenone, confirming that ROS generation was fueled by RET. It should be noted that the addition of succinate rapidly induces reduction of the entire pool of matrix NAD(P) [11]. Therefore, in the steady-state conditions of the succinate oxidase reaction *in vitro*, there is no RET as a classical reduction of matrix nucleotides showed by Britton Chance [11]. In the present paper, we use term RET-like conditions describing the process of oxidation of succinate by a fully competent respiratory chain of intact mitochondria in steady-state.

We estimated the effect of the oxygen concentration on H_2O_2 release during the oxidation of succinate by purging the headspace in our system with nitrogen to quickly reduce the partial pressure of O_2 in the solution. RET-associated H_2O_2 release during succinate oxidation was almost linearly dependent on the oxygen concentration in the medium (Fig. 1B).

As shown in Fig. 2A, during the time course of succinate oxidase reaction in the closed chamber when the oxygen gradually decreases due to the respiratory activity, the rate of H_2O_2 release also declines. We would like to emphasize that the resting respiration rate during the oxidation of succinate stayed almost constant at the wide range of oxygen concentration and dropped sharply only at 20-25 μM [O_2]. In order to evaluate the rate of H_2O_2 emission from mitochondria at constant concentration of oxygen (around 190 μM [O_2]) we carried our similar experiments in the open respirometer chamber. Unexpectedly, we also observed a decline of H_2O_2 release rates during RET in the open chamber (Fig. 2D), while the oxygen concentration in the medium did not change (Fig. 2B).

To further investigate this phenomenon, we tested the effect of amount of mitochondria in the medium to the observed decline of the H_2O_2 emission rate. Representative traces of the H_2O_2 release rate decline in time at different concentrations of mitochondrial protein in the assay are shown in Fig. 3A. The half-time of this decline was around 4.5 min (Fig. 3B) and was not dependent on the concentration of mitochondria in the range of 0.1-0.5 mg of protein/ml. Therefore, the decline was not due to the accumulation of any reaction product in the medium. The inhibitory action of accumulated H_2O_2 under our conditions is not likely, because, upon oxidizing NAD-linked substrates, there is virtually no accumulation of H_2O_2 in the suspension of mitochondria, whereas, during RET-catalyzed H_2O_2 generation, the accumulation is minimal peaking at about 30 nM of H_2O_2 [25]. In agreement with that, preincubating intact mitochondria with 45-90 μM H_2O_2 for 5-10 min before the addition of substrates did not change the parameters of the H_2O_2 rates decline. The addition of 50 U/ml MnSOD to the medium did not change the dynamics of H_2O_2 emission decline (data not presented). Therefore, it is unlikely that in our conditions, ROS

produced during RET had pronounced damaging effect on mitochondrial enzymes involved in RET. We found that the only condition that decreased the rate of decline was temperature. At 25°C in the open chamber, the half-time of the H_2O_2 release rate decline was increased to 10-12 min (not shown).

Several factors directly affects the rate of H_2O_2 release during coupled oxidation of succinate: absolute activitiy of respiratory chain enzymes and the magnitude of membrane potential across the inner mitochondrial membrane. Therefore, first we tested the dependence of H_2O_2 release rate during RET on the potential dependent fluorescence of safranin O [26]. As shown in Fig. 3C, no significant changes in the dye fluorescence was observed during RET, indicating that membrane potential was mainly preserved during this time interval changing only by less than 8%.

Potentially, the rate of H_2O_2 release during RET also depends on the activity of complexes I and II. Therefore, we have assessed the respiratory activities of mitochondria before and after incubation under RET conditions. As shown in Fig. 4A, succinate oxidase activity in both resting and ADP-stimulated state was unaffected by 20 min incubation. Contrary to that, ADP-stimulated respiration on malate and pyruvate was decreased by around 75% (Fig. 4B). This strongly indicates that prolonged RET affected the NADH:ubiquinone reductase activity of complex I. Consequently, it is highly likely that an impairment of complex I function was responsible for the decline in H_2O_2 emission rate during the prolonged oxidation of succinate (Fig. 3A).

The loss of NADH:ubiquinone reductase activity of mitochondrial complex I can be caused by several factors. To elucidate the potential mechanism, we have assessed the changes in the activity of complex I during the time course of the decline with an artificial electron acceptor HAR (NADH:HAR reductase) spectrophotometrically in permeabilized mitochondria (Fig. 5). We found that NADH:HAR activity decreased in parallel with the rate of H_2O_2 release by intact mitochondria. Moreover, the addition of 10μM exogenous FMN had resulted in a partial recovery of NADH:HAR activity. These data unequivocally indicate that the decline in the rate of H_2O_2 generation was due to the loss of complex I flavin caused by prolonged succinate oxidation under RET conditions.

To further support this conclusion, we have assessed flavin fluorescence in intact mitochondria exactly under the same conditions as those employed to measure H_2O_2 emission (Fig. 6A). A fast decline of the fluorescence intensity after additions of substrates indicating a reduction of flavin was observed for the first 2 min. The decline was followed by a slow increase in fluorescence. This could be caused by either an increase in flavin's fluorescence intensity upon its dissociation from the protein or by a fast autoxidation of the reduced flavin in the matrix. The dynamics of the flavin fluorescence intensity (Fig. 6A) followed exactly the inverted pattern of the decline in H_2O_2 emission rate (Fig. 6B). This indicates that in steady-state conditions, the rate of H_2O_2 emission upon RET was directly proportional to the amount of Complex I-bound flavin.

Discussion

In this study, we have shown that prolonged oxidation of succinate by brain mitochondria under RET conditions stimulates dissociation of flavin from mitochondrial Complex I and results in the loss of activity of the enzyme. In turn, this impairs the oxidation of NAD-linked (physiological) substrates as well as the generation of ROS by mitochondria.

The process of RET, first observed more than half a century ago [11, 13] has recently gained significant attention in ischemia/reperfusion studies. First, after a period of oxygen deprivation succinate level is elevated in highly metabolizing tissues such as the brain [14, 15, 27] and heart [18, 28]. Potentially, given the availability of substrate and energization of the inner mitochondrial membrane, RET can take place after oxygen is resupplied to the tissue. Second, succinate-dependent respiration is characterized by the highest rates of ROS production by mitochondria [18, 19, 29-32]. In our study, RET also provided the greatest rate in H_2O_2 release equivalent to around 3.5% of total oxygen consumption, which is very close to what observed for the mitochondria from adult rat brain [26]. Therefore, in postischemic tissue, RET is active and may render injury to the tissue found in several models of tissue ischemia [18, 33].

Mitochondrial complex I is the most sensitive respiratory enzyme to ischemia in the brain [6, 7, 19, 34]. The mechanism of complex I damage during this process is not fully understood. Surprisingly, incomplete ischemia results in greater complex I damage in a rat model of brain oxygen deprivation [35]. Most likely, succinate accumulated in the ischemic core may diffuse to the surrounding tissues and have devastating consequences to the penumbra area, where both substrates (oxygen and succinate) are available. This may explain greater decrease on mitochondrial complex I damage in the models of incomplete brain ischemia.

In our initial experiments, we observed a linear dependence of H_2O_2 release by intact brain mitochondria during RET which is in accordance with the earlier studies on the intact brain *in vivo* [36]. Superoxide anion generation by submitochondrial particles oxidizing succinate [37] also showed a linear dependence of H_2O_2 production upon oxygen concentration. This is in apparent disagreement with recent studies of liver mitochondria [38] and may be due to the different enzymatic composition of the preparations from different tissues.

The unexpected observation in our further study is the gradual decline of H_2O_2 release rate by intact brain mitochondria during oxidation of succinate at constant oxygen level. The rate of decline is not dependent on the concentration of mitochondria (0.1-0.5 mg of protein/ml) and half-time of the process is around 4.5 min. We assessed activities of complexes I and II before and after oxidation of succinate and found that only complex I activity is strongly diminished after mitochondria were exposed to RET-like conditions. We found that this decline is due to the dissociation of the flavin from mitochondrial complex I since NADH:HAR reductase activity of the enzyme declines in simultaneously with ROS production. Artificial acceptor HAR takes electrons directly from the flavin of complex I at the hydrophilic domain and this process does not involve downstream components of the enzyme such as iron-sulfur clusters and ubiquinone-binding site [23, 39]. Moreover, supplementation of exogenous

FMN to the assay medium partially recovered NADH:HAR reductase activity of enzyme supporting the critical role of flavin in the process of RET-induced inactivation.

Under RET conditions, electrons from succinate are transferred to ubiquinone and then directed to the oxygen via downstream complex III and cytochrome c oxidase and partially upstream to complex I. The above process provides respiratory activity and builds up a potential across the mitochondrial membrane, and the latter one is likely to be responsible for the generation of ROS upstream of ubiquinone binding site of complex I. In the steady state, coupled succinate oxidation would maintain high NAD(P)H/NAD(P)$^+$ ratio in the matrix and increases reduction of the tightly-bound FMN of complex I. Non-covalently bound FMN resides on the bottom of a deep cavity of the catalytic subunit NDUFV1 [8] and is accessible to oxygen, therefore, reduced flavin favors ROS formation. FMN has been proposed to be the main site of ROS-production in complex I in several studies [40-44]. However, the FeS clusters of Complex I [45, 46] and semiquinone [47, 48] had also been discussed as the source of ROS origin (see [49] for review). Our experiments strongly support the idea that it is exactly FMN and not the FeS clusters, is the major site of H_2O_2 generation in intact brain mitochondria in conditions of RET during succinate oxidation.

Another important finding of our studies is that FMN quickly dissociates from the enzyme in intact brain mitochondria when it is reduced in the RET-like conditions. Reductive dissociation of FMN from the three-subunit FP-fragment of complex I [50], as well as from the intact bovine and isolated prokaryotic enzyme [51, 52] have been demonstrated *in vitro*. The conditions required for dissociation of FMN in these studies (high pH or the presence of the detergents) are hardly related to any conceivable physiological situation. The significant change in angle of a butterfly-like structure of the isoalloxazine ring [53] and a general loosening of the complex I molecule structure upon reduction [54] may contribute to the strong change in FMN affinity to its binding site. It should be stressed, that release of complex I reduced flavin in the mitochondrial matrix may result not only in complex I impairment, but also may lay at the basis of oxidative stress after IR. Fully reduced flavin reacts avidly (within seconds) with oxygen, and H_2O_2 is the predominant product [55]. After the release, the reduced flavin can be readily oxidized by molecular oxygen resulting in an increase of local ROS generation in the matrix. Oxidized FMN molecule can either be metabolized or reinserted back to complex I. At present, the process of non-covalent inclusion of FMN to complex I and the general mechanisms by which mitochondria can provide their flavin cofactors remains unsolved[56].

Our observation suggests that the main cause of decrease of complex I activity after ischemia is the reversible release of flavin from the enzyme. This is first direct evidence of the mechanism of complex I damage during oxidation of succinate in postischemia-like conditions. Our in vitro findings were corroborated by in vivo data from transient cerebral ischemia by middle cerebral artery occlusion where 35 min ischemia significantly decreased the content of mitochondrial membrane non-covalently bound FMN (Kahl et al., 2017, J.Neurosci., under revision). To our knowledge, the only report describing the loss of mitochondrial flavin during cardiac ischemia in dogs was published more than 35 years ago [57]. Also of note, a high proportion of acute stroke patients manifested riboflavin

deficiency following reperfusion, however, the clinical significance of these findings is not yet known [58]. Further studies are expected to shed light on the mechanism of complex I inactivation in brain ischemia and is currently underway in our laboratory.

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Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' contributions

Anna Stepanova – Acquisition of data, analysis, and interpretation of data, critical revision of the article; Anja Kahl – in vivo studies and data analysis; Csaba Konrad – fluorescent module setting up and programming; Anatoly Starkov – acquisition and analysis of data, critical revision of the article; Vadim Ten – and analysis of data, critical revision of the article; Alexander Galkin – supervisor, conception, acquisition of data, analysis, and interpretation of data, drafting the article, critical revision of the article, final approval.

References

1. Thrift AG, Thayabaranathan T, Howard G, Howard VJ, Rothwell PM, Feigin VL *et al.* Global stroke statistics. *Int J Stroke* 2017; 12(1)**:** 13-32.

2. Kristian T. Metabolic stages, mitochondria and calcium in hypoxic/ischemic brain damage. *Cell Calcium* 2004; 36(3-4)**:** 221-33.

3. Anderson MF, Sims NR. Mitochondrial respiratory function and cell death in focal cerebral ischemia. *J Neurochem* 1999; 73(3)**:** 1189-99.

4. Nakai A, Kuroda S, Kristian T, Siesjo BK. The immunosuppressant drug FK506 ameliorates secondary mitochondrial dysfunction following transient focal cerebral ischemia in the rat. *Neurobiol Dis* 1997; 4(3-4)**:** 288-300.

5. Sims NR, Pulsinelli WA. Altered mitochondrial respiration in selectively vulnerable brain subregions following transient forebrain ischemia in the rat. *J Neurochem* 1987; 49(5)**:** 1367-74.

6. Allen KL, Almeida A, Bates TE, Clark JB. Changes of respiratory chain activity in mitochondrial and synaptosomal fractions isolated from the gerbil brain after graded ischaemia. *J Neurochem.* 1995; 64(5)**:** 2222-9.

7. Canevari L, Kuroda S, Bates TE, Clark JB, Siesjo BK. Activity of mitochondrial respiratory chain enzymes after transient focal ischemia in the rat. *J Cereb Blood Flow Metab* 1997; 17(11)**:** 1166-9.

8. Fiedorczuk K, Letts JA, Degliesposti G, Kaszuba K, Skehel M, Sazanov LA. Atomic structure of the entire mammalian mitochondrial complex I. *Nature* 2016; 537(7622)**:** 644-648.

9. Zhu J, Vinothkumar KR, Hirst J. Structure of mammalian respiratory complex I. *Nature* 2016; 536(7616)**:** 354-8.

10. Galkin AS, Grivennikova VG, Vinogradov AD. $H^+/2e^-$ stoichiometry in NADH-quinone reductase reactions catalyzed by bovine heart submitochondrial particles. *FEBS Lett.* 1999; 451(2)**:** 157-161.

11. Chance B, Hollunger G. Energy-Linked Reduction of Mitochondrial Pyridine Nucleotide. *Nature* 1960; 185(4714)**:** 666-672.

12. Kotlyar AB, Vinogradov AD. Slow active/inactive transition of the mitochondrial NADH- ubiquinone reductase [published erratum appears in Biochim Biophys Acta 1990 Oct 24;1020(1):113]. *Biochim. Biophys. Acta* 1990; 1019(2)**:** 151-158.

13. Klingenberg M, Slenczka W. [Pyridine nucleotide in liver mitochondria. An analysis of their redox relationships]. *Biochemische Zeitschrift* 1959; 331(6)**:** 486-517.

14. Folbergrova J, Ljunggren B, Norberg K, Siesjo BK. Influence of complete ischemia on glycolytic metabolites, citric acid cycle intermediates, and associated amino acids in the rat cerebral cortex. *Brain Res* 1974; 80(2)**:** 265-79.

15. Benzi G, Arrigoni E, Marzatico F, Villa RF. Influence of some biological pyrimidines on the succinate cycle during and after cerebral ischemia. *Biochem Pharmacol* 1979; 28(17)**:** 2545-50.

16. Benzi G, Pastoris O, Dossena M. Relationships between gamma-aminobutyrate and succinate cycles during and after cerebral ischemia. *J Neurosci Res* 1982; 7(2)**:** 193-201.

17. Konig T, Nicholls DG, Garland PB. The inhibition of pyruvate and Ls(+)-isocitrate oxidation by succinate oxidation in rat liver mitochondria. *Biochem J* 1969; 114(3)**:** 589-96.

18. Chouchani ET, Pell VR, Gaude E, Aksentijevic D, Sundier SY, Robb EL *et al.* Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. *Nature* 2014; 515(7527)**:** 431-435.

19. Niatsetskaya ZV, Sosunov SA, Matsiukevich D, Utkina-Sosunova IV, Ratner VI, Starkov AA *et al.* The oxygen free radicals originating from mitochondrial complex I contribute to oxidative brain injury following hypoxia-ischemia in neonatal mice. *J. Neurosci.* 2012; 32(9)**:** 3235-3244.

20. Guaras A, Perales-Clemente E, Calvo E, Acin-Perez R, Loureiro-Lopez M, Pujol C *et al.* The CoQH2/CoQ Ratio Serves as a Sensor of Respiratory Chain Efficiency. *Cell Rep* 2016; 15(1)**:** 197-209.

21. Scialo F, Sriram A, Fernandez-Ayala D, Gubina N, Lohmus M, Nelson G *et al.* Mitochondrial ROS Produced via Reverse Electron Transport Extend Animal Lifespan. *Cell Metab* 2016; 23(4)**:** 725-34.

22. Rosenthal RE, Hamud F, Fiskum G, Varghese PJ, Sharpe S. Cerebral ischemia and reperfusion: prevention of brain mitochondrial injury by lidoflazine. *J. Cereb. Blood Flow Metab.* 1987; 7(6)**:** 752-758.

23. Sled VD, Vinogradov AD. Kinetics of the mitochondrial NADH-ubiquinone oxidoreductase interaction with hexammineruthenium(III). *Biochim. Biophys. Acta* 1993; 1141**:** 262-268.

24. Chinopoulos C, Zhang SF, Thomas B, Ten V, Starkov AA. Isolation and functional assessment of mitochondria from small amounts of mouse brain tissue. *Methods Mol Biol* 2011; 793**:** 311-24.

25. Starkov AA, Andreyev AY, Zhang SF, Starkova NN, Korneeva M, Syromyatnikov M *et al.* Scavenging of H2O2 by mouse brain mitochondria. *J Bioenerg Biomembr* 2014; 46(6)**:** 471-7.

26. Starkov AA, Fiskum G. Regulation of brain mitochondrial H_2O_2 production by membrane potential and NAD(P)H redox state. *J. Neurochem.* 2003; 86(5)**:** 1101-1107.

27. Solberg R, Enot D, Deigner HP, Koal T, Scholl-Burgi S, Saugstad OD *et al.* Metabolomic analyses of plasma reveals new insights into asphyxia and resuscitation in pigs. *PLoS One* 2010; 5(3)**:** e9606.

28. Pisarenko O, Studneva I, Khlopkov V, Solomatina E, Ruuge E. An assessment of anaerobic metabolism during ischemia and reperfusion in isolated guinea pig heart. *Biochim. Biophys. Acta* 1988; 934(1)**:** 55-63.

29. Hinkle PC, Butow RA, Racker E, Chance B. 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.* 1967; 242(22)**:** 5169-5173.

30. Cino M, Del Maestro RF. Generation of hydrogen peroxide by brain mitochondria: the effect of reoxygenation following postdecapitative ischemia. *Arch Biochem Biophys* 1989; 269(2)**:** 623-38.

31. Votyakova TV, Reynolds IJ. $\Delta\Psi_m$-dependent and -independent production of reactive oxygen species by rat brain mitochondria. *J. Neurochem.* 2001; 79(2)**:** 266-277.

32. Quinlan CL, Perevoshchikova IV, Hey-Mogensen M, Orr AL, Brand MD. Sites of reactive oxygen species generation by mitochondria oxidizing different substrates. *Redox. Biol.* 2013; 1**:** 304-312.

33. Ten VS, Starkov A. Hypoxic-ischemic injury in the developing brain: the role of reactive oxygen species originating in mitochondria. *Neurol.Res.Int.* 2012; 2012**:** 542976.

34. Sims NR. Selective impairment of respiration in mitochondria isolated from brain subregions following transient forebrain ischemia in the rat. *J Neurochem* 1991; 56(6)**:** 1836-44.

35. Rehncrona S, Mela L, Siesjo BK. Recovery of brain mitochondrial function in the rat after complete and incomplete cerebral ischemia. *Stroke* 1979; 10(4)**:** 437-46.

36. Yusa T, Beckman JS, Crapo JD, Freeman BA. Hyperoxia increases H2O2 production by brain in vivo. *1985).* 1987; 63(1)**:** 353-8.

37. Vinogradov AD, Grivennikova VG. Generation of superoxide-radical by the NADH:ubiquinone oxidoreductase of heart mitochondria. *Biochemistry (Mosc.)* 2005; 70(2)**:** 120-127.

38. Hoffman DL, Salter JD, Brookes PS. Response of mitochondrial reactive oxygen species generation to steady-state oxygen tension: implications for hypoxic cell signaling. *Am. J. Physiol. Heart Circ. Physiol* 2007; 292(1)**:** H101-H108.

39. Birrell JA, Yakovlev G, Hirst J. Reactions of the flavin mononucleotide in complex I: a combined mechanism describes NADH oxidation coupled to the reduction of $APAD^+$, ferricyanide, or molecular oxygen. *Biochemistry* 2009; 48(50)**:** 12005-12013.

40. Galkin A, Brandt U. Superoxide radical formation by pure complex I (NADH:ubiquinone oxidoreductase) from *Yarrowia lipolytica*. *J. Biol. Chem.* 2005; 280(34)**:** 30129-30135.

41. Grivennikova VG, Vinogradov AD. Generation of superoxide by the mitochondrial complex I. *Biochim. Biophys. Acta* 2006; 1757(5-6)**:** 553-561.

42. Kussmaul L, Hirst J. The mechanism of superoxide production by NADH:ubiquinone oxidoreductase (complex I) from bovine heart mitochondria. *Proc. Natl. Acad. Sci. USA* 2006; 103(20)**:** 7607-7612.

43. Liu Y, Fiskum G, Schubert D. Generation of reactive oxygen species by the mitochondrial electron transport chain. *J. Neurochem.* 2002; 80(5)**:** 780-787.

44. Quinlan CL, Goncalves RL, Hey-Mogensen M, Yadava N, Bunik VI, Brand MD. The 2-oxoacid dehydrogenase complexes in mitochondria can produce superoxide/hydrogen peroxide at much higher rates than complex I. *J. Biol. Chem.* 2014; 289(12)**:** 8312-8325.

45. Fato R, Bergamini C, Leoni S, Strocchi P, Lenaz G. Generation of reactive oxygen species by mitochondrial complex I: implications in neurodegeneration. *Neurochem Res* 2008; 33(12)**:** 2487-501.

46. Herrero A, Barja G. Localization of the site of oxygen radical generation inside the complex I of heart and nonsynaptic brain mammalian mitochondria. *J. Bioenerg. Biomembr.* 2000; 32(6)**:** 609-615.

47. Lambert AJ, Brand MD. Inhibitors of the quinone-binding site allow rapid superoxide production from mitochondrial NADH:ubiquinone oxidoreductase (complex I). *J. Biol. Chem.* 2004; 279(38)**:** 39414-39420.

48. Ohnishi ST, Ohnishi T, Muranaka S, Fujita H, Kimura H, Uemura K *et al.* A possible site of superoxide generation in the complex I segment of rat heart mitochondria. *J. Bioenerg. Biomembr.* 2005; 37(1)**:** 1-15.

49. Andreyev AY, Kushnareva YE, Starkov AA. Mitochondrial metabolism of reactive oxygen species. *Biochemistry (Mosc.)* 2005; 70(2)**:** 200-214.

50. Sled VD, Vinogradov AD. Reductive inactivation of the mitochondrial three subunit NADH dehydrogenase. *Biochim. Biophys. Acta* 1993; 1143**:** 199-203.

51. Gostimskaya IS, Grivennikova VG, Cecchini G, Vinogradov AD. Reversible dissociation of flavin mononucleotide from the mammalian membrane-bound NADH: ubiquinone oxidoreductase (complex I). *FEBS Lett.* 2007; 581(30)**:** 5803-5806.

52. Holt PJ, Efremov RG, Nakamaru-Ogiso E, Sazanov LA. Reversible FMN dissociation from Escherichia coli respiratory complex I. *Biochim. Biophys. Acta* 2016; 1857(11)**:** 1777-1785.

53. Lennon BW, Williams CH, Jr., Ludwig ML. Crystal structure of reduced thioredoxin reductase from Escherichia coli: structural flexibility in the isoalloxazine ring of the flavin adenine dinucleotide cofactor. *Protein Sci* 1999; 8(11)**:** 2366-79.

54. Mamedova AA, Holt PJ, Carroll J, Sazanov LA. Substrate-induced conformational change in bacterial complex I. *J. Biol. Chem.* 2004; 279(22)**:** 23830-23836.

55. Massey V. Activation of molecular oxygen by flavins and flavoproteins. *J. Biol. Chem.* 1994; 269(36)**:** 22459-62.

56. Barile M, Brizio C, Valenti D, De Virgilio C, Passarella S. The riboflavin/FAD cycle in rat liver mitochondria. *Eur J Biochem* 2000; 267(15)**:** 4888-900.

57. Rouslin W, Ranganathan S. Impaired function of mitochondrial electron transfer complex I in canine myocardial ischemia: loss of flavin mononucleotide. *J Mol Cell Cardiol* 1983; 15(8)**:** 537-42.

58. Gariballa S, Ullegaddi R. Riboflavin status in acute ischaemic stroke. *Eur J Clin Nutr* 2007; 61(10)**:** 1237-40.

Figure Legends

Fig. 1. H_2O_2-release during the forward and reverse electron transfer. Representative traces of respiration (top) and generation of H_2O_2 (bottom) by intact mitochondria from mouse brain. A, The reaction was started by addition of mitochondria 0.16 mg of protein/ml to the medium composed of 125 mM KCl, 0.2 mM EGTA, 20 mM HEPES-Tris (pH 7.4), 4 mM KH_2PO_4, 2 mM $MgCl_2$, 2 mg/ml BSA, 10 μM Amplex UltraRed and 4 U/ml horseradish peroxidase at 37°C. Substrates were either 5mM pyruvate and 2mM malate (right) or 5 mM succinate and 1 mM glutamate (left). Black traces correspond to oxygen concentration (top) and Amplex UltraRed fluorescence (bottom). The rate of the change of these signals per minute is shown in red. B, Release of H_2O_2 during coupled oxidation of 5 mM succinate and 1 mM glutamate as a function of oxygen concentration. 100% rate corresponds to 1,400 pmol $H_2O_2 \times min^{-1} \times mg\ protein^{-1}$. Oxygen concentration as measuring directly by the Oroboros respirometer was rapidly varied by continuously purging the headspace with nitrogen as described in Materials and Methods section.

Fig. 2. Consumption of oxygen and H_2O_2 release in closed (A, C) and open (B, D) respirometer chamber. Oxygen concentration (top) and Amplex UltraRed fluorescence (bottom) were measured simultaneously in 125 mM KCl, 0.2 mM EGTA, 20 mM HEPES-Tris, 4 mM KH_2PO_4, pH 7.4, 2 mM $MgCl_2$, 2 mg/ml BSA, 0.25mg/ml of mouse brain mitochondria, 5 mM succinate, 1 mM glutamate 10μM Amplex UltraRed and 4 U/ml horseradish peroxidase. Black traces correspond to oxygen concentration (top) and raw Amplex UltraRed fluorescence (bottom). The rates of the change of these signals per minute are shown in red.

Fig. 3. H_2O_2 release rate decline at different protein concentration and safranin response. A, three different protein concentrations were used to monitor H_2O_2 release in RET-like conditions. Curves 1-3 correspond to 0.5, 0.25, 0.125 mg of protein/ml protein in the measuring assay. Dashed lines show fitting of exponential decay function. B, Half-life of exponential decay of H_2O_2 release rate at difference protein concentrations (n=3-4 for each data point). C, Safranin O fluorescence after addition of 5 mM succinate and 1 mM glutamate to 0.15 mg of protein/ml. Assay conditions are as in Fig. 2B, except 2 μM safranin substituted Amplex UltraRed and horseradish peroxidase in C. Concentration of FCCP was 0.1 μM.

Fig. 4. Effect of incubation of intact brain mitochondria in RET-like conditions on the succinate- and NADH-dependent respiration (A and B, respectively). Mitochondria 0.15-0.2 mg of protein/ml were incubated in the presence of 5 mM succinate and 1 mM glutamate (as in Fig. 2B). After 2min (gray) and 20 min (white) 1 μM rotenone was added for the registration of succinate oxidase only (A). Alternatively, 25 nM atpenin, 5 mM pyruvate and 2 mM malate were added for the NAD^+-dependent respiration (B). After the additions, respirometer chamber was closed and resting respiration (state II) was measured. ADP-stimulated (state III) respiration was measured after addition of 0.5 mM ADP.

Fig. 5. Effect of incubation of intact brain mitochondria in RET-like conditions on NADH:HAR reductase activity. Mitochondria 0.3 mg of protein/ml were incubated in the presence of 5 mM succinate and 1 mM glutamate as in Fig. 2B and H_2O_2 release rate was monitored after reaching the maximum (squares). Small aliquots were taken in time and NADH:HAR reductase activity was assayed as described in Materials and Methods section with or without 10 μM FMN (black and red circles respectively). 100% rate of H_2O_2 release corresponds to 1,530±150 pmol $H_2O_2 \times min^{-1} \times mg\ protein^{-1}$.

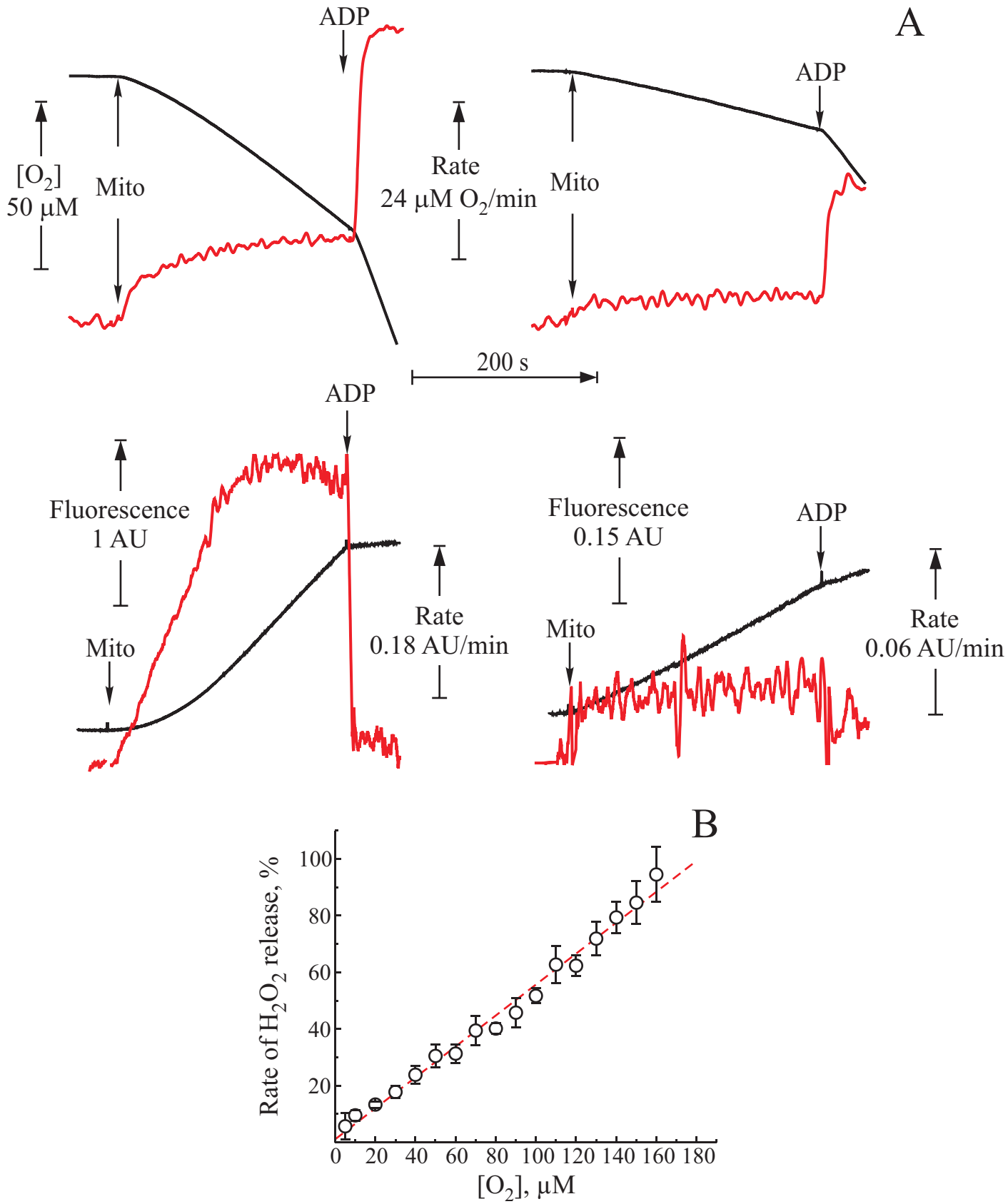
Fig. 6. Flavin fluorescence recovery (A) and dynamics of H_2O_2 release (B) in intact brain mitochondria during oxidation of succinate in RET-like conditions. Parameters of the assay are in Fig. 2, except 0.4 mg of protein/ml mitochondria was used. AmplexUltra Red and horseradish peroxidase were absent from the medium for flavin fluorescence measurements. Black traces correspond to Amplex UltraRed fluorescence and the rate of H_2O_2 release per min per mg of protein is shown in red. Note that fluorescence response (averaged from three measurements) is shown in inverted scale.

Tables

Table 1. Respiration activities and H_2O_2 release by coupled mouse brain mitochondria.

	Respiration, nmol $O_2 \times min^{-1} \times mg^{-1}$	Release of H_2O_2*, pmol $H_2O_2 \times min^{-1} \times mg^{-1}$
Malate +Pyruvate	15.2±6.3	137±18
+ADP (0.5 mM)	107.5±24.8	34±12
Succinate+Glutamate	40±3.1	1,560±300
+ADP (0.5 mM)	201±21.8	55±9
+FCCP (0.2 μM)	165.1±27.7	97±18
+Rotenone (1μM)	35.0±3.2	132±19

* measured simultaneously in 125 mM KCl, 0.2 mM EGTA, 20 mM HEPES-Tris, 4 mM KH_2PO_4, pH 7.4, 2 mM $MgCl_2$, 2 mg/ml BSA, 10 μM Amplex UltraRed and 4 U/ml horseradish peroxidase at 37°C, 0.14-0.19 mg of protein/ml mitochondrial protein. Substrates were either 5mM malate and 2mM pyruvate or 5 mM succinate and 1 mM glutamate. Values are given as mean±SEM. Three different preparations of mitochondria were used, all conditions were repeated in triplicates.



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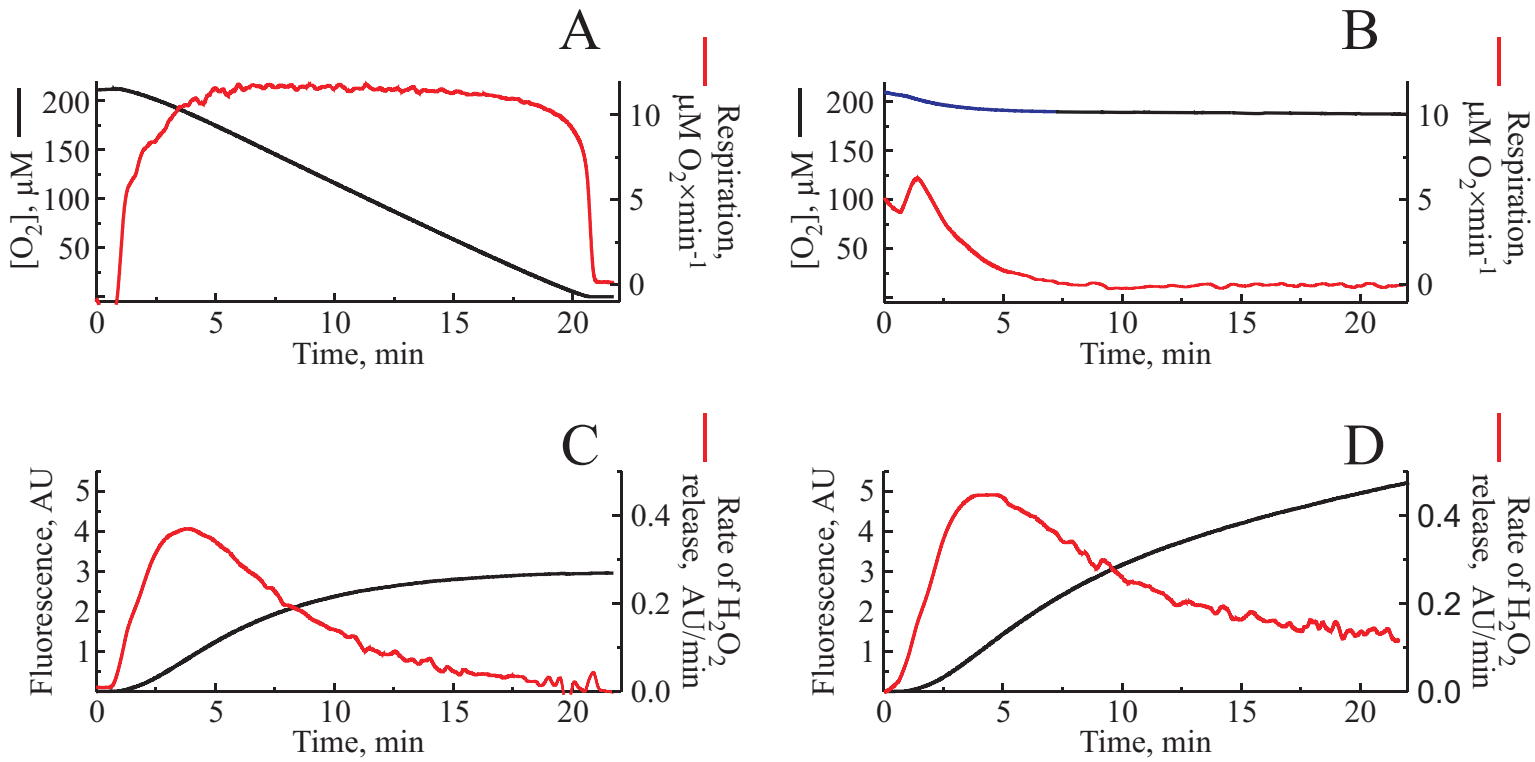

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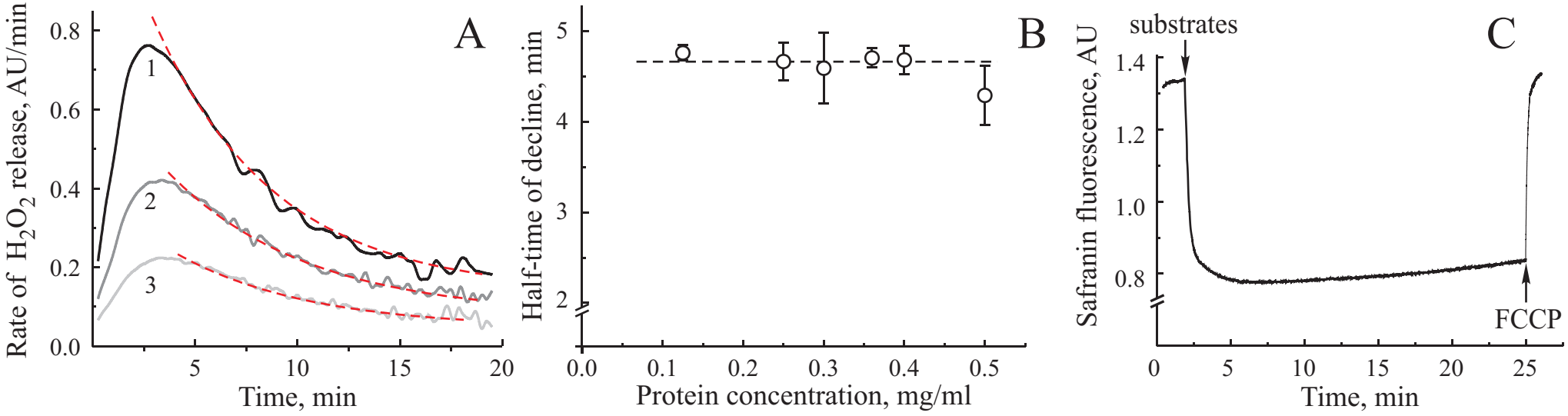

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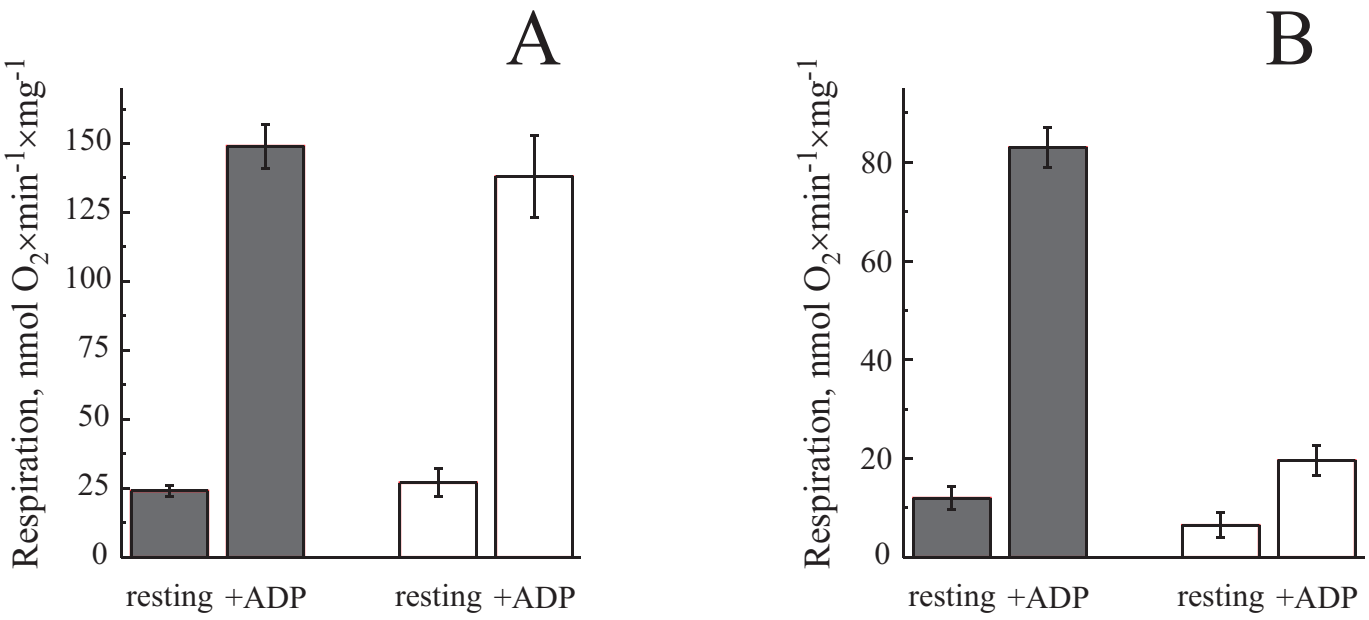

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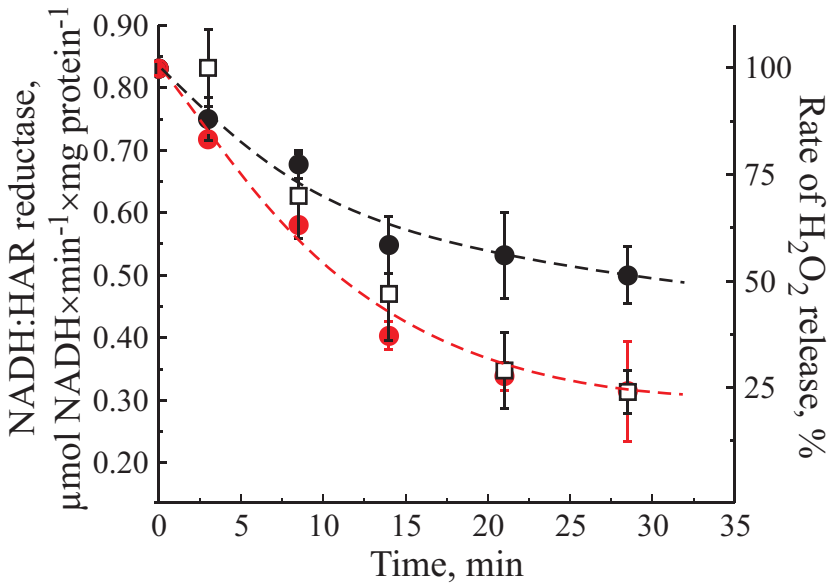

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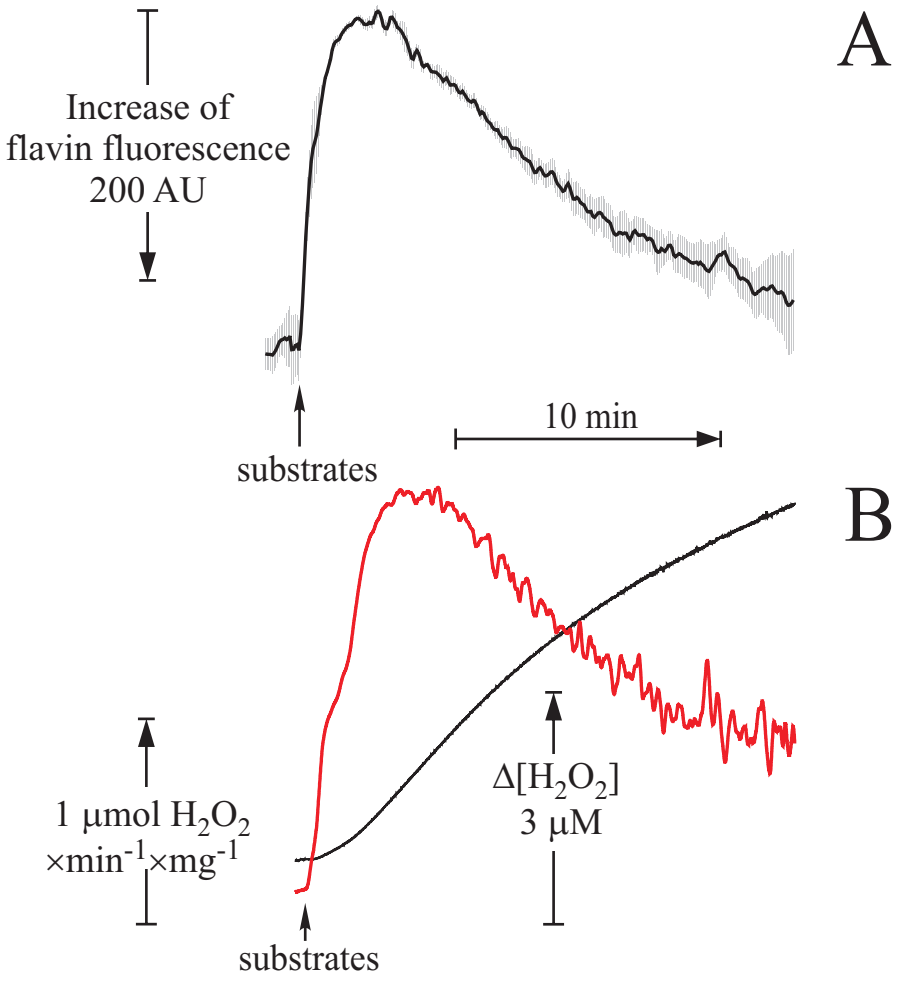

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