Abstract. Some therapeutic and/or adverse effects of drugs may be related to their effects on mitochondrial function. The effects of simvastatin, resveratrol, coenzyme Q₁₀, acetylcysteine, and acetylcarnitine on Complex I-, Complex II-, or Complex IV-linked respiratory rate were determined in isolated brain mitochondrial fraction. The protective effects of these biologically active compounds on the calcium-induced decrease of the respiratory rate were also studied. We observed a significant inhibitory effect of simvastatin on mitochondrial respiration ($IC_{50} = 24.0 \mu M$ for Complex I-linked respiration, $IC_{50} = 31.3 \mu M$ for Complex II-linked respiration, and $IC_{50} = 42.9 \mu M$ for Complex IV-linked respiration); the inhibitory effect of resveratrol was found at very high concentrations ($IC_{50} = 162 \mu M$ for Complex I-linked respiration, $IC_{50} = 564 \mu M$ for Complex II-linked respiration, and $IC_{50} = 1454 \mu M$ for Complex IV-linked respiration). Concentrations required for effective simvastatin- or resveratrol-induced inhibition of mitochondrial respiration were found much higher than concentrations achieved under standard dosing of these drugs. Acetylcysteine and acetylcarnitine did not affect the oxygen consumption rate of mitochondria. Coenzyme Q₁₀ induced an increase of Complex III-linked respiration. The increase of free calcium ions induced partial inhibition of the Complex I+II-linked mitochondrial respiration, and all tested drugs counteracted this inhibition. None of the tested drugs showed mitochondrial toxicity (characterized by respiratory rate inhibition) at drug concentrations achieved at therapeutic drug intake. Resveratrol, simvastatin, and acetylcarnitine had the greatest neuroprotective potential (characterized by protective effects against calcium-induced reduction of the respiratory rate).

Introduction

The predominant mitochondrial function is generation of ATP by oxidative phosphorylation (OXPHOS). Additional mitochondrial functions include generation of reactive oxygen species (ROS), initiation of the intrinsic apoptotic pathway, regulation of cytosolic calcium, and regulation of thermogenesis. Abnormalities in these processes are termed as mitochondrial dysfunctions. Mitochondrial dysfunctions may be included in various neurodegenerative disorders either as a major cause of the disease development or as secondary to other abnormalities (Orth and Shapira, 2001). Novel strategies in the treatment of these diseases and in prevention of negative consequences of aging include regulation of cellular bioenergetics and ROS production via mitochondrial functions.
The mitochondrial electron transfer system (ETS) consists of four enzyme complexes: Complex I (NADH-ubiquinone oxidoreductase; EC 1.6.5.3), Complex II (succinate-ubiquinone oxidoreductase; EC 1.3.5.1), Complex III (ubiquinol-cytochrome c reductase; EC 1.10.2.2), and Complex IV (cytochrome c oxidase, COX; EC 1.9.3.1). Free energy released upon the electron transport is conserved in the pH gradient across the inner mitochondrial membrane, and the resultant electrochemical potential (chemiosmotic potential, proton motive force) drives ATP synthesis using ATP synthase (Complex V; EC 3.6.3.14). The respiratory complexes are thought to be both in their free enzyme forms and associated in supercomplexes within the inner mitochondrial membrane (Genova and Lenaz, 2014).

AbnormalOXPHOS function can affect most of cellular processes, including ATP production, ROS generation, maintenance of the membrane resting potential, disruption of intracellular calcium buffering, and initialization of the intrinsic pathway of apoptosis. Impaired mitochondrial function is an attractive hypothesis to explain the pathophysiology of many diseases with unknown aetiology. Given the extraordinary role of mitochondria in neuronal function, attention is paid to drug effects on the mitochondrial dysfunction in neurodegenerative and psychiatric disorders (Chaturvedi and Flint Beal, 2013).

Different methods are available to measure mitochondrial functions/dysfunctions. A very common approach to addressing mitochondrial bioenergetic dysfunction is measurement of the expression, concentration, or maximum activity of individual mitochondrial respiration complexes or tricarboxylic acid-cycle enzymes. However, due to the complexity and cooperativity of OXPHOS processes, changes in the activity of the candidate complex may have little or no effect on the overall system behaviour, which can be measured as ATP production and oxygen consumption rate (P/O ratio). Mitochondrial respiratory control determined as the increase in respiration rate in response to addition of ADP is the most approved assay for isolated mitochondria (Brand and Nicholls, 2011). If respiration is not significantly affected by a drug, the drug can be considered safe in terms of mitochondrial toxicity. Further, protective effects of the tested drug can be measured against changes in ETS activity induced by other agents such as calcium, inhibitors of OXPHOS enzymes, or uncouplers.

It is assumed that agents enhancing mitochondrial function may be neuroprotective in neuropsychiatric disorders (Dodd et al., 2013). Administration of metabolites and cofactors is the mainstay of real-life therapy and includes both components of the respiratory chain and other natural compounds. There is increasing interest in the administration of ROS scavengers. It is expected that clinically applicable therapies will include techniques to upregulate mitochondrial biogenesis, to modulate Ca\(^{2+}\) homeostasis, to enhance organelar fusion and fission, to shift mtDNA heteroplasmy, and to eliminate the burden of mutant mtDNAs via cytoplasmic transfer (Schon et al., 2010).

**Mitochondria and calcium**

Calcium is a major signalling molecule in cells (Berridge et al., 2000; Fišar and Hroudová, 2010). Neurons have developed a complex calcium signalling system responsible for regulating a number of functions for processing information and changes in synaptic plasticity, which implicate perception, consciousness, learning, and memory.

Concentration of free Ca\(^{2+}\) in the cytosol is maintained at a resting level of about 100 nM. Upon activation, Ca\(^{2+}\) can raise to 1 \(\mu\)M (Berridge et al., 2000). Mitochondrial buffering of cytosolic calcium ions is included in basic mitochondrial functions and constitutes an efficient and versatile mechanism for the control of cellular Ca\(^{2+}\) signals. The outer mitochondrial membrane is permeable to Ca\(^{2+}\); the inner membrane contains specific calcium transporters. The uptake of calcium into the matrix is primarily driven by the mitochondrial membrane potential and mediated by mitochondrial Ca\(^{2+}\) unipporter and a regulatory protein containing a calcium-binding motif (EF-hand). Following Ca\(^{2+}\) uptake into the mitochondria, Ca\(^{2+}\) is slowly released from the matrix via Na\(^+\)/Ca\(^{2+}\) exchanger or via H\(^+\)/Ca\(^{2+}\) exchanger.

Increasing cytosolic Ca\(^{2+}\) stimulates mitochondrial respiration through activation of Ca\(^{2+}\)-regulated mitochondrial carriers and enzymes including ATP synthase, pyruvate dehydrogenase complex, isocitrate dehydrogenase, and \(\alpha\)-ketoglutarate dehydrogenase complex. It has been established that matrix Ca\(^{2+}\) is in the 0.02–2 \(\mu\)M range when the enzymes are activated (McCormack and Denton, 1989). It is assumed that in intact neurons, calcium both activates ATP production through the stimulation of OXPHOS and increases ATP consumption during recovery of the ionic resting state (Llorente-Folch et al., 2015).

High matrix calcium concentrations may lead to Ca\(^{2+}\)-P precipitation, reducing the matrix phosphate (P) concentration and driving the P\(_i\)/H\(^+\) carrier. The calcium-phosphate complex in the matrix effectively buffers the free matrix Ca\(^{2+}\) until excess of Ca\(^{2+}\) has been accumulated, which causes the mitochondrial permeability transition (MPT) (Nicholls, 2009). In the absence of exogenous adenine nucleotides and in the presence of high P\(_i\) or peroxides, mitochondrial Ca\(^{2+}\) overload leads to MPT pore (MPTP) opening (Crompton, 1999); the inner membrane potential is initially rapidly collapsed (as Ca\(^{2+}\) enters the matrix), restored (when Ca\(^{2+}\) uptake is complete) and then collapsed again (as the MPTPs open). ATP synthesis is decreased, ROS production is increased, calcium and apoptogenic proteins are released into the cytosol, and mitochondrial swelling occurs.

Aside of MPT opening, the over-accumulation of Ca\(^{2+}\) in the mitochondrial matrix causes (1) inhibition of OXPHOS, wherein Complex I-linked respiration was found more susceptible to the inhibitory effect of matrix Ca\(^{2+}\) than Complex II-linked respiration (Villalobo and Lehninger, 1980); (2) inhibition of citric acid cycle enzymes, such as \(\alpha\)-ketoglutarate dehydrogenase complex (Lai and Cooper, 1986) or pyruvate dehydrogenase...
complex (Lai et al., 1988); (3) inhibition of ATP-ADP translocase activity; and (4) diffusion limitations for substrate delivery to dehydrogenases in the matrix due to Ca\(^{2+}\)-P precipitation (Chalmers and Nicholls, 2003).

**Coenzyme Q\(_{10}\)**

Coenzyme Q\(_{10}\) (ubiquinone, CoQ\(_{10}\), CoQ) is present primarily in the mitochondria as a component of ETS. CoQ is a lipophilic molecule embedded in the membrane lipid bilayer; it is required for OXPHOS and acts as an antioxidant in cellular membranes (Bentinger et al., 2007). Besides the fundamental role of CoQ as an electron carrier between Complex I and Complex III or between Complex II and Complex III, CoQ is an essential factor in activation of protein uncoupling, controls MPTPs, participates in extramitochondrial electron transport in plasma membranes and lysosomes, controls physicochemical properties of membranes, and acts as an endogenous lipid antioxidant (Rauchová and Vokurková, 2009).

CoQ is partly synthesized in the body and partly taken with food. Aging is associated with a decrease in the content of CoQ in mitochondria and increased production of ROS. Several mitochondrial diseases due to the primary or secondary CoQ deficiency have been described (Quinzi and Hirano, 2010). CoQ and its synthetic analogues are the only agents that have shown some therapeutic benefit to patients with mitochondrial respiratory chain disorders (Hargreaves, 2014). Nevertheless, CoQ is not approved for the treatment of diseases; it is available as a dietary supplement. It shows minimal toxicity, excellent tolerance, and no significant side effects.

**Simvastatin**

Simvastatin is an effective drug lowering cholesterol. Simvastatin and other 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) have been demonstrated to reduce mortality and the risk of major cardiovascular events. However, statins can have been demonstrated to reduce mortality and the risk of metabolic disease and neurodegeneration. So far, clinical studies have shown inconsistent results for metabolic and cardiovascular diseases.

**Resveratrol**

Resveratrol (3,5,4’-trihydroxy-trans-stilbene) is a natural antioxidant and derivative of stilbene. Food sources of resveratrol include the shells and kernels of blue vine and blackcurrant, grapefruit peels, peanuts, cocoa, blueberries, and mulberries. Resveratrol is studied for its therapeutic potential in various diseases, from cancer to metabolic disease and neurodegeneration. For so, clinical studies have shown inconsistent results for metabolic and cardiovascular diseases.

The major supposed biological activities of resveratrol include inhibition of lipid peroxidation, chelation of copper, free-radical scavenging, alteration of eicosanoid synthesis, inhibition of platelet aggregation, anti-inflammatory activity, vaso-relaxing activity, modulation of lipid metabolism, anticancer activity, and oestrogenic activity (Frémont, 2000). Resveratrol has been shown to have beneficial effects in cardiovascular disease, metabolic disease, cancer, and neurodegeneration. Improvement of mitochondrial function is one of the important metabolic effects of resveratrol. Metabolic effects of resveratrol (Bitterman and Chung, 2015) are supposed to be associated with (1) activation of AMP-activated kinase (AMPK) (Park et al., 2012), master regulator of cellular energy homeostasis; (2) activation of sirtuin 1 (SIRT1), which has been studied for the effects of caloric restriction and lifespan extension (Bitterman and Chung, 2015); (3) inhibition of ATP synthase, where the beneficial effects may come from preventing mitochondrial ATP synthesis in tumour cells, thereby inducing apoptosis (Gledhill et al., 2007).

Pathways for activation of AMPK may include an increase of the AMP/ATP ratio and/or cAMP-dependent processes. Resveratrol directly inhibits cAMP-specific phosphodiesterases, leading to elevated concentration of cAMP. The exchange protein activated by cAMP (Epac1) was identified as a key mediator of the effects of resveratrol, which lead to the activation of AMPK and SIRT1. Briefly, activation of Epac1 leads to the activation of phospholipase C\(_\gamma\) resulting in calcium release from the endoplasmic reticulum and activation of calmodulin-dependent protein kinase kinase-\(\beta\), which in turn can phosphorylate and thereby activate AMPK. AMPK increases mitochondrial biogenesis and function by increasing peroxisome proliferator-activated receptor \(\gamma\) coactivator 1-\(\alpha\) (PGC-1\(\alpha\)) expression, NAD\(^+\) levels, and SIRT1 activity (Park et al., 2012). An important regulatory mechanism of PGC-1\(\alpha\) transcription complex is through acetylation and SIRT1-mediated lysine deacetylation under low nutrient conditions (Rodgers et
However, additional pathways are supposed to contribute to the resveratrol action.

**Acetylcysteine**

Acetylcysteine (N-acetylcysteine) is primarily used as a mucolytic agent and in the management of paracetamol overdose. It has been successfully tried as a treatment for a number of neuropsychiatric disorders, such as addiction (marijuana dependence, nicotine addiction, cocaine addiction, pathological gambling), obsessive-compulsive disorders, schizophrenia, bipolar disorder, depression, autism, drug-induced neuropathy, progressive myoclonic epilepsy, Alzheimer’s or Parkinson’s disease, etc. (Deepmala et al., 2015).

Acetylcysteine has been hypothesized to exert beneficial effects through its modulation of glutamate and dopamine neurotransmission, antioxidant glutathione, neurotrophins, apoptosis, mitochondrial function, and inflammatory pathways (Dean et al., 2011; Berk et al., 2013). Acetylcysteine corrects mitochondrial dysfunctions by modifying calcium dynamics within the mitochondria and by decreasing cytosolic Ca²⁺ (SanMartin et al., 2012). It also reverses mitochondrial toxicity, which in turn decreases the ROS production by mitochondria. Acetylcysteine is a potent antioxidant that both acts as a direct free radical scavenger and supports glutathione homeostasis. Acetylcysteine also dampens the inflammatory response by decreasing production of cytokines and in this way decreases cellular stress and mitochondrial dysfunction (Berk et al., 2013; Deepmala et al., 2015). Treatment with an amide form of N-acetylcysteine with higher bioavailability maintained mitochondrial bioenergetics and normalized glutathione levels following spinal cord injury (Patel et al., 2014) or traumatic brain injury (Pandya et al., 2014).

**Acetylcarnitine**

Acetylcarnitine (acetyl-L-carnitine) is a molecule derived from acetylation of carnitine inside mitochondria. Acetylcarnitine is broken down to carnitine, which is used by cells to transport fatty acids into the mitochondria via carnitine shuttle; the fatty acids then undergo β-oxidation. The mitochondrial content of endogenous acetylcarnitine is an indicator of mitochondrial metabolism of acetyl-CoA (Rosca et al., 2009).

Acetylcarnitine is described as having several properties that may be beneficial in the treatment of dementia. These include effects on integrity of the lipid environment of the inner mitochondrial membrane (increase of cardiolipin content), control of mitochondrial protein synthesis, increase of fat utilization as a metabolic fuel, antioxidant properties, and antiapoptotic effect (Rosca et al., 2009).

Acetylcarnitine has shown to be beneficial in animal models of Parkinson’s disease (Beal, 2003) and may be effective in the treatment of depression (Wang et al., 2014). The clinical efficiency of acetylcarnitine was studied in the treatment of patients with Alzheimer’s disease. So far, however, no evidence for recommending routine use of acetylcarnitine in clinical practice has been found (Hudson and Tabet, 2003).

**Aim**

The aim of our study was to measure the effects of selected biologically active compounds on mitochondrial respiration and evaluate their mitochondrial toxicity by measurement of both inhibiting/stimulating action on mitochondrial respiration and protective effects on calcium-induced damage of mitochondrial respiration. We focused on simvastatin, coenzyme Q₁₀, resveratrol, acetylcysteine, and acetylcarnitine as potential agents in preventing age-related defects. High-resolution respirometry with isolated pig brain mitochondria was used for the study of direct effects of these drugs on mitochondrial respiration linked to Complex I, Complex II, or Complex IV. The protective effect of the drugs on calcium-induced decrease of Complex I+II-linked respiration was investigated.

**Material and Methods**

**Media, chemicals**

Buffered sucrose (sucrose 0.32 M, HEPES 4 mM, pH 7.4) was used both as isolation medium and preservative medium for crude mitochondrial fraction (CMF), mitochondria purified in sucrose gradient (PMS), and mitochondria purified in Percoll gradient (PMP). The mitochondrial respiration medium (MiR05) consisted of sucrose 110 mM, K⁺-lactobionate 60 mM, tauroine 20 mM, MgCl₂·6H₂O 3 mM, KH₂PO₄ 10 mM, EGTA 0.5 mM, BSA essentially fatty acid free 1 g l⁻¹ and HEPES 20 mM, adjusted to pH 7.1 with KOH (Gnaiger et al., 2000). Substrates, inhibitors, and uncouplers were used in concentrations described previously (Pesta and Gnaiger, 2012; Gnaiger, 2014). The following stock solutions were used: 10 mg ml⁻¹ digitonin in dimethyl sulphoxide (DMSO), 5 mM simvastatin in DMSO, 10 mM and 100 mM resveratrol in ethanol, 50 mM CoQ in acetone, 5 mM and 100 mM acetylcysteine in water, and 5 mM and 100 mM acetylcarnitine in water. The chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO).

**Pig brain mitochondria preparation**

Brains were obtained from slaughter pigs and immediately transported in ice-cold saline buffer to the laboratory and further processed as described previously (Fišar, 2010). Crude mitochondrial fraction (CMF) was isolated from the brain cortex by the standard differential centrifugation technique (Whittaker, 1969; Fišar, 2010), which ensured removal of whole cells or nuclei and minimization of contamination by microsomes, plasma membranes, lysosomes and cytosol. Synaptosomes and other contaminants are present in CMF (Whittaker, 1969; Wieckowski et al., 2009). Therefore, purified mitochondria were separated from CMF by centrifugation in sucrose gradient (PMS) (Whittaker 1969; Pinna et al., 2003) or in Percoll gradient (PMP) (Graham,
2001). Samples were stored in buffered sucrose 0.32 M on ice at total protein concentration 10–40 mg·ml⁻¹. Protein concentration was determined by the method of Lowry et al. (1951), with bovine serum albumin as the standard.

**High-resolution respirometry**

The OROBOROS Oxygraph-2k (O2k; OROBOROS INSTRUMENTS, Innsbruck, Austria) and the automatic titration-injection micropump TIP2k were used for high-resolution respirometry measurements. Samples in a volume of 2 ml were measured in two glass chambers equipped with Clark polarographic oxygen electrodes. Common experimental conditions include physiological temperature 37 °C, stirring, closed-chamber mode of operation, oxygen solubility factor of the mitochondrial respiration medium MiR05 0.92, calibration of the polarographic oxygen sensor before each measurement, and periodic measurement of instrumental background oxygen consumption (Gnaiger, 2014).

Most measurements were time consuming (60–120 min per one experiment) and some drugs were not soluble in water; thus, sample titration with solvent without drug was simultaneously measured in one of the two chambers for correcting both the solvent effect and temporal changes in the respiration rate.

Experimental protocols for various respirometric titration regimes are shown in Tables 1–4. Each protocol consists of the sequence of substances added to the sample after MiR05 saturation with atmospheric oxygen, closing the chamber, mitochondria addition, and digitonin addition. All samples were treated with optimal concentrations of digitonin before substrate addition: 573 µg digitonin per 1 mg protein for CMF, or 140 µg digitonin per 1 mg protein for PMS or PMP. Final values were corrected for both non-mitochondrial (residual) oxygen consumption (ROX) and the effect of the solvent, sample dilution, and temporal changes. Control ratio was calculated as the ratio of oxygen consumption rate before/after drug addition.

**Protocols for the effect of drugs on mitochondrial respiration**

Following substrate addition (malate + pyruvate + ADP for Complex I-linked respiration, ADP + rotenone

| Table 1. Protocol for evaluating the effect of the drug concentration on Complex I-linked respiration in pig brain mitochondria |
|---|---|---|---|---|
| Order | Chamber | Substance | Abbr. | Stock conc. (solvent) | Volume added (µl) | Final concentration |
| 1 | AB | Malate | M | 0.8 M (H₂O) | 5 | 2 mM |
| 2 | AB | Pyruvate | P | 2 M (H₂O) | 5 | 5 mM |
| 3 | AB | ADP | D | 0.5 M (H₂O) | 4 | 1 mM |
| 4 | A | Solvent | titration: 0.5–100 |
| 4 | B | Drug | titration: 0.5–100 |
| 5 | AB | Rotenone | ROT | 1 mM (EtOH) | 1 | 0.5 µM |

| Table 2. Protocol for evaluating the effect of the drug concentration on Complex II-linked respiration in pig brain mitochondria |
|---|---|---|---|---|
| Order | Chamber | Substance | Abbr. | Stock conc. (solvent) | Volume added (µl) | Final concentration |
| 1 | AB | ADP | D | 0.5 M (H₂O) | 4 | 1 mM |
| 2 | AB | Rotenone | ROT | 1 mM (EtOH) | 1 | 0.5 µM |
| 3 | AB | Succinate | S | 1 M (H₂O) | 20 | 10 mM |
| 4 | A | Solvent | titration: 0.5–100 |
| 4 | B | Drug | titration: 0.5–100 |
| 5 | AB | Antimycin A | ANTI | 0.5 mg·ml⁻¹ (EtOH) | 5 | 1.25 µg·ml⁻¹ |

| Table 3. Protocol for evaluating the effect of the drug concentration on Complex IV-linked respiration in pig brain mitochondria |
|---|---|---|---|---|
| Order | Chamber | Substance | Abbr. | Stock conc. (solvent) | Volume added (µl) | Final concentration |
| 1 | AB | ADP | D | 0.5 M (H₂O) | 4 | 1 mM |
| 2 | AB | Antimycin A | ANTI | 0.5 mg·ml⁻¹ (EtOH) | 5 | 1.25 µg·ml⁻¹ |
| 3 | AB | Ascorbate | ASC | 1 M (H₂O) | 4 | 2 mM |
| 4 | AB | TMPD | TMPD | 200 mM (EtOH) | 5 | 0.5 mM |
| 5 | A | Solvent | titration: 0.5–100 |
| 5 | B | Drug | titration: 0.5–100 |
| 6 | AB | Azide | AZD | 4 M (H₂O) | 100 | 200 mM |

TMPD – N,N,N',N'-tetramethyl-p-phenylenediamine
Inhibition of respiration rate was analysed using the four-parameter logistic regression with SigmaPlot software (Systat Software, Inc., Richmond, CA) to establish the half maximal inhibitory concentration \((IC_{50})\), which represents the concentration of a substance that is required for 50% inhibition of mitochondrial respiration, the Hill slope, reflecting the degree of cooperativity of the ligand binding to the enzyme, and the residual activity at high drug concentration.

Statistical analyses were performed using the STATISTICA data analysis software system (StatSoft, Inc., Tulsa, OK). One-sample \(t\)-test for single means was used to determine whether the oxygen consumption rate in the sample with the drug and/or calcium added was significantly different from the control (100%). Data are presented as the mean ± standard deviation (SD) or the mean ± standard error (SE).

**Results**

Illustrative OROBOROS Oxygraph-2k runs are shown for the simvastatin inhibitory effect on Complex II-linked respiration measured according to the protocol given in Table 2 (Fig. 1) and for the resveratrol protective effect on \(Ca^{2+}\)-induced changes in Complex I+II-linked respiration measured according to the protocol given in Table 4 (Fig. 2).

The effects of simvastatin, resveratrol, CoQ, acetyl-cysteine, and acetylcarnitine on mitochondrial respiration rate linked to Complex I, Complex II or Complex IV were measured (Figs. 3–5). The effect of free calcium alone and the protective effects of the drugs on calcium-induced decline of oxygen consumption rate were measured for Complex I+II-linked respiration (Figs. 6 and 7).

**Effect of drugs on mitochondrial respiration**

**Simvastatin**

A strong inhibitory effect of simvastatin was found for mitochondrial respiration linked to Complex I (Fig. 3), Complex II (Fig. 4) as well as Complex IV (Fig. 5). To evaluate the effect of the procedure of mitochondria isolation, the effects of simvastatin in PMS, PMP, and CMF were compared for Complex I-linked and Complex II-linked respiration. Equality of respiration in the sample with the drug to respiration in the sample without drug was rejected by \(t\)-test at simvastatin concentrations ≥ 10 \(\mu\)M for CMF, ≥ 5 \(\mu\)M for PMS, and ≥ 5 \(\mu\)M for PMP.

### Table 4. Protocol for evaluating the protective effect of drugs on the \(Ca^{2+}\)-induced change of Complex I+II-linked respiration in pig brain mitochondria

<table>
<thead>
<tr>
<th>Order</th>
<th>Chamber</th>
<th>Substance</th>
<th>Abbr.</th>
<th>Stock conc. (solvent)</th>
<th>Volume added (µl)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AB</td>
<td>Malate</td>
<td>M</td>
<td>0.8 M (H(_2)O)</td>
<td>5</td>
<td>2 mM</td>
</tr>
<tr>
<td>2</td>
<td>AB</td>
<td>Pyruvate</td>
<td>P</td>
<td>2 M (H(_2)O)</td>
<td>5</td>
<td>5 mM</td>
</tr>
<tr>
<td>3</td>
<td>AB</td>
<td>ADP</td>
<td>D</td>
<td>0.5 M (H(_2)O)</td>
<td>4</td>
<td>1 mM</td>
</tr>
<tr>
<td>4</td>
<td>AB</td>
<td>Succinate</td>
<td>S</td>
<td>1 M (H(_2)O)</td>
<td>20</td>
<td>10 mM</td>
</tr>
<tr>
<td>5</td>
<td>A</td>
<td>Solvent</td>
<td></td>
<td></td>
<td>2–4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>B</td>
<td>Drug</td>
<td></td>
<td></td>
<td>2–4</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>AB</td>
<td>CaCl(_2)</td>
<td>CA</td>
<td>100 mM (H(_2)O)</td>
<td>titration: 2–60</td>
<td>0.1–3.0 mM</td>
</tr>
<tr>
<td>15 min</td>
<td></td>
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</table>

+ succinate for Complex II-linked respiration, and ADP + antimycin A + ascorbate + TMPD for Complex IV-linked respiration), the sample was titrated by the drug in the first chamber and by the solvent in the second chamber (Tables 1–3). The added volume was between 0.5 and 100 µl, to give a final drug concentration of 5–150 µM for simvastatin, 2.5–5000 µM for resveratrol, 12.5–1000 µM for CoQ\(_{10}\), 2.5–5000 µM for acetylcysteine, and 2.5–5000 µM for acetylcarnitine. Finally, inhibitors of the respiratory complexes (rotenone, antimycin A, or azide) were added to determine ROX.

Note that for Complex IV-linked respiration, the dependence of oxygen consumption on oxygen concentration was measured after Complex IV inhibition by azide and used for correction on non-mitochondrial oxygen consumption (Kuznetsov and Gnaiger, 2015).

**Protocol for the protective effect of drugs on calcium-induced changes**

The protective effects of the drugs on calcium-induced decrease of Complex I+II-linked respiration rate were determined according to the protocol given in Table 4. Following addition of digitonin and substrates, the sample was incubated with the drug (or solvent) for 15 min. The final drug concentration was 25 µM for simvastatin, 100 µM for resveratrol, 100 µM for CoQ, 100 µM for acetylcysteine, or 100 µM for acetylcarnitine. Note that a lower simvastatin concentration was used due to a strong inhibitory action of simvastatin at 100 µM. Then, the sample was titrated by CaCl\(_2\) and residual oxygen consumption was determined after antimycin A addition.

**Data analysis**

DatLab software (OROBOROS INSTRUMENTS) was used for respirometry data acquisition and analysis. Mass-specific oxygen flux (pmol·s\(^{-1}·mg\(^{-1}\)) was based on protein concentration in the samples.

Inhibition of respiration rate was analysed using the four-parameter logistic regression with SigmaPlot software (Systat Software, Inc., Richmond, CA) to establish the half maximal inhibitory concentration \((IC_{50})\), which represents the concentration of a substance that is required for 50% inhibition of mitochondrial respiration, the Hill slope, reflecting the degree of cooperativity of the ligand binding to the enzyme, and the residual activity at high drug concentration.
In PMS, Complex I-linked respiration was inhibited with \( IC_{50} = 24.0 \pm 0.8 \mu M \), Hill slope = 3.13 ± 0.29 and residual respiration at high drug concentration = –1.0 ± 1.7 %. Complex II-linked respiration was inhibited with \( IC_{50} = 31.3 \pm 1.6 \mu M \), Hill slope = 2.43 ± 0.24, and residual respiration at high drug concentration = 9.7 ± 2.7 %. Complex IV-linked respiration was inhibited with \( IC_{50} = 42.9 \pm 5.6 \mu M \), Hill slope = 1.44 ± 0.22, and residual respiration at high drug concentration = –1.5 ± 5.2 % (all values are means ± SE).

In PMP, Complex I-linked respiration was inhibited with \( IC_{50} = 19.7 \pm 1.4 \mu M \), Hill slope = 2.18 ± 0.31, and residual respiration at high drug concentration = 0.1 ± 3.6 %. Complex II-linked respiration was inhibited with \( IC_{50} = 43.5 \pm 1.2 \mu M \), Hill slope = 2.64 ± 0.18, and residual respiration at high drug concentration = 7.7 ± 2.0 %.

In CMF, Complex I-linked respiration was inhibited with \( IC_{50} = 44.6 \pm 2.1 \mu M \), Hill slope = 2.43 ± 0.23, and residual respiration at high drug concentration = –1.5 ± 3.0 %. Complex II-linked respiration was inhibited with \( IC_{50} = 46.4 \pm 1.5 \mu M \), Hill slope = 2.81 ± 0.22, and residual respiration at high drug concentration = –0.9 ± 2.7 %.

**Resveratrol**

The inhibitory effect of high resveratrol was found for mitochondrial respiration linked to Complex I (Fig. 3) with \( IC_{50} = 162 \pm 11 \mu M \), Hill slope = 1.00 ± 0.07, and residual respiration at high drug concentration = 8.0 ± 2.0 %. Complex II-linked respiration was also inhibited at high drug concentrations (Fig. 4) with \( IC_{50} = 564 \pm 67 \mu M \), Hill slope = 0.62 ± 0.03, and residual respiration at high drug concentration = –2.3 ± 2.9 %. Inhibition of Complex IV-linked respiration was found at very high resveratrol concentration (Fig. 5) with \( IC_{50} = 1454 \pm 124 \mu M \) (mean ± SE). Equality of respiration in the sample with resveratrol to respiration in the sample without resveratrol was rejected by \( t \)-test at drug concentrations ≥ 25 µM for Complex I-linked respiration, at ≥ 5 µM for Complex II-linked respiration, and at ≥ 100 µM for Complex IV-linked respiration.

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Fig. 1. Effect of simvastatin on Complex II-linked respiration in the crude mitochondrial fraction (CMF) at sample concentration 0.20 mg protein·ml\(^{-1}\). Illustrative Oroboros Oxygraph-2k runs are displayed for the performed protocol (Table 2) and DatLab software. After addition of digitonin (DIG), adenosine diphosphate (D), rotenone (ROT), and succinate (S), samples were titrated with (A) DMSO, (B) simvastatin. Finally, oligomycin (O) and antimycin A (ANTI) were added to determine the residual oxygen consumption.

Grey thin line: oxygen concentration (nmol·ml\(^{-1}\)); black thick line: oxygen flux per mass (pmol·s\(^{-1}·mg\(^{-1}\)).
Coenzyme Q₁₀
The increase of Complex I-linked respiratory rate was associated with CoQ addition (Fig. 3), but no effect of CoQ was observed for Complex II- or Complex IV-linked respiration (Figs. 4, 5). Equality of respiration in the sample with CoQ to respiration in the sample without CoQ was rejected by t-test at drug concentrations ≥ 25 μM for Complex I-linked respiration.

Acetylcysteine and acetylcarnitine
Both acetylcysteine and acetylcarnitine did not affect mitochondrial respiration up to concentration of about 1 mM. Millimolar drug concentrations showed a small inhibitory effect (Figs. 3, 4, 5).

Protective effects of drugs on calcium-induced changes
Initially, a dose-dependent direct effect of free calcium on Complex I+II-linked respiration was measured in PMS. The respiration medium MiR05 contains 0.5 mM EGTA; therefore, free Ca²⁺ concentrations were computed using the Schoenmakers’ method (Schoenmakers et al., 1992; http://maxchelator.stanford.edu/CaEGTA-TS.htm). We observed an increase of oxygen consumption rate of PMS when free Ca²⁺ was increased from 1 mM to 17 mM after one titration step by CaCl₂ (data not shown). This increase was temporary and was followed by a rapid decline in the respiratory rate, probably in response to increasing calcium concentrations in the matrix. Increasing free Ca²⁺ inhibited mitochondrial respiration with \( IC_{50} = 66 ± 30 \) mM, Hill slope = 0.41 ± 0.05, and residual respiration 34.0 ± 5.3 % (means ± SE, N = 23) (Fig. 6). Protective effects of all drugs on calcium-induced inhibition of mitochondrial Complex I+II-linked respiration were observed when PMS were preincubated with drugs and then titrated with CaCl₂ (Fig. 7).

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Fig. 2. Effect of resveratrol and calcium on Complex I+II-linked respiration in purified mitochondria (PMS) at sample concentration 0.05 mg protein ml⁻¹. Illustrative Oroboros Oxygraph-2K runs are displayed for the performed protocol (Table 4) and DatLab software. After addition of digitonin (DIG), malate (M), pyruvate (P), adenosine diphosphate (D), and succinate (S), samples were treated with 2 μl (A) ethanol, (B) resveratrol in final concentration 100 μM. After incubation for 15 min, samples were titrated by CaCl₂. Finally, antimycin A (ANTI) was added to determine the residual oxygen consumption.

Grey thin line: oxygen concentration (nmol·ml⁻¹); black thick line: oxygen flux per mass (pmol·s⁻¹·mg⁻¹).
Fig. 3. Effects of simvastatin, resveratrol, coenzyme Q_{10}, acetylcysteine, and acetylcarnitine on mitochondrial Complex I-linked respiration rate in mitochondria purified in sucrose gradient (PMS). The effect of simvastatin was also measured in mitochondria purified in Percoll gradient (PMP) and in the crude mitochondrial fraction (CMF). Final sample concentration was in the range 0.03–0.15 mg protein·ml⁻¹. Complex I-linked respiration was measured as a basal value and the sample was then titrated by the drug or solvent according to the protocol given in Table 1. Data are shown as means of 3–8 measurements ± SD.

Fig. 4. Effects of simvastatin, resveratrol, coenzyme Q_{10}, acetylcysteine, and acetylcarnitine on mitochondrial Complex II-linked respiration rate in mitochondria purified in sucrose gradient (PMS). The effect of simvastatin was also measured in mitochondria purified in Percoll gradient (PMP) and in the crude mitochondrial fraction (CMF). Final sample concentration was in the range 0.03–0.15 mg protein·ml⁻¹. Complex II-linked respiration was measured as a basal value and the sample was then titrated by the drug or solvent according to the protocol given in Table 2. Data are shown as means of 3–5 measurements ± SD.
Fig. 5. Effects of simvastatin, resveratrol, coenzyme Q$_{10}$, acetylcysteine, and acetylcarnitine on mitochondrial Complex IV-linked respiration rate in mitochondria purified in sucrose gradient (PMS). Final sample concentration was in the range 0.03–0.05 mg protein ml$^{-1}$. Complex IV-linked respiration was measured as basal value and the sample was then titrated by the drug or solvent according to the protocol given in Table 3. Data are displayed as a ratio of oxygen consumption rate before/after drug addition (means of 3–5 measurements ± SD).

Fig. 6. Calcium-induced inhibition of mitochondrial Complex I+II-linked respiration in purified mitochondria (PMS). Final sample concentration was in the range 0.03–0.15 mg protein ml$^{-1}$. Digitonin, malate 2 mM, pyruvate 5 mM, ADP 1 mM, and succinate 10 mM were added and Complex I+II-linked respiration was used as basal value. The sample was then titrated in the first chamber by CaCl$_2$ and in the second chamber by water. Residual oxygen consumption was determined after antimycin A 1.25 µg ml$^{-1}$ addition. Values were corrected for residual oxygen consumption, the effect of sample dilution, and temporal changes. The relative respiration rate is displayed, which was calculated as a ratio of respiration rate before and after addition of CaCl$_2$. Data are shown as means of 23 measurements ± SD. The line represent the best fitted curve using the four-parameter logistic function.
The oxygen consumption rate is one of important parameters enabling highly sensitive measurement of the impact of drugs on mitochondrial function, especially on the activity of ETS. Previously, we confirmed that pig brain mitochondria might serve as an appropriate biological model for studying in vitro effects of drugs on cellular energetics (Hroudová and Fišar, 2012; Fišar et al., 2014; Singh et al., 2015).

The effects of simvastatin on mitochondrial respiration in PMS, PMP, and CMF were compared for Complex I- and Complex II-linked respiration. Higher \( IC_{50} \) which was observed in CMF compared with purified mitochondria, probably reflects the decrease of free simvastatin concentration due to its interaction with non-mitochondrial membranes in CMF. CMF appears completely satisfactory for assessment of qualitative changes and has the added advantage in rapid preparation, higher yield, and less damage to the mitochondrial membrane caused by the isolation procedure. We assume that the purified mitochondria are preferable for quantitative analysis of drug effects on the respiratory rate. Thus, PMS was used in all other measurements presented in this paper.

We found simvastatin-induced inhibition of the mitochondrial oxygen consumption rate with \( IC_{50} \) in tens of micromoles. It is supposed that some pleiotropic effects of simvastatin are related to its action on mitochondrial functions. The majority of pleiotropic effects of statins were observed at micromolar statin concentrations, which may be misleading (Björkhem-Bergman et al., 2011), because \( IC_{50} \) of simvastatin for HMG-CoA reductase inhibition is 18.1 nM only (Gazziero et al., 2012) and the mean concentration of simvastatin in human serum is about 10\(^3\) nM (Keskitalo et al., 2009). There is some accumulation of simvastatin in the liver tissue (less than threefold), but not in the brain tissue (about one quarter of that in the serum) (Thelen et al., 2006), and the intracellular concentration of simvastatin is unknown. Thus, it is unlikely that a micromolar concentration needed to effectively inhibit mitochondrial respiration could be achieved (under normal conditions). However, simvastatin synergy with other drugs or high local concentrations of simvastatin in pathological conditions cannot be excluded.

Compared to simvastatin, resveratrol effectively inhibited mitochondrial respiration only at very high concentrations. Different \( IC_{50} \) and Hill slopes indicate a different mechanism of action of simvastatin and resveratrol on mitochondrial respiration. We can speculate that simvastatin affects the interaction of membrane lipids and complexes, whereas high resveratrol rather influences assembly of subunits of Complex I. The mean plasma concentration of therapeutically administered resveratrol is in the micromolar range (10\(^3\)–10\(^4\) μM).

**Discussion**

**Fig. 7.** Protective effects of drugs on calcium-induced inhibition of mitochondrial Complex I+II-linked respiration in purified mitochondria (PMS). Final sample concentration was in the range 0.05–0.10 mg protein-mL\(^{-1}\). Complex I+II-linked respiration was measured in samples preincubated with the drug or solvent and used as basal value; the sample was then titrated by CaCl\(_2\) according to the protocol given in Table 4. Ratios were calculated of respiration rates in the presence and absence of the drug at various concentrations of free Ca\(^{2+}\). Displayed data are shown as means of 3–8 measurements ± SD.
(Howells et al., 2011), which is insufficient for effective inhibition of mitochondrial respiration.

The increased Complex I-linked respiratory rate at high concentrations of CoQ suggests that there is support of electron transfer between Complex I and Complex III, probably through incorporation of CoQ into the inner mitochondrial membrane. The relatively high CoQ concentration required to produce this effect is probably due to the insolubility of CoQ in water, when precipitation occurs after addition to an aqueous sample, leading to limited incorporation of CoQ into the membranes.

Total carnitine and acetyl carnitine concentrations were about 71 µM and 14 µM, respectively, in whole blood of healthy adults (Minkler et al., 2008). The mean concentration of total plasma N-acetylcysteine was also found in micromolar range (Fisher and Bostom, 1997). We found that mitochondrial respiration was not affected even at millimolar concentrations of acetyl carnitine or acetylcarnitine, which indicates no mitochondrial toxicity of these substances.

Dose- and time-dependent inhibition of mitochondrial respiration by calcium was observed at about 1 µmol Ca²⁺ per mg protein without influencing mitochondrial enzyme activities (Pandya et al., 2013). In accordance with these data, we observed partial inhibition of mitochondrial respiration with increasing free Ca²⁺. Preincubation with simvastatin, resveratrol, CoQ, acetyl cysteine, or acetylcarnitine led to lower calcium-induced inhibition of the oxygen consumption rate. The ratio of respiration rate in the presence and absence of the drug at higher concentrations of free calcium was found greater than one for all the tested drugs. It can be interpreted as the protective effect of drugs on the imbalance caused by high concentrations of free calcium. From this perspective, resveratrol showed the most protective effects, acetylcarnitine and simvastatin were less effective, and acetyl cysteine and CoQ showed the least protective effects. Our results indicate that all tested drugs may participate in the fine tuning of Ca²⁺ signals, which play a key role in the cellular bioenergetics, while resveratrol, acetylcarnitine, and simvastatin seem to be the most efficient.

Conclusions

Measurement of the drug effect on the kinetics of mitochondrial oxygen consumption and/or protective effects of drugs against calcium-induced inhibition of the mitochondrial respiration can be used for the study of mitochondrial toxicity and neuroprotective effects of drugs. Different in vitro effects of simvastatin, resveratrol, CoQ, acetyl cysteine, and acetylcarnitine on oxygen consumption rate support the view for an independent mode of mitochondrial action of these drugs. We found direct in vitro inhibitory effects of simvastatin and resveratrol on mitochondrial respiration. CoQ₁₀ had a stimulatory effect and acetyl cysteine and acetylcarnitine did not have a significant impact on the oxygen consumption rate. Furthermore, we observed protective effects of resveratrol, acetylcarnitine and simvastatin against a calcium-induced decrease in the respiratory rate. Supposing that the drug-induced inhibition of mitochondrial respiratory rate is associated with neurotoxicity, and that the protective effects against calcium-induced reduction of the respiratory rate contribute to neuroprotection, our results indicate that (i) there is no neurotoxic effect of CoQ₁₀, acetyl cysteine, and acetylcarnitine even at very high drug concentrations; (ii) resveratrol, simvastatin, and acetyl carnitine have higher neuroprotective potential. Although the effects of the tested compounds on mitochondrial respiration were significant only at their high concentrations, their effects on mitochondrial function under pathological conditions or during interactions with other drugs should be taken into account.

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References


