Ischemia-Reperfusion Induces Inhibition of Mitochondrial Protein Synthesis and Cytochrome c Oxidase Activity in Rat Hippocampus

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Summary

Dysfunction of mitochondria induced by ischemia is considered to be a key event triggering neuronal cell death after brain ischemia. Here we report the effect of ischemia-reperfusion on mitochondrial protein synthesis and activity of cytochrome c oxidase (EC 1.9.3.1, COX). By performing 4-vessel occlusion model of global brain ischemia, we have observed that 15 min of global ischemia led to the inhibition of COX subunit I (COXI) synthesis to 56 % of control. After 1, 3 and 24 h of reperfusion, COXI synthesis was inhibited to 46, 50 and 72 % of control, respectively. Depressed synthesis of COXI was not a result of either diminished transcription of COXI gene or increased proteolytic degradation of COXI, since both Northern hybridization and Western blotting did not show significant changes in COXI mRNA and protein level. Thus, ischemia-reperfusion affects directly mitochondrial translation machinery. In addition, ischemia in duration of 15 min and consequent 1, 3 and 24 h of reperfusion led to the inhibition of COX activity to 90.3, 80.3, 81.9 and 83.5 % of control, respectively. Based on our data, we suggest that inhibition of COX activity is rather caused by ischemia-induced modification of COX polypeptides than by inhibition of mitochondrial translation.

Key words

Cytochrome c oxidase • Global ischemia • Mitochondria • Translation

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Introduction

Inhibition of global protein synthesis after brain ischemia is well known phenomenon already documented in several studies (for review see Hossmann 1993, DeGracia *et al.* 2002). However, little is known about the effect of brain ischemia-reperfusion injury on mitochondrial protein synthesis. Despite the involvements of mitochondria in both neuronal cell survival (Nicholls and Budd 2000) and ischemic cell death (Fiskum 2000) there is only one study dealing with the effect of brain ischemia on mitochondrial translation (Smialek and Hamberger 1970). Last two decades, mitochondria are attracting more interests due to recognition of their important role in the process of apoptosis (Nicholls and Budd 2000, Polster and Fiskum 2004) and activation of apoptosis after brain ischemia has been documented in several studies (Nitatori *et al.* 1995, Charriaut-Marlangue *et al.* 1996, Honkainiemi *et al.* 1996, Krajewski *et al.* 1999, Perez-Pinzon *et al.* 1999, Sugawara *et al.* 1999, Cao *et al.* 2003, Endo *et al.* 2006). Recent studies have postulated that apoptosis is closely linked to mitochondrial dysfunction, including disturbances of mitochondrial translation. Inhibition of mitochondrial protein synthesis led to increased sensitivity of cells to nitric oxide-induced apoptosis and to inhibition of mitochondrial respiratory chain complexes (Ramachandran *et al.* 2002). In turn, inhibition of respiratory chain complexes provoked the activation of mitochondrial-dependent apoptotic machinery by direct triggering of cytochrome c release from mitochondria (Clayton *et al.* 2005) or indirect

induction of Bax-dependent apoptosis through mitochondrial oxidative damage (Perier *et al.* 2005). Since core subunits of crucial mitochondria respiratory chain complexes are encoded by mitochondrial DNA, inhibition of mitochondrial protein synthesis might have a great impact on brain functions. This has been documented in some types of mitochondrial encephalopathies, which are often associated with point mutations in mitochondrial genes and consequent disturbance in synthesis and functions of respiratory chain complexes (Taanman 1999, Wallace 1999, Chomyn *et al.* 2000, Smeitink *et al.* 2001, Tryoen-Thot *et al.* 2003). Despite these important facts, little is known about the effect of ischemia on mitochondrial translation. To our knowledge, there is only one study dealing with the effect of global brain ischemia on mitochondrial translation (Smialek and Hamberger 1970) showing that ischemia led to an increase of both mitochondrial translation rate and activity of COX. Therefore the main aim of our study was to investigate the effect of global brain ischemia and consequent reperfusion on mitochondrial protein synthesis in more details. In addition to mitochondrial translation, the effect of ischemia-reperfusion on transcription of mitochondrial DNA, mitochondrial protein levels and activity of COX was investigated.

Methods

Ischemia-reperfusion

Animal studies were performed under a protocol approved by the State Veterinary and Food Department of Slovak Republic. A total of 30 adult male Wistar rats from the breeding house of the Institute of Experimental Pharmacology of Slovak Academy of Science (Dobrá voda, Slovak Republic) weighing 320-380 g were used. All animals were maintained on a 12/12 h light/dark cycle. Food and water were available *ad libitum* until the beginning of the experiment. The animals were anesthetized by inhalation of 2 % halothane, 30 % O_2 and 68 % N_2O mixture. Transient global cerebral ischemia was produced using the four-vessel occlusion model. Briefly, on day 1, both vertebral arteries were irreversibly occluded by coagulation through the alar foramina. On day 2, the animals were again anesthetized by inhalation of 2 % halothane, 30 % O_2 and 68 % N_2O mixture followed with surgical preparation of both common carotid arteries. Two min before carotid occlusion, the halothane was removed from the mixture. Then, both

common carotid arteries were occluded for 15 min by small surgical clips. Body temperature was maintained using a homoeothermic blanket. Reperfusion in duration 1, 3, or 24 h was induced by a release of clips. After 15 min of ischemia and 1, 3, or 24 h of reperfusion following 15 min of global ischemia, animals were sacrificed by decapitation and both hippocampi were dissected and processed immediately. Control animals underwent the same procedure except of carotid occlusion. Isolations of mitochondria and measurements of mitochondrial translations were carried out on experimental and control tissue simultaneously to reduce variability. Numbers of analyzed animals per experimental group are indicated in figure legends.

Isolation of mitochondria

A protocol adapted from Lee *et al.* (1993) with some modifications was used to prepare metabolically active mitochondria from rat hippocampus. Dissected tissue was homogenized in ice-cold homogenization buffer (25 mM 4-morpholinepropanesulfonic acid, pH 7.4, 250 mM sucrose, 4 mM $MgCl_2$, 0.05 mM EGTA) using Potter Teflon-glass homogenizer. Homogenate was centrifuged at 400 x *g* for 5 min and supernatant was collected. Supernatant was then centrifuged at 12000 x *g* for 10 min. Resulting sediment was resuspended in homogenization buffer and centrifuged at 12000 x *g* for 10 min. Final sediment was resuspended in homogenization buffer and stored on ice. Protein concentration was determined by protein Dc assay kit (Bio-Rad).

Western blot analysis

Membrane protein fractions were prepared by homogenization of tissue in homogenization buffer using a Potter Teflon-glass homogenizer. Cell membranes were sedimented by centrifugation at 30000 x *g* for 20 min. Membrane pellets were resuspended in homogenization buffer and membrane proteins were solubilized by addition of 20 % sodium dodecylsulfate (SDS) to final concentration of 10 %. Protein concentration was determined by protein Dc assay kit (Bio-Rad). Proteins were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5 % acrylamide-bis acrylamide gels, and then transferred on nitrocellulose membranes using a semi-dry transfer protocol. The membranes were controlled for even load and possible transfer artifacts by staining with Ponceau Red solution. After blocking with 2.5 % of Topo-block

(Fluka) solution in Tris-buffered saline with addition of 0.05 % of Tween 20 (TBS-T), membranes were incubated with primary antibodies against either COXI (1 µg/ml 1D6 Molecular Probes) and cytochrome c oxidase subunit Vb (COXVb) (2 µg/ml, 6E9 Molecular Probes) for 90 min and cytochrome c oxidase subunit II (COXII) (2.5 µg/ml 12C4 Molecular Probes) for 5 h at room temperature. All used antibodies were dissolved in TBS-T solution containing 1 % of protease-free bovine serum albumin (BSA). Incubation of membranes with primary antibodies was followed by extensive washing using TBS-T solution and consequently by incubation of membranes with biotinylated anti-mouse antibodies (1:10000 in TBS-T, Vector Laboratories). After washing, membranes were incubated with avidin-biotin conjugated peroxidase (Vector Laboratories) solution in TBS-T, washed again in TBS-T solution four times 15 min and then incubated in SuperSignal West Pico Chemiluminescent Substrate (Pierce) solution for 1 min. The bands corresponding to particular proteins were visualized by exposition on Biomax MR film (Kodak). The pictures were digitalized and the bands were integrated using GeneTools software (SynGene).

RNA isolation and Northern blot analysis

Total RNA was isolated from rat hippocampi using the Trizol reagent (Invitrogen) following the manufacturer's protocol. Total RNA (5 µg) was separated by denaturing electrophoresis on formaldehyde-containing 1.2 % agarose gels, then transferred on positively charged nylon membranes (Roche) using the capillary downstream method (Chomczynski 1992). Membranes were controlled for even load and possible transfer artifacts by staining with methylene blue solution. The position of 18S and 28S rRNA was marked on the membranes in order to estimate molecular sizes of particular signals. Membranes were prehybridized in ExpressHyb solution (Clontech) for 30 min at 60 °C and then hybridized with digoxigenin-labeled cDNA probe for 60 min at 60 °C. The COXI probe used in the study was synthesized by amplification of rat brain cDNA using the PCR probe digoxigenin synthesis kit (Roche) applying the manufacturer's protocol. Specific front 5´- CGA GCC TAC TTT ACA TCT GCC-3´ and reverse 5´-GAG TAA CGA CGA GGT ATC CCT-3´ primers were used to amplify part of COXI cDNA. After hybridization, membranes were first washed twice with 0.1 % SDS in double saline sodium citrate buffer (SSC, 0.3 M NaCl, 30 mM sodium citrate, pH=7.0) for 5 min at

room temperature. The next step comprised a double washing with 0.1 % SDS in 0.1x SSC (0.015 M NaCl 1.5 mM sodium citrate, pH 7.0) for 15 min at 58 °C. After washing, membranes were shortly rinsed in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) and then blocked in 1 % blocking reagent (Roche) solution in maleic acid buffer for 30 min at room temperature. Membranes were then incubated with anti-digoxigenin alkaline phosphatase conjugated antibodies (1:4000, Roche) for 30 min. Membranes were washed twice with 3 % Tween-20 solution in maleic acid buffer for 15 min and then preincubated in detecting buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5) for 5 min. Finally, membranes were incubated in detecting buffer-containing CDP-star substrate (1:100, Roche). The bands corresponding to COXI mRNA were visualized by exposing of Northern blot to Biomax (Kodak) film. The pictures were digitalised and the bands were integrated using GeneTools software (SynGene).

In vitro translation

Mitochondrial protein synthesis was performed according to McKee e*t al.* (1990) with some modifications. Mitochondrial translation was initiated by resuspending of isolated mitochondria (3.6 mg/ml of mitochondrial protein) in a prewarmed translation buffer (the final composition of 0.05 ml of translation mixture was: 20 mM 4-morpholine-propanesulfonic acid pH 7.4, 90 mM sucrose, 90 mM KCl, 5 mM MgCl$_2$ 2.5 mM KH$_2$PO$_4$ 16 mM ADP, 5 mM succinate, 5 mM pyruvate, 0.02 mM EGTA, 0.05 mM unlabeled amino acids (except of methionine), 0.5 mg/ml fatty acid free BSA, 0.1 mg/ml cycloheximide and 0.74 MBq of [^{35}S]-methionine (ICN)). Translation mixture was then incubated at 30 °C. At the indicated time intervals translation was analyzed either by determination of [^{35}S]-methionine incorporation or by SDS-PAGE (see Figure legend).

Measurement of cytochrome c oxidase activity

Activity of cytochrome c oxidase was measured by monitoring of oxidation of ascorbate reduced cytochrome c. Membrane proteins were resuspended in reaction buffer (50 mM Tris pH=8.0, 0.01 % n-dodecyl β-D-maltoside). The reaction was initialized by addition of reduced cytochrome c to the final concentration of 0.05 mmol/l. Oxidation of cytochrome c was monitored spectrophotometrically at 550 nm by UltrospecIII (Pharmacia-Amersham) and the rate of oxidation was calculated from the slope of absorbance dependence on
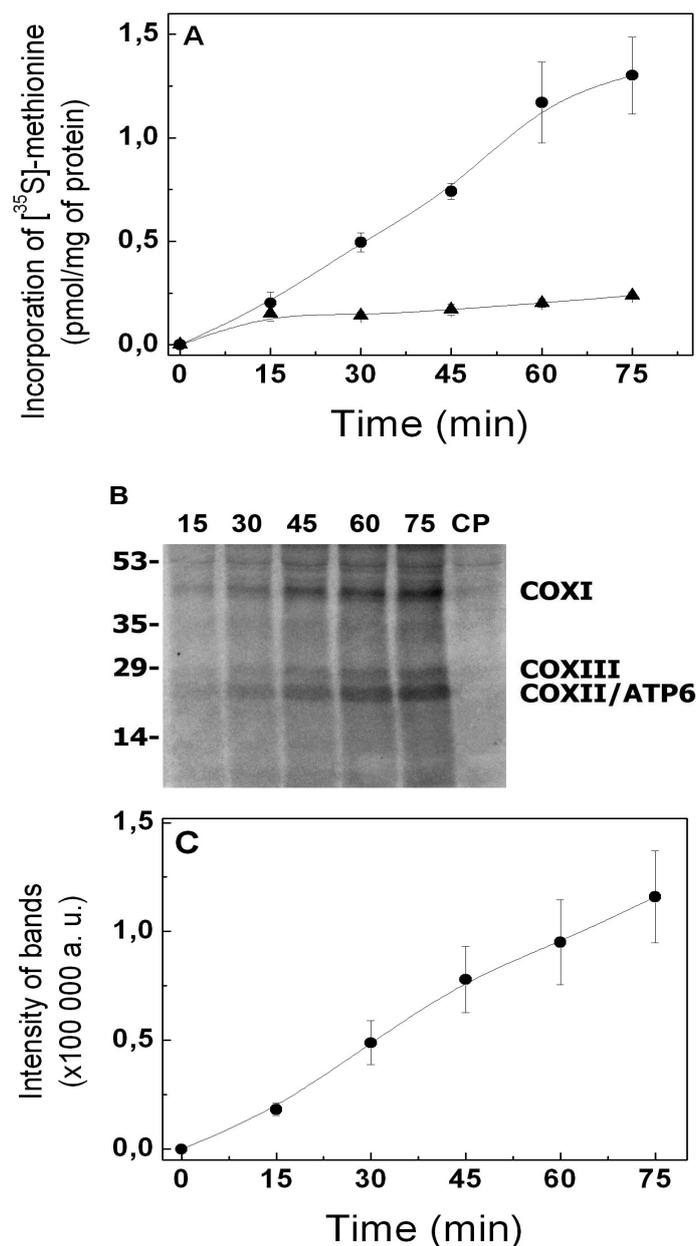
Fig. 1. Time course of mitochondrial protein synthesis. A) Time course of [^{35}S]-methionine incorporation in the absence (●) and presence of 0.05 mg/ml of chloramphenicol (▲). Mitochondria isolated from rat hippocampus were incubated for 15, 30, 45, 60 and 75 min in translation mixture as described in Material and Methods. Protein synthesis rates were determined by measuring of [^{35}S]-methionine incorporation by liquid scintillation counting. Aliquots of translation mixture (corresponding to 0.02 mg of mitochondrial proteins) were dissolved in 1M NaOH/2 % hydrogen peroxide and incubated at 37 °C for 10 min. At the end of the incubation, 2.25 ml of ice-cold 25 % trichloroacetic acid/2 % casamino acid (BD Science) were added to precipitate the translation products. The mixture was further incubated on ice for 30 min and then immediately filtered through membrane filters (Millipore HA, 0.45 μm) Filters were washed with 4 x 5 ml of ice-cold washing solution (5 % trichloroacetic acid) and dried. Radioactivity was measured by liquid scintillation spectrometer TriCarb 2200 CA. Number of moles of incorporated [^{35}S]-methionine per mg of mitochondrial protein was calculated for each time point. Results are presented as mean ± standard deviation of three independent experiments. **B)** Electrophoretic patterns of newly synthesized mitochondrial translation products after incubation of mitochondria in translation mixture for 15, 30, 45, 60 and 75 min in the absence or 75 min in the presence of 0.05 mg/ml of chloramphenicol (CP). Mitochondria isolated from rat hippocampus were incubated for 15, 30, 45, 60 and 75 min in translation mixture as described in Material and Methods. Translation was terminated by addition of 6X SDS-PAGE sample buffer (300 mM Tris-HCl pH=6.8, 300 mM dithioerythritol, 12 % SDS, 60 % glycerol, 0.6 % bromophenol blue) and consequent incubation at 95 °C for 5 min. The products of translation were separated by SDS-PAGE. Aliquots of translation mixture (corresponding to 0.05 mg of mitochondrial proteins) were loaded on 12.5 % acrylamide-bis acrylamide gels. Synthesized proteins were visualized by exposition of dried gels to Biomax film (Kodak). Molecular mass in kDa of standard proteins is indicated. **C)** Semi-quantitative analysis from three independent experiments. Resulted autoradiograms were digitalized and the intensities of particular bands for each time point were integrated using GeneTools software (SynGene). Results are presented as mean ± standard deviation of three independent experiments.

incubation time using the value of 19.6 M^{-1}cm^{-1} as molar extinction coefficient of oxidized cytochrome c at 550 nm. The net value of the complex IV activity was calculated by subtracting the rate of cytochrome c oxidation determined in the presence of 7.5 mM sodium azide (non-specific cytochrome c oxidation) from that obtained in the absence of sodium azide (total cytochrome c oxidation). COX activities are expressed as the number of nanomoles of oxidized cytochrome c per mg of protein per min.

Statistical analysis

Statistical analyses were done using GrafPhad InStat V2.04a (GrafPhad Software). For the comparison of ischemia-induced changes either one-way or two-way ANOVA test (see figure legend) was performed. Additionally, Tukey's test was carried out to demonstrate significant differences between data. Significance levels were set at $p < 0.05$.

Results

The main aim of our work was to study the effect of global brain ischemia-reperfusion on mitochondrial protein synthesis in more details. Based on previous works (McKee *et al.* 1990), we have performed in vitro translation using mitochondria isolated from rat hippocampus and [^{35}S]-methionine labeling. Incorporation of [^{35}S]-methionine into trichloroacetic acid-insoluble material was linear within at least 75 min
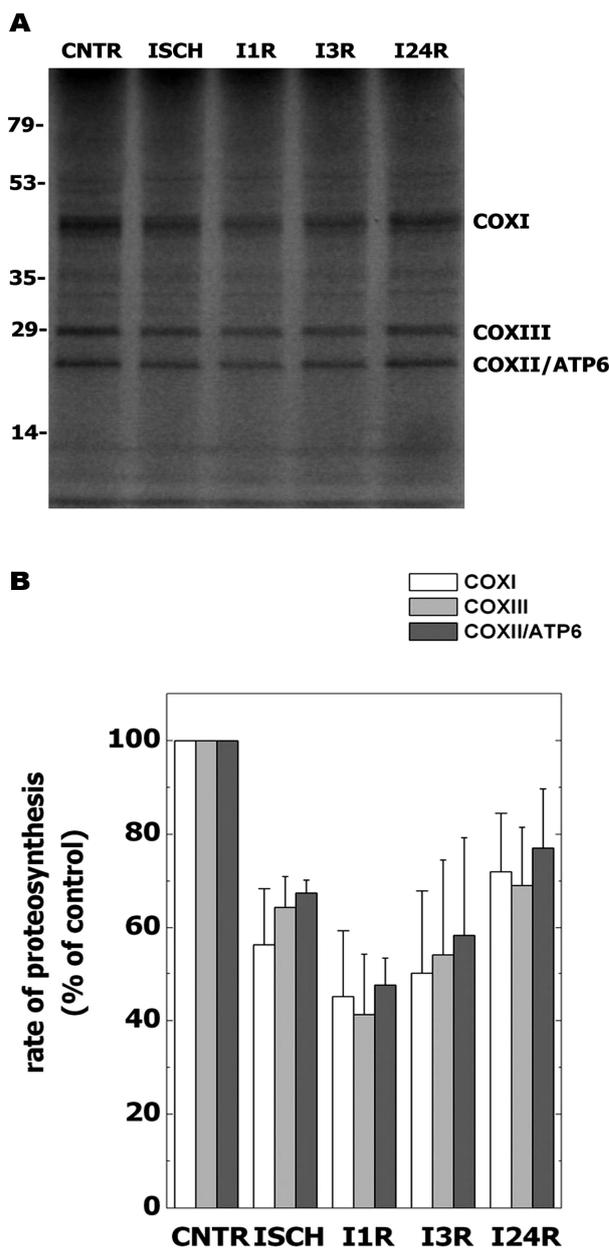
A

B

Fig. 2. Effect of ischemia-reperfusion on mitochondrial protein synthesis. A) Electrophoretic patterns of newly synthesized mitochondrial translation products. Mitochondria isolated from hippocampus of control animals (**CNTR**), animals subjected to 15 min of global ischemia (**ISCH**) and animals subjected to 15 min of global ischemia followed with reperfusion in duration of 1 (**I1R**), 3 (**I3R**) and 24 (**I24R**) h were incubated for 1 h in translation mixture as described in Material and Methods. Translation was terminated by addition of 6X SDS-PAGE sample buffer and consequent incubation at 95 °C for 5 min. The products of translation were separated by SDS-PAGE. Aliquots of translation mixture (corresponding to 50 µg of mitochondrial proteins) were loaded on 12.5 % acrylamide-bis acrylamide gels. Synthesized proteins were visualized by exposition of dried gels to Biomax film (Kodak). Molecular mass in kDa of standard proteins is indicated. **B**) Semi-quantitative analysis from 3 animals per experimental group and samples were quantified from 3 independent SDS-PAGE separations. Resulted autoradiograms were digitalized and the intensities of bands corresponding to COXI, COXIII and COXII/ATP6 were integrated using GeneTools software (SynGene). Levels of COXI, COXIII and COXII/ATP6 in control group were set to be 100 %. Results are presented as mean ± S.E.M. For the comparison of ischemia-induced changes two-way ANOVA test was performed. Additionally, Tukey's test was carried out to demonstrate significant differences between data. P values calculated with Tukey's test are **COXI**: $p<0.01$ (CNTR vs. ISCH), $p<0.001$ (CNTR vs. I1R), $p<0.001$ (CNTR vs. I3R) and $p>0.05$ (CNTR vs. I24R); **COXIII**: $p<0.05$ (CNTR vs. ISCH), $p<0.001$ (CNTR vs. I1R), $p<0.01$ (CNTR vs. I3R) and $p>0.05$ (CNTR vs. I24R); **COXII/ATP6**: $p<0.05$ (CNTR vs. ISCH), $p<0.001$ (CNTR vs. I1R), $p<0.01$ (CNTR vs. I3R) and $p<0.05$ (CNTR vs. I24R).

and was negligible in the presence of 0.05 mg/ml chloramphenicol, which is strong inhibitor of mitochondrial protein synthesis (Fig. 1A). Average rate of [^{35}S]-methionine incorporation (1.13±0.04 pmol/mg of protein/h) was approximately one order of magnitude smaller than that obtained for global protein synthesis (Garcia *et al.* 2004). Thus despite the use of cycloheximide as inhibitor of cytoplasmic translation, the results of mitochondrial protein synthesis might be significantly influenced by cytoplasmic translation. To avoid misinterpretations, we have also performed electrophoretic analysis of translation products. Analysis of labeled mitochondrial translation products demonstrated that bona fide mitochondrial peptides were

synthesized (Fig. 1B). We have identified all three subunits of COX encoded by mitochondrial DNA (COXI, COXII and COXIII) and subunit 6 of mitochondrial ATPase (ATP6) by comparison of our results to previously published data (Polosa and Attardi 1991).

These proteins were not detected if translation was performed in the presence of 0.05 mg/ml of chloramphenicol (Fig. 1B). In order to quantify translation based on electrophoretic analysis, resulted radiograms were digitalized and the intensities of particular bands at different time points were integrated. The results of semi-quantitative analysis showed that intensities of bands are linear at least within 75 min (Fig. 1C). Based on these results, we have investigated
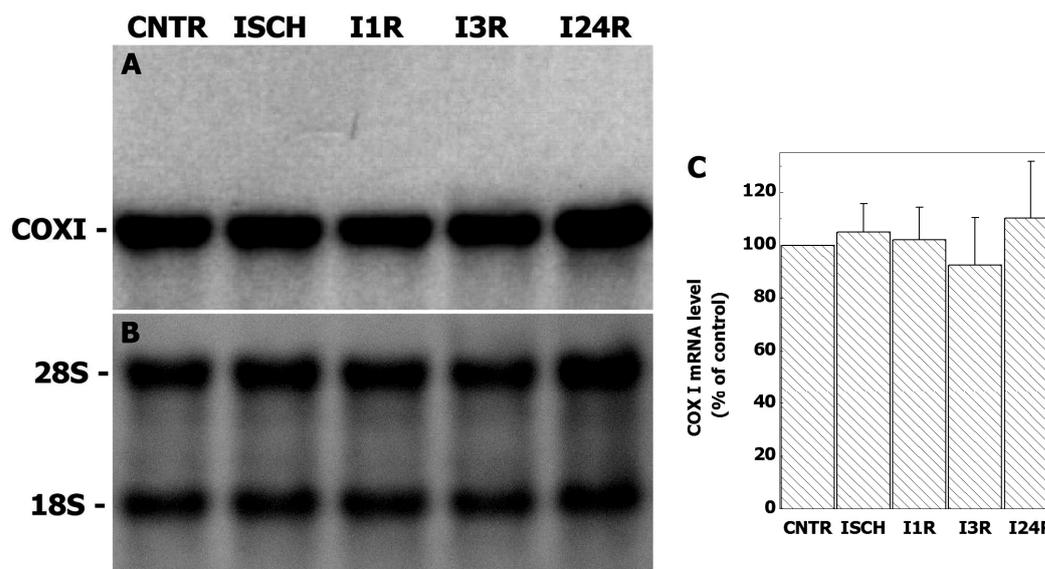
Fig. 3. Effect of ischemia-reperfusion on COXI mRNA level. A) Representative Northern blot of four independent experiments. Total RNA isolated from hippocampus of control animals (**CNTR**), animals subjected to 15 min of global ischemia (**ISCH**) and animals subjected to 15 min of global ischemia followed with reperfusion in duration of 1 (**I1R**), 3 (**I3R**) and 24 (**I24R**) h was analyzed by Northern blot as described in Material and Methods. No significant differences in the COXI mRNA levels were detected. **B**) Staining of membrane by methylene blue used as a loading control. **C**) Semi-quantitative analysis of COXI mRNA from four independent experiments. Results are presented as mean ± S.E.M. For the comparison of ischemia-induced changes one-way ANOVA test was performed.

the effect of ischemia-reperfusion on mitochondrial protein synthesis by performing densitometric analysis of radiograms obtained after electrophoretic analysis of mitochondrial translation in duration of 60 min (Fig. 2A). Our results showed that 15 min of global ischemia led to the inhibition of COXI synthesis to 56 % of control (p<0.01) (Fig. 2B). Rates of COXI synthesis observed 1 and 3 h after 15 min of global ischemia were 46 % (p<0.001) and 50 % (p<0.001) of control, respectively. Reperfusion in duration of 24 h following 15 min of global ischemia led to partial recovery of COXI synthesis to 72 % (p>0.05) of control. The extent COXIII and COXII/ATP6 synthesis inhibition is comparable to the extent of COXI synthesis inhibition (Fig. 2B).

In order to investigate whether depressed mitochondrial translation is not a result of diminished transcription we have estimated the level of COXI mRNA. We have focused our attention to this subunit since ischemia-induced decrease of COXI mRNA level has been documented in hippocampal CA1 neurons by *in situ* hybridisation (Abe *et al.* 1998). However, by performing Northern hybridization, we did not reveal significant changes in COXI mRNA level after ischemia as well as after all investigated intervals of reperfusion following 15 min of global ischemia (Fig. 3).

In addition to the rate of transcription and translation, level of proteins is significantly affected by

velocity of protein turnover. Increased protein degradation due to stimulation of different proteolytic enzymes was documented after ischemia-reperfusion in several studies (for review see Lipton 1999). Degradation of COXII subunit by mitochondrial protease Lon was also reported under the conditions of endoplasmic reticulum stress (Hori *et al.* 2002), which is often implicated as pathophysiological mechanisms associated with brain ischemia-reperfusion injury (Paschen and Doutheil 1999). Therefore, we have performed semi-quantitative Western blot analysis of the level of COXI and COXII proteins. However, our results did not show any significant changes in both COXI and COXII protein levels after ischemia in duration of 15 min as well as after 1, 3 and 24 h of reperfusion following 15 min of global ischemia (Fig. 4). We have also investigated the effect of ischemia-reperfusion on expression of regulatory COXVb subunit (Burke and Poyton 1998), which is encoded by nuclear DNA. As it is shown in Figure 4, we did not observe significant changes in the level of protein after ischemia and after all investigated intervals of reperfusion.

Since subunits of COX were identified as mitochondrial translation products, the effect of ischemia-reperfusion on activity of COX was investigated as well. We have observed that global ischemia in duration of 15 min led to a significant inhibition of COX activity to
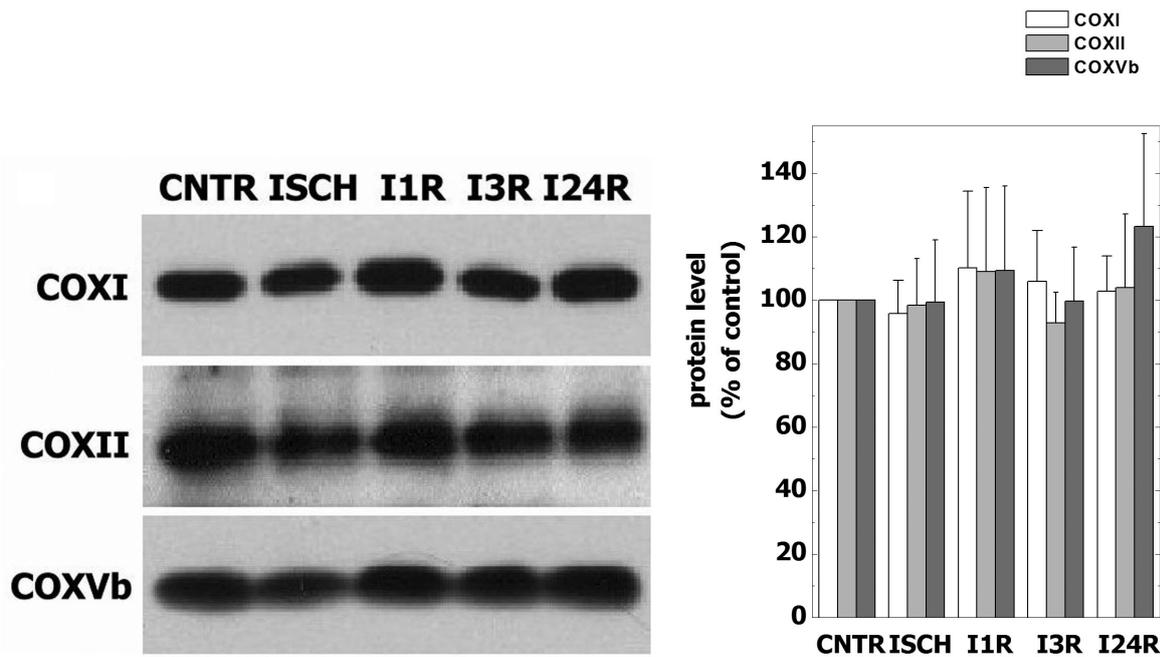
Fig 4. Effect of ischemia-reperfusion on COXI, COXII and COXVb protein level. Representative Western blots of COXI, COXII and COXVb and semi-quantitative analysis from four independent experiments. Membrane proteins were isolated from the hippocampus of control animals (**CNTR**), animals subjected to 15 min of global ischemia (**ISCH**) and animals subjected to 15 min of global ischemia followed with reperfusion in duration of 1 (**I1R**), 3 (**I3R**) and 24 (**I24R**) h and analyzed by Western blot as described in Material and Methods. No significant differences in the COXI, COXII and COXVb protein levels were detected. Results are presented as mean ± S.E.M. For the comparison of ischemia-induced changes one-way ANOVA test was performed.

90.3 % of control ($p < 0.001$). Similarly to the inhibition of mitochondrial protein synthesis, the minimal COX activity, 80.3 % of control ($p < 0.001$), was observed one hour following 15 min of global ischemia. Reperfusions in duration of 3 and 24 h led to inhibition of COX activity to 81.9 % ($p < 0.001$) and 83.5 % ($p < 0.001$) of control, respectively (Fig. 5).

Discussion

The main finding of our study was that ischemia-reperfusion affects directly mitochondrial translation and induces inhibition of mitochondrial protein synthesis in rat hippocampus. Three sites of protein synthesis (cytoplasm, rough endoplasmic reticulum and mitochondria) were recognized in mammalian cells (Kapp and Lorsch 2004) including neurons. The ischemia-induced inhibition of global protein synthesis has already been documented in several studies (Bodsch and Takahashi 1984, Kiessling *et al.* 1986, Thilmann *et al.* 1986, Nowak 1990, Hossmann 1993). To our knowledge, there is only one study dealing with effect of global brain ischemia on mitochondrial translation (Smialek and Hamberger 1970). Unlike our study, they showed that ischemia led to increased mitochondrial translation and activity of COX. The

discrepancies between results could be attributed to the different models of ischemia, since bilateral occlusion of common carotid arteries of rabbits without occlusion of vertebral arteries was used in previous study.

Decreased mRNA level due to depressed transcription of particular gene or decreased mRNA stability is often the reason of decreased translation. In fact, ischemia-induced decline of COXI mRNA was observed in pyramidal CA1 neurons using *in situ* hybridization (Abe *et al.* 1993, Abe *et al.* 1998). In addition, down-regulation of mitochondrial transcription and mRNA in both mitochondrial-encoded proteins (COXI and II) and nuclear-encoded proteins (COXIV and Vb) was observed in PC12 cells exposed to hypoxia for 10 h (Vijayasarathy *et al.* 2003). Reoxygenation nearly completely reversed hypoxia-mediated changes in COX mRNA contents and rate of mitochondrial transcription. However, our Northern blot analysis did not reveal significant changes in mRNA of COXI, indicating that depressed mitochondrial translation observed in our study was not due to diminished transcription of mitochondrial genes. Unaltered level of COXI mRNA observed in our study represents only apparent discrepancy to the results published by Abe *et al.* (1993, 1998). Since COX is expressed ubiquitously and CA1 pyramidal neurons represent only a small fraction of rat hippocampus cells,
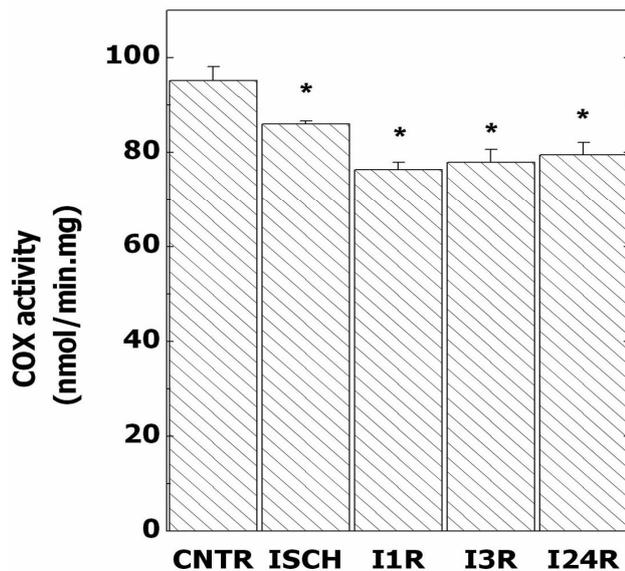
Fig. 5. Effect of ischemia-reperfusion on COX activity. Membrane proteins were isolated from hippocampus of control animals (**CNTR**), animals subjected to 15 min of global ischemia (**ISCH**) and animals subjected to 15 min of global ischemia followed with reperfusion in duration of 1 (**I1R**), 3 (**I3R**) and 24 (**I24R**) hours. Activity of cytochrome c oxidase was determined as described in Materials and Methods. Results are presented as mean ± SD of four independent experiments performed in duplicate. For the comparison of ischemia-induced changes one-way ANOVA test was performed. Additionally, Tukey's test was carried out to demonstrate significant differences between data. * $P < 0.001$.

it is likely that changes observed selectively in CA1 cells are masked by unaltered level of COXI mRNA in the rest of hippocampal cells. Increased proteolysis would be another plausible explanation of decreased level of mitochondrial proteins detected after PAGE analysis. Since our Western blot analysis did not reveal any changes in COXI and COXII protein level, we assume that decreased level of newly synthesized COXI and COXII subunits observed after ischemia was not due to ischemia-induced stimulation of their proteolytic degradation but due to decreased rate of their synthesis. This finding was in particular important with respect to COXII. Degradation of COXII by mitochondrial protease Lon was reported under the conditions of endoplasmic reticulum stress (Hori *et al.* 2002), which is often implicated in pathophysiological mechanisms associated with brain ischemia-reperfusion injury (Paschen and Doutheil 1999). The observed differences between depression of mitochondrial translation and unaltered level of COXI and COXII might be attributed to stability of these proteins.

Based on unaltered transcription and protein degradation, our results indicate that brain ischemia-

reperfusion affects directly mitochondrial translation machinery. Modifications of some translation factors has been recognized as the major cause of ischemia/reperfusion-induced depression of cytosolic translation (Hu and Wieloch 1993, Burda *et al.* 1994, Neumar *et al.* 1995, DeGracia *et al.* 1996, Neumar *et al.* 1998, Martin de la Vega *et al.* 2001, Althausen *et al.* 2001, Mengesdorf *et al.* 2002). Mitochondrial translation is governed by translation factors, which are derived from bacterial translation factors sharing 30-40 % homology to mammalian cytosolic translation factors (Ma and Spremulli 1995). Whether ischemia-reperfusion affects the mitochondrial translation factors in the same way as cytosolic translation factors is unclear and remains to be further investigated. Cytoplasmic translation is almost completely inhibited by ischemia-reperfusion (Hossmann 1993). Thus, our results showing moderate inhibition of mitochondrial protein synthesis might indicate that distinct mechanisms are involved in ischemia/reperfusion-induced inhibition of cytoplasmic and mitochondrial translation.

In addition, we showed that ischemia induced inhibition of COX activity without significant changes of COXI and COXII proteins. It was demonstrated by a histochemical method that COX activity without exogenous cytochrome c decreased in the CA1 neurons from 1 hour after ischemia, but was restored by the addition of exogenous cytochrome c in the following 6 h after ischemia (Nakatsuka *et al.* 2000). These results suggest that it is not COX activity but endogenous cytochrome c that is changed in the early phase after ischemia, and that COX activity begins to decrease 9 h after ischemia. Since our measurements were done in the presence of exogenous cytochrome c, this possibility does not seem to be a plausible explanation of our results. In addition to mitochondrial subunits, Western blot quantification of nuclear-encoded COXVb did not reveal any ischemia-induced changes at the level of this subunit. In yeast, COXVb is a hypoxic gene the transcription of which is increased and exclusively expressed under hypoxic conditions and the increase of COXVb level elevates overall COX activity (Burke and Poyton 1998). However, it seems that in mammals COXVb is transcribed and expressed at high level also under full oxygen availability (Racay *et al.* 2006). Similarly to COXVb, the level of another nuclear-encoded subunit COXIV was not changed one day after global brain ischemia (Sugawara *et al.* 1999). The unaltered level of nuclear-encoded subunits, despite strong depression of

nuclear gene translation observed after global brain ischemia, further supports the view about high stability of COX subunits. On the other hand, we cannot exclude that ischemia-reperfusion does not affect another components of such complex structure of COX. For example, in PC12 cells a 38 % decrease in heme aa3 content was seen after 10 h of hypoxia, while COX activity was reduced only marginally (Vijayasarathy *et al.* 2003). In cultured cerebellar granular cells, significant changes in kinetic parameters of COX (decrease of turnover number, increase of Hill coefficient and increase of K_m of cytochrome c) were documented after 3 h of hypoxia (Horvat *et al.* 2006). These changes were attributed to increased expression of COX subunit IV-2 isoform. In gerbil brain, COX activity was not affected by brain ischemia and short-term reperfusion (Almeida *et al.* 1995). Significant inhibition of COX observed after 120 min of reperfusion was attributed to free radical production and consequent modification of COX (Almeida *et al.* 1995). In fact, recent studies have postulated the effect of posttranslational modifications of COX subunits on COX inhibition. Several mechanisms, like nitrosylation, phoshorylation or oxidative modification by free radicals, were documented. One of the hallmarks of brain ischemia-reperfusion injury is overproduction of nitric oxide (Lipton 1999), which induces irreversible inhibition of COX activity (Zhang *et al.* 2005). In addition, inhibition of COX activity due to either AMP-dependent phosphorylation of COXI (Lee *et al.* 2005) or modification of COX subunits by hydroxynonenal, product of lipid peroxidation, was documented (Chen *et al.* 2001, Kaplan *et al.* 2007). However, structure of COX is extremely complex and its biogenesis involves the coordinated action of two genomes, the three mitochondrial DNA encoded subunits of the catalytic core plus another 10 nuclear DNA encoded subunits and requiring more than 20 additional nuclear-encoded factors (Fontanesi *et al.* 2006, Stibůrek *et al.* 2006). Therefore, possible mechanisms associated with observed ischemia-induced decrease of COX activity should be further investigated using comprehensive proteomic approach.

In conclusion, our results showed that ischemia-reperfusion affects mitochondrial translation machinery leading to inhibition of mitochondrial protein synthesis in rat hippocampus. Although, mitochondria-encoded core subunits I, II and III of COX are translated in mitochondria, based on our data we suggest that inhibition of COX activity is caused by ischemia-induced modification of COX polypeptides rather than by inhibition of mitochondrial translation. Since whole hippocampi were used in our study, observed inhibition of both mitochondrial protein synthesis and activity of COX seems to be a general result of brain ischemia, affecting also other hippocampal cells than the most vulnerable CA1 pyramidal neurons. We assume that generally observed changes together with CA1 specific alterations, observed by morphological approach (Abe *et al.* 1993, 1998, Nakatsuka *et al.* 2000), might significantly aggravate the ischemia-induced mitochondrial dysfunction in these vulnerable neurons.

Conflict of Interest
There is no conflict of interest.

Acknowledgements

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