

Effect of Ageing and Ischemia on Enzymatic Activities Linked to Krebs' Cycle, Electron Transfer Chain, Glutamate and Aminoacids Metabolism of Free and Intrasynaptic Mitochondria of Cerebral Cortex

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Abstract The effect of ageing and the relationships between the catalytic properties of enzymes linked to Krebs' cycle, electron transfer chain, glutamate and aminoacid metabolism of cerebral cortex, a functional area very sensitive to both age and ischemia, were studied on mitochondria of adult and aged rats, after complete ischemia of 15 minutes duration. The maximum rate (V_{max}) of the following enzyme activities: citrate synthase, malate dehydrogenase, succinate dehydrogenase for Krebs' cycle; NADH-cytochrome *c* reductase as total (integrated activity of Complex I–III), rotenone sensitive (Complex I) and cytochrome oxidase (Complex IV) for electron transfer chain; glutamate dehydrogenase, glutamate–oxaloacetate- and glutamate–pyruvate transaminases for glutamate metabolism were assayed in non-synaptic, perikaryal mitochondria and in two populations of intra-synaptic mitochondria, i.e., the light and heavy mitochondrial fraction. The results indicate that in normal, steady-state cerebral cortex, the value of the same enzyme activity markedly differs according (a) to the different populations of mitochondria, i.e., non-synaptic or intra-synaptic light and heavy, (b) and respect to ageing. After 15 min of complete ischemia, the enzyme activities of mitochondria located near the *nucleus* (perikaryal mitochondria) and in

synaptic structures (intra-synaptic mitochondria) of the cerebral tissue were substantially modified by ischemia. Non-synaptic mitochondria seem to be more affected by ischemia in adult and particularly in aged animals than the intra-synaptic light and heavy mitochondria. The observed modifications in enzyme activities reflect the metabolic state of the tissue at each specific experimental condition, as shown by comparative evaluation with respect to the content of energy-linked metabolites and substrates. The derangements in enzyme activities due to ischemia is greater in aged than in adult animals and especially the non-synaptic and the intra-synaptic light mitochondria seems to be more affected in aged animals. These data allow the hypothesis that the observed modifications of catalytic activities in non-synaptic and intra-synaptic mitochondrial enzyme systems linked to energy metabolism, amino acids and glutamate metabolism are primary responsible for the physiopathological responses of cerebral tissue to complete cerebral ischemia for 15 min duration during ageing.

Keywords Brain energy metabolism · Ischemia · Enzymes · Glutamate and amino acids · Non-synaptic and intra-synaptic mitochondria · Ageing

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Introduction

Damage due to brain ischemia represents one of the greatest challenges of today research in Neuroscience. Among the cellular compartments of high functional significance damaged by ischemia are mitochondria of different populations termed non-synaptic (free) and intra-synaptic (light and heavy) with respect to their subcellular localization in vivo.

Enzymes related to both energy metabolism and amino acid metabolism are linked to non-synaptic perikaryal and intra-synaptic structures highly sensitive to changes of the biological environment. In this context, “physiological” ageing has been found to modify enzyme activities differently in the mitochondria populations: increases in malate dehydrogenase, succinate dehydrogenase, NADH-cytochrome *c* reductase (both total and rotenone sensitive) and glutamate–pyruvate transaminase in the heavy type of intra-synaptic mitochondria, whereas no changes were found in the other mitochondrial population studied [1] largely confirming results of the former investigations on normal ageing [2–4]. More pronounced abnormalities in mitochondria-linked enzyme activities were found under defined experimental conditions such as brain hypoxia and ischemia [5–7].

Ageing has been demonstrated to be the most important risk factor for brain ischemia. We, therefore, were interested to study the effect of age on the ischemia-induced damage on specific enzyme activities linked to energy and amino acid metabolism of non-synaptic and intra-synaptic mitochondria. A 15-min complete cerebral ischemia induced preferentially changes in enzyme activities linked to energy metabolism in non-synaptic mitochondria in adult animals. The ischemic damage was found to be more pronounced in aged animals.

Materials and Methods

Animals Care

The experiments were performed on 1-year and 2-year old male Wistar rats provided by the Zentralinstitut für Versuchstierzucht, Hannover, FRG. The selection of the animals and the time course of physiopathological experimental condition of ischemia of 15 min duration were established by permutation tables and the animals were allotted randomly to the respective control and ischemic groups. At the start of the experiment, each group comprised 8–10 animals.

Rats may be designated as “aged” when their strain has a 50% survival rate and when their survival curve is more or less rectangular. In male Wistar rats, this deflection point was found to be at the age of 24 months [8]. The period of complete cerebral ischemia of 15 min duration was chosen to induce characteristics and long-lasting metabolic aberrations.

Preparative Procedures for Experimental Ischemia

One week prior to the ischemic procedure, the animals were anesthetized by intraperitoneal injection with

chloralhydrate ($8 \text{ mg} \times \text{kg}^{-1}$ body weight of a 4% solution). Both vertebral arteries were exposed at the alar foramina of the atlas and closed by electrocoagulation. The common carotid arteries were also exposed and wound around with threads without damaging the vessels. The incision were sutured and the animals were left quiet for a further week. They had free access to food and water.

For the final experiment, anaesthesia was started with 3.0 vol% halothane, and tracheotomy and intubation were performed. Anesthesia was then continued with 1.5% halothane and nitrous oxide/oxygen 70:30. The animals were immobilized with pancuronium bromide ($2 \text{ mg} \times \text{kg}^{-1}$, b. wt.) and artificially ventilated. Femoral arteries and veins were exposed and catheterized to measure arterial blood pressure by means of a blood pressure transducer and a Hellige polygraph and to sample arterial blood for gas analysis and for the measurements of hemoglobin, hematocrit, glucose and lactate concentrations. If necessary, bicarbonate was applied via the venous catheter to maintain acid-base equilibrium. The skull was sagittally incised, and a skin funnel was formed for later freezing of the brain. After this operation procedure, anesthesia was continued with 0.5 vol% halothane and nitrous oxide/oxygen 70:30, until steady state conditions were established. Halothane application then ceased and anesthesia was continued with nitrous oxide/oxygen 70:30 only.

Within five minutes, expired halothane concentration fell from 0.5 vol% to 0.07 vol% (WTI Anesthetic Gas Monitor, Jaeger and Koellisch, Munich, FRG: single). Anesthesia with 0.5 vol% halothane and nitrous oxide/oxygen 70:30 may be assumed to be appropriate in animal experiments requiring steady state conditions. Painful stimuli and autonomic reflexes are avoided.

Nitrous oxide in the concentration used as in the present study was not found to influence either cortical cerebral blood flow or overall glucose utilization in the brain [9, 10]. Although halothane in as low concentration as 0.6 vol% reduced the cerebral metabolic rate of oxygen by 25% [11], no variations in glycolytic flux and in cerebral energy state, except for a decrease of glucose concentration, could be observed under anesthesia with 1.0 vol% halothane [12, 13]. However, (a) to completely avoid the influence of halothane on ischemic tissue damage reported by Smith [14] and Michenfelder and Milde [15] for higher halothane concentrations in monkeys and dogs, (b) to avoid a possible negative effect on mitochondrial respiration in steady-state control condition and (c) to be able to perform studies on ischemia of 15 min duration without any anesthetic side effects, halothane anesthesia was discontinued with the onset of the steady state in all groups investigated.

Induction of Experimental Complete Cerebral Ischemia

A steady state of arterial normotension (mean arterial blood pressure MABP: 100–120 mmHg), normocapnia (paCO₂: 35–42 mmHg), normoxemia and slight hyperoxemia, respectively (paO₂: 100–180 mmHg) and normothermia (body temperature: 37.0–37.5°C) was maintained over 20 min. After this period, complete cerebral ischemia (CCI) was induced for 15 min by looping the threads wound around the common carotid arteries. This device allows the occlusion of the arteries with only minimal damage to the vessels. To avoid retrograde perfusion of the brain via the anterior spinal artery, MABP was reduced to 30–40 mmHg by hypovolemic hypotension. This procedure gives rise to a total circulatory arrest in the brain [16]. Otherwise, a respiration controlled arterial hypotension of MABP 30–40 mmHg caused only minimal changes in arterial oxygen tension in an early phase of shock, because of remarkably small changes in lung mechanics with a drop of venous admixture to arterial blood [17].

Two other groups of 1-year and 2-year-old animals were sham operated 1 week prior to the final experiment. The brains of these control animals were frozen in situ in liquid nitrogen and the samples of fronto-parieto-temporal cerebral cortex were removed rapidly and prepared in an appropriate isolation medium at 0–4°C (see after), under the same steady state conditions mentioned above. Animals in which the experimental conditions could not be maintained were excluded.

For biochemical assays of 1-year and 2-year old animals, after this ischemic period of 15 min duration, the brains were frozen in situ by liquid nitrogen, as indicated previously; thereafter, they were removed and the samples of fronto-parieto-temporal cerebral cortex were isolated and immersed in an appropriate isolation medium at 0–4°C (see after). This is the best way to preserve not only metabolites, but also the catalytic activity of enzymes, allowing the sub-fractionation of the cerebral tissue, as previously reported [2, 7, 18–24].

Preparation of Synaptosomal Fraction and Non-Synaptic Mitochondria

Non-synaptic (free) and intra-synaptic (two types) mitochondria were prepared from cerebral cortex of single animal by the method of Lai et al. [25], as modified and described in details for analytical evaluations by Villa et al. [4, 26].

The rats aged 12 and 24 months from the various experimental groups were sacrificed as previously indicated and the subsequent procedures were performed at 0–4°C. The samples of fronto-parieto-temporal cerebral cortex were immediately placed in the isolation medium (0.32 M

sucrose, 1.0 mM EDTA-K⁺, 10 mM Tris-HCl, pH 7.4). The homogenate (usually 5 ml final volume) was obtained using a Teflon-glass homogenizer (Braun S Homogenizer) by five up and down strokes of the pestle (total clearance: 0.1 mm) rotating at 800 rpm × min⁻¹, with electronic control of the pestle speed. The homogenate was then centrifuged at 3.6 × 10³ g × min (2.37 × 10⁷ ω²t) in a Beckman J2–21 Supercentrifuge, rotor JA-17.

The nuclear pellet was washed twice and the three combined supernatants were centrifuged at 288 × 10³ g × min (189.2 × 10⁷ ω²t) to yield the “crude” mitochondrial pellet. The “crude” mitochondrial pellet containing the synaptosomes was washed by resuspending in the isolation medium and centrifuged in the same conditions. The “crude” mitochondrial fraction containing synaptosomes was re-suspended by soft homogenization in the isolation medium and was placed on discontinuous Ficoll-sucrose gradients (7.5%–12%, w/w), the Ficoll dissolved in stock solution consisting of 0.32 M sucrose, 50 μM EDTA-K⁺, 10 mM Tris-HCl, pH 7.4. This gradient was centrifuged at 175.2 × 10⁴ g × min (123.7 × 10⁸ ω²t) in a OTD-65B Sorvall Ultracentrifuge (AH-650 type rotor).

After centrifugation, the myelin fraction was suckled off and the synaptosomal fraction at the interface of the 7.5–12% Ficoll-sucrose interphase was collected by aspiration, then diluted threefold with isolation medium and centrifuged at 288 × 10³ g × min (189.2 × 10⁷ ω²t), rotor JA-17. The purified “free” mitochondrial fraction was re-suspended in buffered sucrose solution 0.32 M, pH 7.4, and pelleted at 162.4 × 10³ g × min (106.7 × 10⁷ ω²t); the pellet was then re-suspended by soft homogenization in a small volume of 0.32 M sucrose buffered solution (pH 7.4) for the assay of the catalytic activity of enzymes.

Preparation of Intra-Synaptic Mitochondria

The synaptosomal pellet previously isolated from Ficoll-sucrose gradient, was lysed by resuspension in 6 mM Tris-HCl, pH 8.1, by soft homogenization [26].

After osmotic shock, the lysate was centrifuged at 399 × 10³ g × min (262.1 × 10⁷ ω²t) and the pellet obtained from lysed synaptosomes was again re-suspended and centrifuged at 192.6 × 10³ g × min (126.5 × 10⁷ ω²t); at the end of this centrifugation, the pellet was re-suspended in a medium (3% w/w Ficoll, 0.12 M mannitol, 30 mM sucrose, 25 μM EDTA-K⁺, 5 mM Tris-HCl, pH 7.4). This suspension was layered on a Ficoll discontinuous gradient consisting of two layer 4.5% w/w Ficoll in 0.24 M mannitol, 60 mM sucrose, 50 μM EDTA-K⁺, 10 mM Tris-HCl, pH 7.4 and, at the bottom, 6% w/w Ficoll in the same solution. This gradient was centrifuged at 280.2 × 10³ g × min (197.3 × 10⁷ ω²t), rotor AH-650.

At the end of this centrifugation, the upper phase, the “light” intra-synaptic mitochondrial fraction, was sucked off and pelleted at $166.5 \times 10^3 g \times \text{min}$ ($109.4 \times 10^7 \omega^2 t$); the pellet of this centrifugation and that from the gradient, the “heavy” intra-synaptic mitochondrial fraction, were separately re-suspended in 0.32 M sucrose buffered solution (pH 7.4) and centrifuged at $162.4 \times 10^3 g \times \text{min}$ ($106.7 \times 10^7 \omega^2 t$). The washed pellets were finally re-suspended in the same washing solution for the assay of the catalytic activity of enzymes.

Enzyme Assays

On the different subcellular mitochondrial fractions, the maximum rate (V_{max}) of the following enzyme activities was evaluated: citrate synthase (citrate oxaloacetate-lyase, EC 4.1.3.7) [27]; malate dehydrogenase (L-malate: NAD + oxidoreductase, EC 1.1.1.37) [28]; succinate dehydrogenase (succinate: oxidoreductase, EC 1.3.99.1) [29] for Krebs’ cycle; NADH-cytochrome *c* reductase (NADH-cytochrome *c* oxidoreductase, EC 1.6.99.3) [30] and cytochrome oxidase (ferrocytochrome *c*: oxygen oxidoreductase, EC 1.9.3.1) [31–33] for electron transfer chain (ETC); glutamate dehydrogenase (L-glutamate: NAD + oxidoreductase deaminating, EC 1.4.1.3) [27], glutamate-oxaloacetate transaminase (L-aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1) [25], glutamate-pyruvate transaminase (L-alanine: 2-oxoglutarate aminotransferase, EC 2.6.1.2) [34]. In some instance, catalytic enzyme activity was assayed in presence of inhibitors (rotenone), Triton X-100 and/or Tween 20 in the assay medium, at concentrations useful to release the maximum activity of each specific enzyme.

The protein concentration of the tested subfractions was determined using crystalline bovine serum albumin as standard, according to Lowry et al. [35].

Enzyme Activity Calculation

Enzyme catalytic activities were measured by graphic recordings for at least 3 min in a double beam recorder Spectrophotometer (Perkin–Elmer 554, 541S) or by automatic calculation of absorbance variations for at least 5 min using a Beckman DU-8 Spectrophotometer equipped with Kinetics II systems and temperature control. Each value was calculated from two blind determinations on the same sample and catalytic activities were expressed as specific activities in $(\text{micromoles of substrate transformed}) \times \text{min}^{-1} \times (\text{mg of mitochondrial protein})^{-1}$ of the subfraction tested.

This value is an extensive quantity and the quantities derived from it include “specific catalytic activity” and “catalytic activity”; hence, the values calculated may be: (a) enzyme specific activity (S.A.) if expressed as

$(\text{micromoles of substrate transformed}) \times \text{min}^{-1} \times (\text{mg of protein})^{-1}$; (b) enzyme catalytic activity (SI Units), if expressed as nanokatal: $(\text{nanomoles of substrate transformed}) \times \text{sec}^{-1}$ [3, 26].

Statistical Analysis

The two ways ANOVA test was used to evaluate the comparisons of enzyme activity between the different mitochondrial populations as well as of the effects of ischemia (15 min) and after recovery times. The ANOVA test for multiple comparisons was used to evaluate the interactions between the values of control group (sham operated animals) versus ischemic groups of animals of both ages, for each mitochondrial sub-fractions, for each individual enzyme activity and for each biochemical parameter tested. The homogeneity of variance was checked by Bartlett’s test and post hoc tests were used to compare the differences between individual groups, controlling statistical evaluation by the Tukey’s and Dunnett’s tests.

Ethics

The experimental protocol was approved by the review committee for animal experimentation of the Medical Faculty of the University of Heidelberg and by responsible Germany Government Agency.

Results

The maximum rates (V_{max}) of some mitochondrial enzyme activities related to energy and aminoacid metabolism such as citrate synthase (CS), malate dehydrogenase (MDH) and succinate dehydrogenase (SDH) for Krebs’ cycle (TCA), the last being also an ETC-linked enzyme (Complex II); NADH-cytochrome *c* reductase total activity (NADH-CCRT) and rotenone sensitive NADH-cytochrome *c* reductase (NADH-CCRS) as integrated activity of Complex I–III [36] and cytochrome *c* oxidase (COX, Complex IV) for the electron transfer chain (ETC); glutamate dehydrogenase (GIDH), glutamate-oxaloacetate-(GOT) and glutamate-pyruvate-(GPT) transaminases were evaluated in non-synaptic (or free, perikaryal) mitochondria and in intra-synaptic mitochondria (light and heavy ones) from rat fronto-parieto-temporal cerebral cortex. These three types of mitochondria were isolated from brain of rats aged 12 and 24 months.

In general, in rat cerebral cortex the biochemical machinery, as evaluated by enzyme activity, was differently expressed in non-synaptic free and in intra-synaptic light and heavy mitochondrial sub-populations at each

single age. With respect to the enzyme patterns of these three types of mitochondria non-synaptic (free) and intra-synaptic (two types: light and heavy) the different maximum rates of almost all enzymes were observed, these activities being lower in the synaptic heavy mitochondrial sub-populations than in free and intra-synaptic light mitochondrial sub-population during ageing (Figs. 1, 2, 3, 4, 5), with some exceptions (for a complete discussion see 1).

In steady state control conditions, i.e., in sham operated rats, enzyme activity values of adult animals were chosen as the reference value to evaluate the effect of ageing per se.

Briefly, citrate synthase activities were not different between the different types of mitochondria of two age

groups, but the activity was lower in heavy mitochondria of adult animals respect to non-synaptic mitochondria, as malate dehydrogenase activity (Fig. 1). Succinate dehydrogenase activities (Complex II) were found to be lower especially in intra-synaptic heavy mitochondria of adult animals (and in intra-synaptic light of aged ones), as compared to non-synaptic and intra-synaptic light mitochondria (Fig. 2). Also NADH-cytochrome *c* reductase as total (Complex I–III) and rotenone sensitive NADH-cytochrome *c* reductase (Complex I) activities were lower especially in heavy mitochondria of adult animals, as compared to non-synaptic and intra-synaptic light mitochondria (Fig. 2). The exceptions are related to an increased activity for both NADH-cytochrome *c* reductase

Fig. 1 Specific enzymatic activities (expressed as micromoles of substrate transformed) $\times \text{min}^{-1} \times (\text{mg of mitochondrial protein})^{-1}$) of citrate synthase and malate dehydrogenase of “free” (FM) and intra-synaptic “light” (LM) and “heavy” (HM) mitochondria from *cerebral cortex* of “adult” (1 year-old) and “aged” (2 year-old) male Wistar rats in control animals. Results are the mean \pm standard error of the mean of $n = 7$ –10 animals. Statistical analysis performed by ANOVA and control-test; 1 symbol: $P < 0.05$; 2 symbols: $P < 0.01$. Symbols of comparisons: # controls 1 year versus controls 2 years; § control adult or aged animals versus ischemia for all types of mitochondria

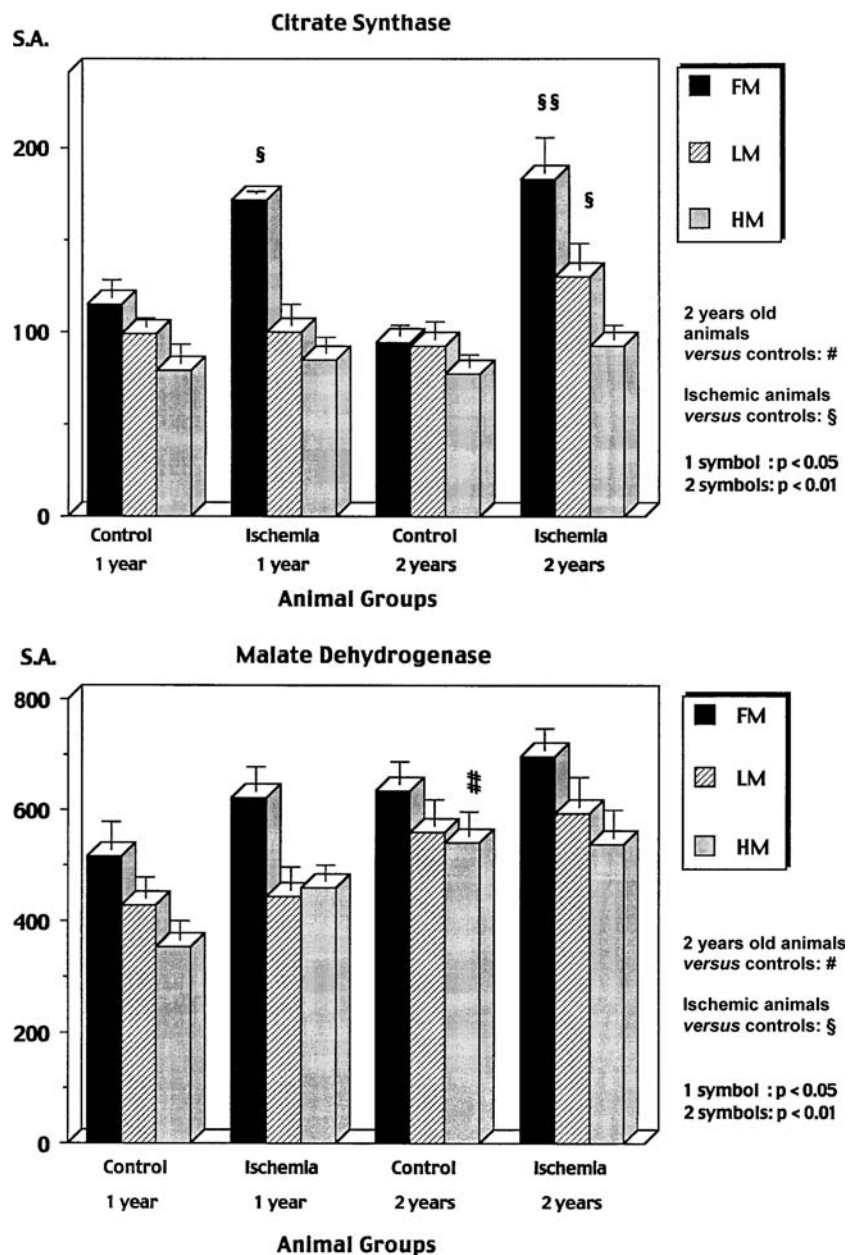
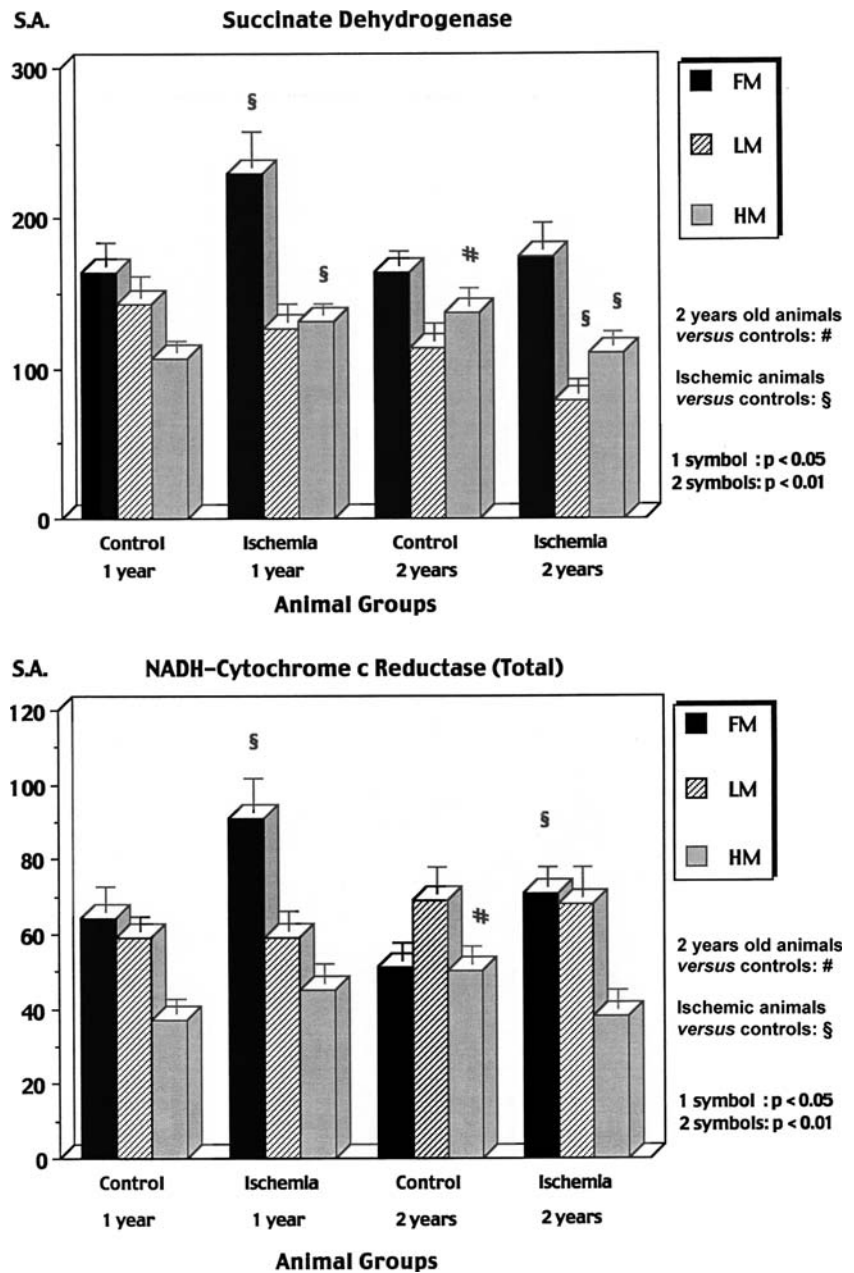


Fig. 2 Specific enzymatic activities of succinate dehydrogenase (expressed as micromoles of $K_3Fe(CN)_6$ or substrate transformed, respectively) $\times \text{min}^{-1} \times (\text{mg of mitochondrial protein})^{-1}$ and “total” NADH-cytochrome *c* reductase (expressed as micromoles of substrate transformed) $\times \text{min}^{-1} \times (\text{mg of mitochondrial protein})^{-1}$ of “free” (FM) and intra-synaptic “light” (LM) and “heavy” (HM) mitochondria from cerebral cortex of “adult” (1 year-old) and “aged” (2 year-old) male Wistar rats in control animals. Results are the mean \pm standard error of the mean of $n = 7-10$ animals. Statistical analysis performed by ANOVA and control-test; 1 symbol: $P < 0.05$; 2 symbols: $P < 0.01$. Symbols of comparisons: # controls 1 year versus controls 2 years; § control adult or aged animals versus ischemia for all types of mitochondria



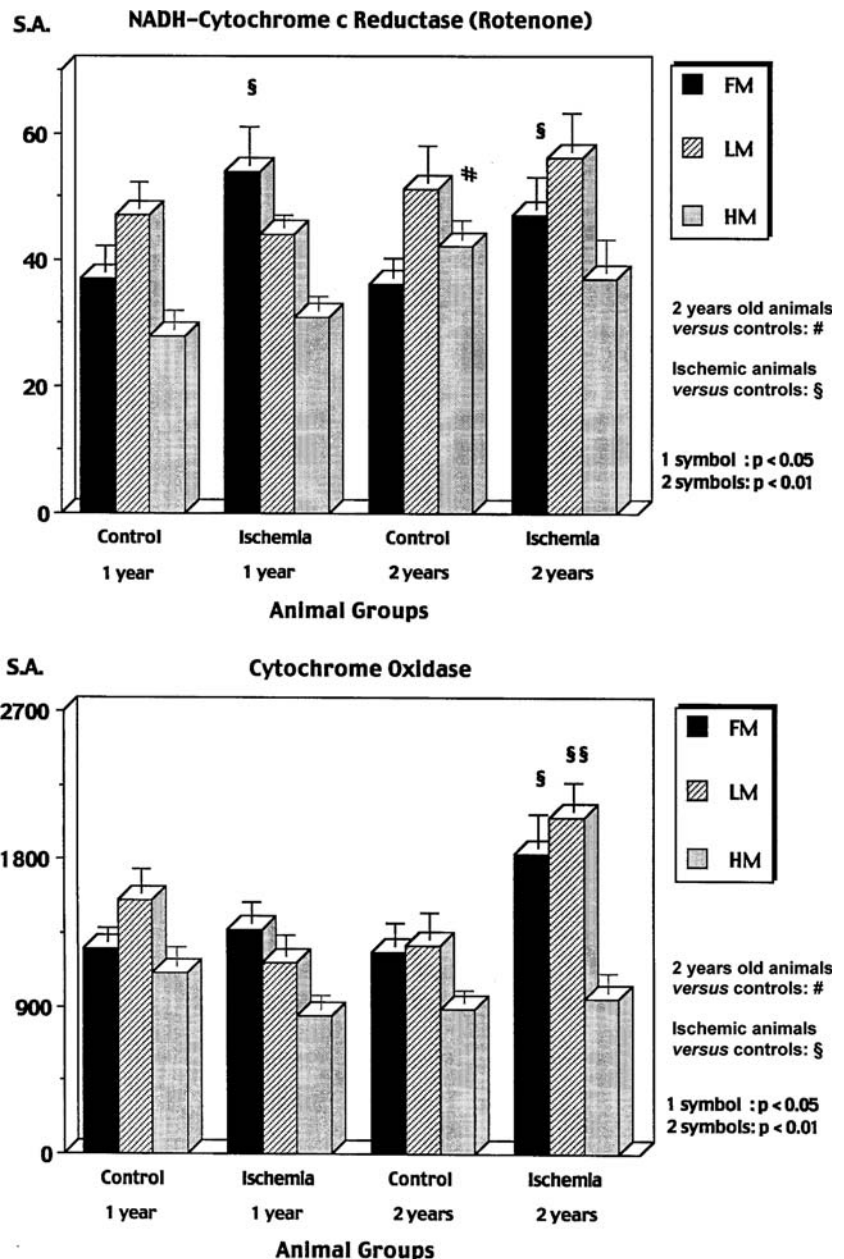
enzymes in the light fraction (aged animals) and for the activity of Complex IV, because cytochrome oxidase is higher in light mitochondria from adult animals respect to that of non-synaptic mitochondria (Fig. 3), as described in details elsewhere [1].

Glutamate dehydrogenase activity was lower in intra-synaptic heavy mitochondria as compared to free in adult animals or to light mitochondria of both ages (Fig. 4). Also glutamate-oxaloacetate-transaminase activities were lower in light and in heavy mitochondria as compared to free of both ages (Fig. 4), while glutamate-pyruvate-transaminase was higher in heavy mitochondria as compared to free (aged) when assayed on intact mitochondria (Fig. 5), being

not different when the enzyme activity was assayed in Triton X-100 treated mitochondria (“total” activity GPT-TX, Fig. 5), as described in details elsewhere [1].

With respect to the age factor, as regards the catalytic differences in enzyme activities between the non-synaptic free (FM) mitochondria and intra-synaptic “light” (LM) and “heavy” (HM) mitochondria in control animals it was observed (Figs. 1, 2, 3) that the activities of malate dehydrogenase, succinate dehydrogenase, NADH-cytochrome *c* reductase as total, and rotenone sensitive NADH-cytochrome *c* reductase of heavy mitochondria from aged animals were higher than in the corresponding type of mitochondria of adult ones, while no differences were

Fig. 3 Specific Enzymatic Activities (expressed as micromoles of substrate transformed) \times min⁻¹ \times (mg of mitochondrial protein)⁻¹ of NADH cytochrome *c* reductase rotenone sensitive and cytochrome oxidase of “free” (FM) and intra-synaptic “light” (LM) and “heavy” (HM) mitochondria from *cerebral cortex* of “adult” (1 year-old) and “aged” (2 year-old) male Wistar rats in control animals. Results are the mean \pm standard error of the mean of $n = 7$ –10 animals. Statistical analysis performed by ANOVA and control-test; 1 symbol: $P < 0.05$; 2 symbols: $P < 0.01$. Symbols of comparisons: # controls 1 year versus controls 2 years; § control adult or aged animals versus ischemia for all types of mitochondria



observed for citrate synthase and cytochrome oxidase activities (Figs. 1, 2, 3).

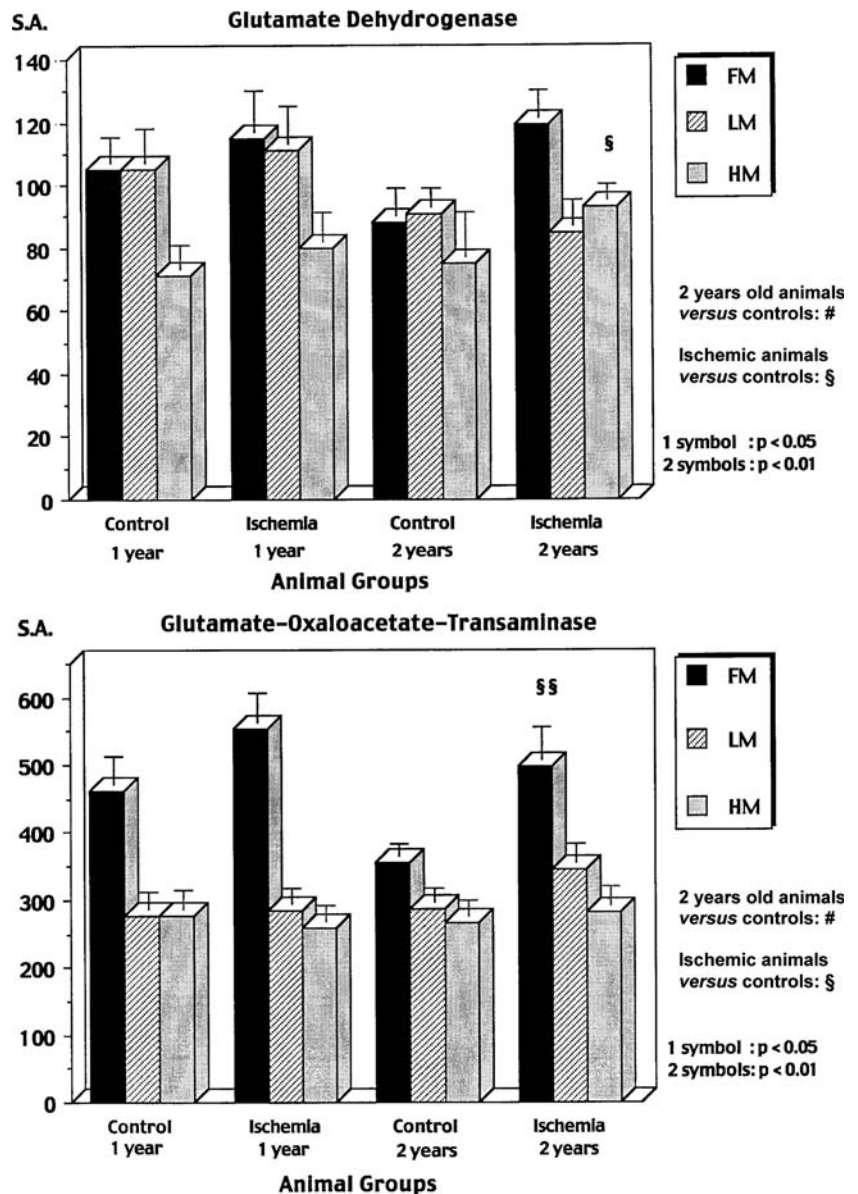
As far as glutamate metabolism and the catalytic differences in enzyme activities between the non-synaptic “free” (FM) mitochondria and intra-synaptic “light” (LM) and “heavy” (HM) mitochondria in control adult animals are concerned with respect to those of the corresponding types of mitochondria from aged animals, the only difference was related to glutamate-pyruvate transaminase “total” activity (Triton X-100), greater in heavy mitochondria of aged animals (Fig. 5).

With regard to the effect of ischemia in adult and aged animals, a period of 15-min of complete cerebral ischemia

increased citrate synthase activity in non-synaptic mitochondria at both ages and only in aged animals in intra-synaptic light ones, while malate dehydrogenase was unaffected (Fig. 1). Succinate dehydrogenase (only adult animals, Fig. 2), NADH cytochrome *c* reductase as total (Fig. 2) and rotenone sensitive (Fig. 3) activities on non-synaptic mitochondria of both ages were increased by ischemia, while cytochrome oxidase activity was increased in non-synaptic and in intra-synaptic light mitochondria from aged animals (Fig. 3).

In intra-synaptic mitochondria, the decrease of succinate dehydrogenase activity was observed (Fig. 2) in both types of intra-synaptic light and heavy mitochondria from

Fig. 4 Specific enzymatic activities (expressed as micromoles of substrate transformed) \times min⁻¹ \times (mg of mitochondrial protein)⁻¹ of glutamate dehydrogenase and glutamate-oxaloacetate-transaminase of “free” (FM) and intra-synaptic “light” (LM) and “heavy” (HM) mitochondria from cerebral cortex of “adult” (1 year-old) and “aged” (2 year-old) male Wistar rats in control animals. Results are the mean \pm standard error of the mean of $n = 7-10$ animals. Statistical analysis performed by ANOVA and control-test; 1 symbol: $P < 0.05$; 2 symbols: $P < 0.01$. Symbols of comparisons: # controls 1 year versus controls 2 years; § control adult or aged animals versus ischemia for all types of mitochondria



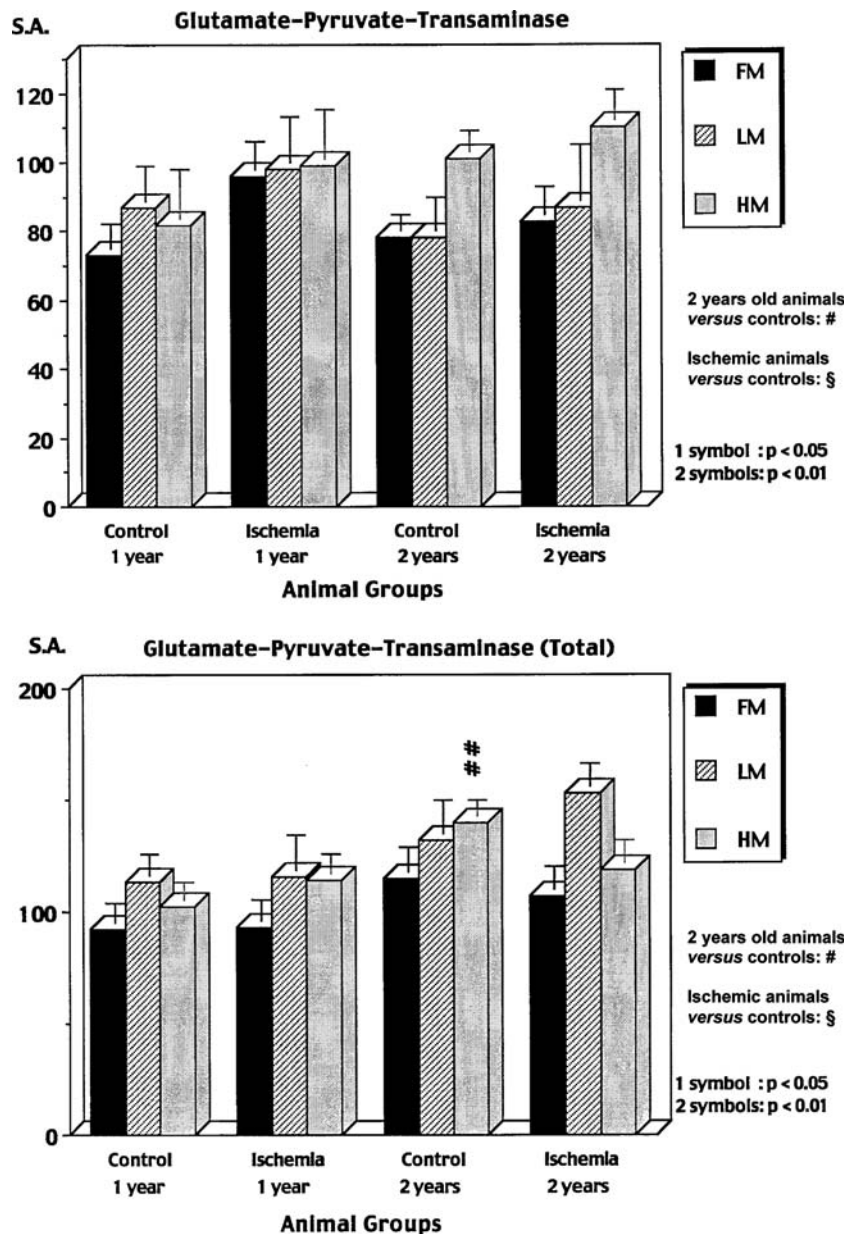
aged animals, while the protein concentration and content remained unaltered in any case (Fig. 6). All the others evaluated enzymatic activities (Fig. 4, 5) were unchanged by complete cerebral ischemia (CCI) of 15 min duration, with the exception of an increased activity of glutamate dehydrogenase (aged synaptic heavy mitochondria) and glutamate-oxaloacetate transaminase (aged non-synaptic mitochondria, Fig. 4).

The enzyme’s activities of all these enzymes were also assayed in presence of Triton X-100 aimed to evaluate the “total” activity released after the disintegration of mitochondrial membranes, and the given results were not different; as an example of the effect of Triton X-100, in Fig. 5 GPT activities are reported.

In summary, ageing affected enzyme activities by complete ischemia lasting 15 min: for the Krebs’ cycle enzymes, citrate synthase activity was stimulated particularly in non-synaptic mitochondria (and in light mitochondria from aged brain), succinate dehydrogenase activity was increased on non-synaptic and heavy mitochondria from adult animals. Interestingly, the activity was decreased in intra-synaptic light and heavy ones from aged animals. NADH-cytochrome *c* reductase activities were increased in non-synaptic mitochondria at both ages, like cytochrome oxidase in non-synaptic and intra-synaptic light mitochondria (only aged brain).

As regards the enzymes related to amino acid metabolism, only glutamate dehydrogenase and glutamate-

Fig. 5 Specific enzymatic activities (expressed as μM of substrate transformed) $\times \text{min}^{-1} \times (\text{mg}$ of mitochondrial protein) $^{-1}$) of glutamate-pyruvate-transaminase assayed in absence and in presence of Triton-X100 of “free” (FM) and intra-synaptic “light” (LM) and “heavy” (HM) mitochondria from cerebral cortex of “adult” (1 year-old) and “aged” (2 year-old) male Wistar rats in control animals. Results are the mean \pm standard error of the mean of $n = 7$ –10 animals. Statistical analysis performed by ANOVA and control-test; 1 symbol: $P < 0.05$; 2 symbols: $P < 0.01$. Symbols of comparisons: # controls 1 year versus controls 2 years; § control adult or aged animals versus ischemia for all types of mitochondria



oxaloacetate-transaminase were stimulated only in aged animals and on different types of mitochondria (Fig. 4). Both protein concentration and content were not modified by ischemia in adult and aged rats (Fig. 6).

Thus, perikaryal, non-synaptic mitochondria seem to be more affected in adult and particularly in aged animals, while the non-synaptic light mitochondria of adult animals were not affected by ischemia (with the exception of the increased succinate dehydrogenase activity in heavy mitochondria); the heavy mitochondria, particularly in aged mitochondria, were affected by ischemia and age but to a lesser extent than the non-synaptic ones (Table 1).

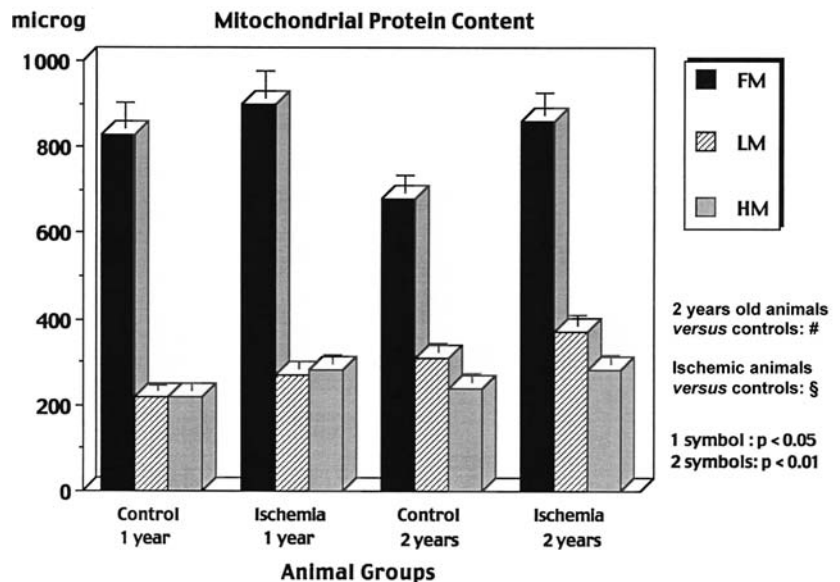
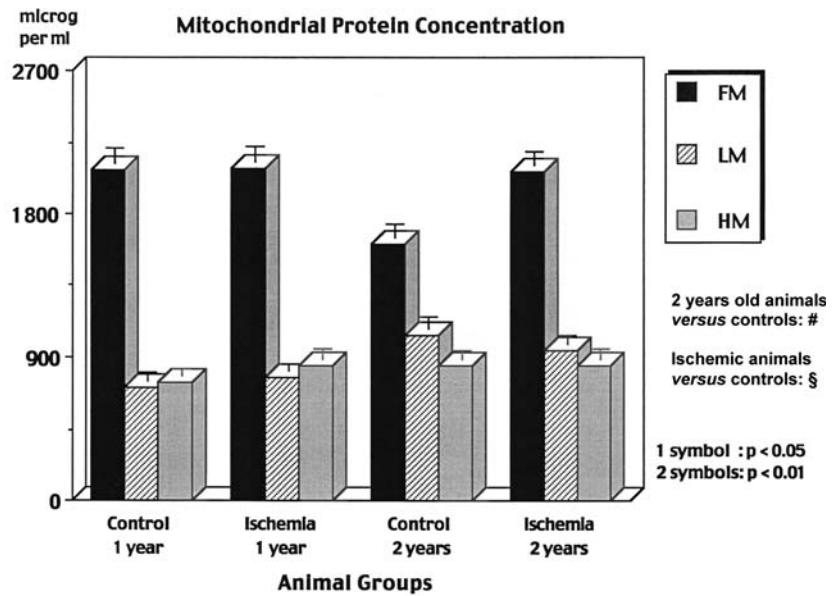
Considering all these data, it may be concluded that the biochemical machinery of mitochondria related to the

energy linked enzymes and those of amino acid and glutamate metabolism appears to be differently affected during ageing and particularly in their catalytic sensitivity to ischemia. In fact, when analyzing these results in detail, it clearly appears that the activities of these enzymes possess distinct catalytic power depending on the age, ischemia and particularly on the different type of mitochondrial populations on which the activity was detected.

Discussion

These results confirm that during the metabolic steady-state a different maximum rate of enzyme activities of Krebs'

Fig. 6 Protein concentration (expressed as mg of mitochondrial protein \times ml⁻¹) or content (multiplying \times 0.3, expressed as μ g) of “free” (FM) and intra-synaptic “light” (LM) and “heavy” (HM) mitochondria from cerebral cortex of “adult” (1 year-old) and “aged” (2 year-old) male Wistar rats in control animals. Results are the mean \pm standard error of the mean for $n = 7$ –10 animals. Statistical analysis performed by ANOVA and control-test; 1 symbol: $P < 0.05$; 2 symbols: $P < 0.01$. Symbols of comparisons: # controls 1 year versus controls 2 years; § control adult or aged animals versus ischemia for all types of mitochondria



cycle, electron transfer chain, glutamate and aminoacid metabolism occurs in various types of cerebral cortex mitochondria showing also that these biochemical enzyme systems undergo diversified changes in ageing [1], in agreement with previously data published by us on Sprague-Dawley strain in “young”, “adult” and “aged” rats [3, 21].

The major interest of our finding is that the distinct metabolic profiles of these mitochondrial populations appear to be not a subtle and unclear problem of biochemical micro-heterogeneity, but a possible relevant factor with respect to ageing processes, both in physiological and pathological brain, allowing a model to study physiopathological changes in functional proteomics of

mitochondria by a large *spectrum* analysis, also respect to sensitivity to drug’s treatment [4].

In particular, *as regards the age factor* in steady-state condition, the complete discussion about the relationships between catalytic enzyme activities up-to-24-months during aging evaluated at 2 months intervals is very intriguing and the theoretical implications about energy metabolism suggest that specific biochemical situations take place at each single age, an observation of physio-pathological and pharmacological significance (for a review see 1, 21).

Thus, the major point in this research was to assess if during physiopathological conditions cerebral mitochondria retained the catalytic power of their specific enzyme systems to yield energy (TCA, ETC) and to metabolize

Table 1 Comparative scheme of increased (↑) or decreased (↓) catalytic activities of indicated enzymes evaluated on “perikaryal” non-synaptic mitochondria, intra-synaptic “light” and intra-synaptic “heavy” mitochondria of fronto-parietal-temporal cerebral cortex from brain of Wistar rats aged 1 and 2 years after 15 min of complete cerebral ischemia respect to control “steady-state condition” (qualitative changes)

Perikaryal mitochondria	1 Year ischemia 15 min	2 Year Ischemia 15 min	Intrasynaptic light mitochondria	1 Year ischemia 15 min	2 Year ischemia 15 min	Intrasynaptic heavy mitochondria	1 Year ischemia 15 min	2 Year ischemia 15 min
CS	↑	↑↑	CS		↑	CS		
MDH			MDH			MDH		
SDH	↑		SDH		↓	SDH	↑	↓
CCRT	↑	↑	CCRT			CCRT		
CCRS	↑	↑	CCRS			CCRS		
COX		↑	COX		↑↑	COX		
GIDH			GIDH			GIDH		↑
GOT		↑↑	GOT			GOT		
GPT			GPT			GPT		
GPT-TX			GPT-TX			GPT-TX		
Protein								

Legend: Significance of changes: (↑) means increased or (↓) decreased activity versus control; statistical significance: 1 symbol: $P < 0.05$; 2 symbols; $P < 0.01$

glutamate and aminoacid, evaluating the potential catalytic power and/or the derangements induced by ischemia on the catalytic properties of enzymes.

The results showed that 15 min of ischemia stimulated the activity of citrate synthase, that exerts one of the major point of metabolic control in the Krebs' cycle (ΔG (kJ/mol) = negative; ΔG^0 (kJ/mol) = -31.5), only in perikaryal mitochondria of adult animals, and in perikaryal or intra-synaptic light mitochondria in aged animals, confirming the stimulation of Krebs' cycle in ischemia. In addition, the increased activity of GOT and GIDH was detected in aged cerebral cortex mitochondria, in perikaryal and intra-synaptic heavy mitochondria, respectively, also indicating an influence on glutamate metabolism.

On cerebral tissue, the changes undergone by energy metabolites during ischemia are influenced by age, in that the effects on the metabolic sequences of TCA cycle is accentuated at the beginning, but interestingly the drop of Energy Charge Potential (ECP) was less in aged animals and ATP seems to be less utilized in aged rats [37, 38]. In particular, this last observation well correlates with enzymatic data on Na^+ , K^+ -ATPase in adult and particularly in aged animals, being these activities not different for the former or decreased for the latter in *hippocampus* as indicated by our results on ATP-ases during ageing and ischemia [7].

In particular, Hoyer and Krier [37] have shown that in the *fronto-parieto-temporal cerebral cortex*, during 15 min of complete occlusion of the circulation, the concentrations of many metabolic intermediates of glycolysis, tricarboxylic acid cycle (TCA) and energy-rich compounds (ATP,

ADP, CrP, ECP and the sum of adenine nucleotides AN) exhibited remarkable changes, while no significant differences in brain substrate concentrations were found in steady-state between adult and aged control rats, except succinate concentration, lower in aged animals [37]. A 15 min of complete cerebral ischemia (CCI) caused the drop of cortical glucose, pyruvate, citrate, α -ketoglutarate, malate, oxaloacetate, ATP and CrP, whereas lactate, succinate and AMP increased and particularly succinate rose more in aged animals. Comparing these results on metabolites with those of the evaluated enzymes in different types of mitochondria, an interesting picture emerges, also because the experimental model of CCI is similar.

In fact, *as regards the different types of mitochondria*, the non-synaptic mitochondria are much more affected by ischemia than intra-synaptic ones at both ages (1 or 2 years) during the acute phase of ischemia, increasing the activity of CS, SDH (Complex II), CCRT (Complex I–III), CCRS (Complex I) in adult animals and of CS, CCRT, CCRS, COX (Complex IV) in aged ones. In intra-synaptic light mitochondria, citrate synthase (ΔG (kJ/mol) = negative; ΔG^0 (kJ/mol) = -31.5), and cytochrome oxidase were increased in aged animals; only SDH was *increased* by ischemia in heavy intra-synaptic mitochondria of adult animals, and *decreased* in both types of intra-synaptic mitochondria from aged animals.

These data clearly suggest that energy metabolism of perikaryal mitochondria, located *in vivo* near the *nucleus* in the soma of neurons (i.e., post-synaptic), are primarily affected by acute phase of ischemia and that the decreased concentrations of Krebs' intermediates well correlate with

the stimulated activity of TCA enzymes, in particular succinate dehydrogenase (ΔG (kJ/mol) = ≈ 0 ; $\Delta G^{o'}$ (kJ/mol) = + 6.0).

The observed *increase* due to ischemia in succinate concentration by 226%, together with the loss of ATP, stimulated the activity of SDH in adult animals, that almost partially is able to metabolize succinate to compensate the TCA stimulation; on the contrary, the increased concentration of succinate by 548% in aged cerebral cortex [37] is explained now on proteomic functional basis, because of the *decreased* catalytic activity of succinate dehydrogenase in intra-synaptic mitochondria of aged animals suggests that the pre-synaptic compartment is severely compromised for SDH activity, unable to compensate the higher succinate concentrations. Of note, in control brain the baseline activity of malate dehydrogenase and succinate dehydrogenase are greater in heavy mitochondria of aged that adult brain (Figs. 2, 3), suggesting that the catabolism or the production of succinate is a critical point also in steady-state conditions.

It is important to underline that these considerations are impossible to be drawn on data on metabolite's concentrations, being impossible to distinguish between subcellular compartments. Thus, the massive succinate concentrations in aged is due (a) to the decreased activity of SDH and (b) these are also accumulated as end product of anaerobic catabolism of glucose during ischemia from the "succinate cycle" by anaplerotic reactions [39], contributing to more pronounced tissue acidosis in aged than in adult animals [37].

As far as the ischemia-induced depletion of ATP and CrP found to be extreme in both age groups [37], it seems that the compensative stimulation of Complex I–III activity and COX recordered on enzymatic basis (Table 1), could be ineffective for ATP synthesis in mitochondria of ischemic animals of both ages, because of the lack of oxygen that functions as an electrons acceptor.

On the other hand, the major contribution to recovery phase from ischemia is made by the part of the tissue that survives and some data indicate that after cerebral ischemia new neurons are directed toward the sites of brain injury, participating to brain repair and functional recovery [40], probably changing the metabolic and enzymatic assessment of non-synaptic and intra-synaptic mitochondria.

As regards glutamate and aminoacid metabolism, it may be concluded that this metabolism is not affected in perikaryal and intra-synaptic mitochondria in adult animals after 15 min of ischemia, whereas it seems compromised (not severely) in the acute phase in cerebral cortex only in aged animals, as shown by the increased activity of glutamate dehydrogenase ($\Delta G^{o'} = + 30$ kJ/mol) in intra-synaptic heavy mitochondria and of glutamate–oxaloacetate transaminase activity in perikaryal mitochondria of

aged animals after 15 min of ischemia (Figs. 4, 5; Table 1).

This observation is of interest because glutamate may be metabolized by transamination and deamination reactions, which participants are: (a) aspartate in GOT reaction (at the expense of oxaloacetate), (b) alanine in GPT reaction (at the expense of pyruvate) and (c) the net metabolism of aminoacid pool is made by oxidative deamination to glutamate, highly favoured *in vivo*, due to $\Delta G^{o'}$ value (and *viceversa*). All these reactions lead to the formation of α -ketoglutarate, depending on the ratio of the concentrations of these compounds: all the enzymes catalyzing near equilibrium reactions, i.e., GOT and GPT, act very fast and the net catalytic rates are efficiently regulated and adaptable to the varying biochemical situations that takes place in the tissue *in vivo*.

Thus, the increased activities of GIDH and GOT lead to the decrease of their own substrates, i.e., glutamate and aspartate, according to Krebs' theorem or, alternatively, the lack of substrates blocks the reactions in the corresponding way, even if these assumptions cannot be interpreted in a straightforward way. In fact, if the α -ketoglutarate decreased when TCA cycle is stimulated, this could be caused either by a shift in the direction of GIDH and/or GOT equilibria, but all these considerations explain their initial increase of activity in ischemic (15 min) aged animals.

In addition, because TCA cycle, aminoacid and glutamate metabolism are interrelated, these data, taken together, also well explain the increased activity of citrate synthase in free mitochondria of aged animals. The GOT activity was stimulated in the direction of aspartate formation which concentration increased in ischemia, but this enzyme, at the same time, enters in competition with citrate synthase for oxaloacetate. This indicates that the Borst' cycle (malate–aspartate shuttle) is operative in brain [21] because in this way the concentrations of oxaloacetate are maintained to a low concentration in mitochondria, allowing the NADH/NAD⁺ ratio greater in mitochondria than in cytoplasm, necessary to maintain the electron transport. In fact, "total" and rotenone sensitive NADH-cytochrome *c* reductase activities at both ages and cytochrome oxidase activity in aged mitochondria increased in ischemia (Table 1).

In intra-synaptic mitochondria, glutamate dehydrogenase activity is stimulated for the production of α -ketoglutarate to yield energy in Krebs' cycle, being ATP an allosteric blocker (ADP activator) for this reaction in the direction of α -ketoglutarate formation.

Brain cellular levels of glutamate and aspartate, increase rapidly following the onset of ischemia, remain at elevated levels during ischemia, and then decline over 20–30 min following reperfusion, with the important observation

about the delayed decrease of excitatory aminoacid neurotransmitters in parieto-temporal cortex and *hippocampus* after complete cerebral ischemia in aged rats, as discussed by Muldner and Hoyer [41]; the elevated levels of these excitotoxic amino acids are thought to contribute to ischemia-evoked neuronal injury.

However, studying the glutamate content in the nervous cell gap after 15, 30 min and 1 h focal cerebral ischemia by middle cerebral artery (MCA) occlusion and during recovery after 3, 24, 48 and 96 h, it was concluded that glutamate content in the *hippocampus* (and *hypothalamus*) was increased after 1 h ischemia reaching the highest value [42]. Recovery of pre-ischemic levels of aminoacid requires their uptake by high affinity sodium dependent transporters that are energy dependent processes, again indicating the central role of requiring the restoration at first of energy metabolism and related resting potentials and ionic gradients [7].

The molecular mechanisms that are operating in increasing glutamate levels are different *respect to the times of ischemia and/or reperfusion*: calcium-evoked release accounts for the initial (1–2 min) release, with non-vesicular release responsible for much of the subsequent efflux, i.e., the opening of swelling-induced chloride channels along the concentration gradients, while extracellular calcium-independent release is mediated by the reversal of sodium-dependent aminoacid transporters of the plasma membranes. Consequently, the real-time measurements of ischemia-evoked glutamate release in the cerebral cortex showed that glutamate levels in the interstitial space are enhanced during a low flow state of CBF and glutamate transients are seen to occur during the first 90 s of reperfusion [43].

The ATP synthesis from oxidative phosphorylation derives from the coupled functioning of the mitochondrial respiratory chain (ETC) and the ATP synthase activity; interestingly, the mitochondrial respiratory chain is one of the main sites of oxygen reactive species formation (ROS). It was shown that antioxidants administration before the onset of permanent MCA occlusion, increased ATP and Complex I–III activity and respiration, probably removing the inhibition of ETC by endogenous ROS, also inhibiting the glutamate release [44], pointing out the central role of mitochondria in cerebral ischemia and in glutamate moiety.

At this regards, the major modifications on ETC enzyme activities were observed in the perikaryal post-synaptic mitochondria (both ages) and mainly in aged animals in intra-synaptic light and heavy mitochondrial fraction, the mitochondrial population that represents the oldest intra-synaptic mitochondria [45–47], and structural damages induced by peroxidation also in normal brain [19, 47], stressing again the importance of mitochondrial enzymes systems.

The different behaviour of enzymes depending on the subcellular fractions on which they are *in vivo* located, is not surprising, because the same plasticity was also observed for ATP-ases activities during ageing in synaptic plasma membranes [48–50], during the recovery from hypoxic hypoxia cycles [51–53] and in others models of cerebral ischemia [5, 6, 54].

In summary, the following points are evidenced in this study: (a) 15 min of cerebral complete ischemia affected primarily the enzyme systems of perikaryal post-synaptic mitochondria, increasing the activity of Krebs' cycle and stimulating the ETC complexes, tentatively increasing the energy production (ATP) in adult animals, even if this could be ineffective in terms of energy (ATP) production, that remains impaired because of the lack of the electrons acceptor (oxygen); (b) the above mentioned changes occur also in aged animals, in pre-synaptically located light intra-synaptic mitochondria, and the activity of succinate dehydrogenase was severely compromised in synaptic mitochondria; (c) the ETC Complexes I–III and II (succinate dehydrogenase) are more sensitive to ischemia leading to the conclusion that (d) the major adaptive response to the acute phase of ischemia of the brain proteomic efficiency of mitochondrial systems in biochemical terms is the link between the Krebs' cycle for the production of electron equivalents to be adequately inserted in the ETC both for the first enter point into the chain (Complex I), as well as at the level of the linkage to Complex II (succinate); (e) this metabolic picture may be correlated to the compensatory increased activity of cytochrome oxidase (Complex IV) observed in perikaryal mitochondria and in intra-synaptic light mitochondria aged old animals. In fact, during ischemia, the loss of transmembrane gradients stimulated the activity of Na^+ , K^+ -ATPase of synaptic plasma membranes to restore the transmembrane ions gradients and electrochemical potential (thermodynamically unfavourable), all events that imply an increased consumption of ATP [7] that stimulated the activity of cytochrome oxidase.

Discussing the present results on enzyme activities linked to glutamate and aminoacid metabolism in the perspective of glutamate toxicity theory, it seems that glutamate-related non-synaptic and intra-synaptic enzymes' catalytic properties undergone by ischemia are working to cause primary important physiological and/or physiopathological modifications in cerebral tissue *in vivo*, as was previously hypothesized by us also for these subcellular systems [55] especially as regards the responsiveness to noxious stimuli and particularly during the recovery period from ischemia [7], as the changes undergone on metabolites and substrates demonstrated [37, 38].

In conclusion, this *in vivo* study on ischemia, indicates that (a) specific biochemical situations take place after

15 min time of complete ischemia in the different types of mitochondria, probably underlying specific biochemical assessments or plasticity of the ischemic tissue during the acute phase and (b) that these modifications in energy metabolism and glutamate metabolism are of primary importance for the physio-pathological development and/or maturation of neuronal damage, especially for recovery and for the biochemical basis of pharmacological therapy and treatment of ischemic disease.

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