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Ischemia decreases the content of the adenine nucleotide translocator in mitochondria of rat kidney

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The activity of the adenine nucleotide translocator is decreased at ischemia. Studies were undertaken to elucidate changes in the adenine nucleotide translocator by determining its content in mitochondria of ischemic rat kidney. After 60 min of ischemia, the content of the adenine nucleotide translocator amounted only to about 55%, of that measured in control mitochondria. At the same time, the flux control coefficient was increased. These changes paralled the well-known effects of ischemia: the decrease in oxidative phosphorylation and in adenine nucleotides. It is supposed that the decrease in the adenine nucleotide translocatar content accounts, at least partially, for the ischemia-induced impairment of mitochondria.

Introduction

Ischemia markedly depresses the oxidative phosphorylation capacity of mitochondria [1–3]. Several mechanisms have been proposed to account for this mitochondrial impairment and some of them involve the adenine nucleotide translocator.

The adenine nucleotide translocator warrants the energy flux between mitochondria and cytosol by exchanging equimolar amounts of ATP and ADP [4]. Applying the principles of control theory developed by Kacser and Burns [5] and Heinrich and Rapoport [6], respectively, it has been shown that the adenine nucleotide translocator has a considerable share in flux control of the respiration [7,8]. Therefore, elucidation of changes of this translocating reaction should be helpful in understanding basic mechanisms of mitochondrial dysfunction induced by ischemia.

The transport activity of the translocator was found to be diminished after ischemia [9–12]. It has been suggested that accumulation of long chain acyl-CoA esters, deenergization of the inner mitochondrial membrane and loss of adenine nucleotides, respectively, may cause this activity loss [13–15]. It has been shown by computer simulations, using a comprehensive mathematical model of the mitochondrial energy transduction, that a decrease in the translocator content may cause a lower respiratory rate [16].

Therefore, the present study was prompted to elucidate a further possible mechanism of the ischemia-induced impairment of translocator activity: the decrease of the mitochondrial content of the translocator protein. This mechanism was investigated by titrating the state 3 respiration of mitochondria from ischemic rat kidney cortex with carboxyatractyloside (CAT). The CAT titration method has often been used in the past to determine the mitochondrial content of the adenine nucleotide translocator [7,8,17–21].

Materials and Methods

Materials. Carboxyatractyloside was purchased from Boehringer, Mannheim, F.R.G. Nucleotides, nucleosides and nucleobases used as standards for HPLC were from Sigma, Deisenhofen, F.R.G. and Boehringer, Mannheim, F.R.G., respectively.

Animals. Wistar rats (200–250 g) were obtained from VEB Versuchstierproduktion Schönwalde, G.D.R. Renal ischemia was induced as followed: rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and then infused with heparin (100 U, i.v.) for 10 min. Subsequently, the abdomen was incised and the right kidney was occluded by placing a microsurgical clip

Abbreviation: CAT, carboxyatractyloside.

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across the renal artery. After different time intervals of ischemia both the right ischemic and the left non-ischemic kidney were removed to prepare mitochondria. Recovery experiments were performed by restoring the renal blood flow after 60 min of ischemia and suturing the abdomen. At given days of blood reflow, the rats were used for mitochondria preparation.

Isolation of mitochrondria. Mitochondria were isolated from the cortical tissue of the kidney as described previously [3]. Renal cortex was homogenized in a medium containing 250 mM mannitol/70 mM sucrose/1 mM EDTA/traces of Tris (pH 7.4). The mitochondria were separated by differential centrifugation at $600 \times g$ for 10 min and at $10000 \times g$ for 5 min. The final pellet was resuspended in the isolation medium. In experiments to determine adenine nucleotides, EDTA was omitted.

For CAT-titration experiments with ischemic mitochondria, up to three kidneys were pooled to obtain optimal respiratory rates. Protein determination was made by a Biuret method with human serum albumin as standard [3].

Measurement of respiratory rate. Respiratory rates were measured with a Clark-type oxygen electrode from Metra, Radebeul, G.D.R. at 25 °C in a closed oxygraph cell [3]. The medium contained 210 mM sucrose/10 mM KCl/10 mM KH_2PO_4/0.5 mM EDTA/60 mM Tris-HCl (pH 7.4)/10 mM succinate/0.2 mM ADP.

Extraction of purine compounds. Mitochondrial suspensions were precipitated with 0.1 vol. of 0.33 M ice-cold perchloric acid. After centrifugation at $11000 \times g$ for 2 min, the supernatants were neutralized with 0.15 vol. of a solution containing 2 M K_2CO_3/0.5 M triethanolamine. The extracts were cleaned by centrifugation as before and by fitration using 0.45 μm cellulose nitrate filters from Sartorius, Göttingen, F.R.G.

Measurement of purine compounds. Purine compounds were measured by ion-pair reversed phase high-performance liquid chromatography (HPLC) as described recently [22]. The 1090 M HPLC system of Hewlett Packard, Vienna, Austria, consisting of a ternary DR 5 solvent-delivery system, a diode array detector, an autosampler, an autoinjector and a 79994 A LC workstation was used. Columns applied were a microbore column (100×2.1 mm i.d.) prepacked with 5 μm ODS Hypersil (Shandon, U.K.), a guard column (20×2.1 mm i.d.) filled with 10 μm ODS material from the Academy of Sciences, Berlin, Germany, both manufactured by Hewlett Packard.

Solvent A contained 10 mM $NH_4H_2PO_4$ (pH 5.0)/2 mM tetrabutylammonium bromide and solvent B 10 mM $NH_4H_2PO_4$ (pH 6.5)/0.5 mM tetrabutylammonium bromide/25% (v/v) acetonitrile. The gradient elution started with 7% of solvent A, increased lineary to 30% of B during 12 min and continued for 2 min to 80% of B. The reequilibration step required 10 min, the flow rate was 0.1 ml/min, and 5 μl of the extract were injected.

The purine compounds were identified by comparing retention times as well as spectra acquired during the run by the diode array detector with those of external standards stored in the library of the LC workstation. The peak purity was routinely checked using spectra corresponding to the upslope, apex and downslope of the desired peak.

Determination of translocator content. The content of the adenine nucleotide translocator was determined by titrating state 3 respiration with CAT. Mitochondria were preincubated with CAT in the medium used for the measurment of respiratory rate for 1 min. Starting with succinate the reaction was traced for 1 min after reaching the steady state. The content of the active adenine nucleotide translocator was determined by that amount of CAT sufficient to completely inhibit the state 3 respiration and the flux control coefficient was calculated according to Groen et al. [7] using the initial slope of the CAT titration curve.

Results

Patterns of titrating the state 3 respiration of mitochondria isolated from nonischemic as well as ischemic kidneys are shown in Fig. 1. Mitochondria from nonischemic kidney were characterized by a translocator content of about 375 pmol/mg of mitochondrial protein. A value of about 0.2 was estimated for the flux control coefficient of the translocator reaction. The translocator content decreased with increasing time of ischemia and amounted to be about 190 pmol/mg protein at 60 min of ischemia, whereas the flux control

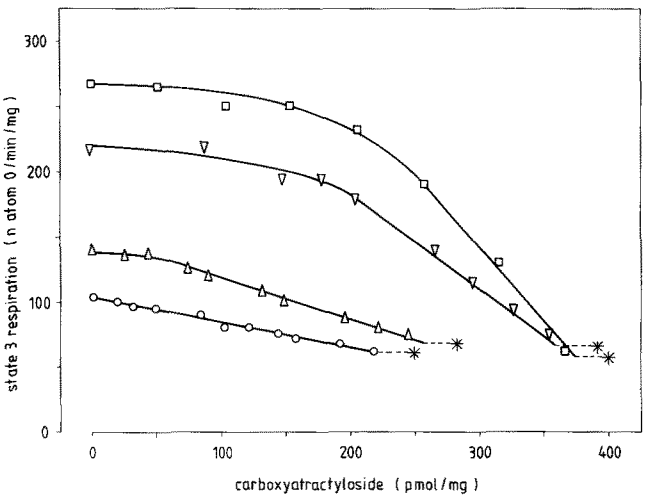

Fig. 1. Titration of state 3 respiration by carboxyatractyloside at different time periods of ischemia. For experimental conditions see section 'Materials and Methods'. Control, □. Ischemia: ▽, 15 min; △, 30 min; ○, 60 min; *, respiration at complete inhibition. Results of typical experiments.

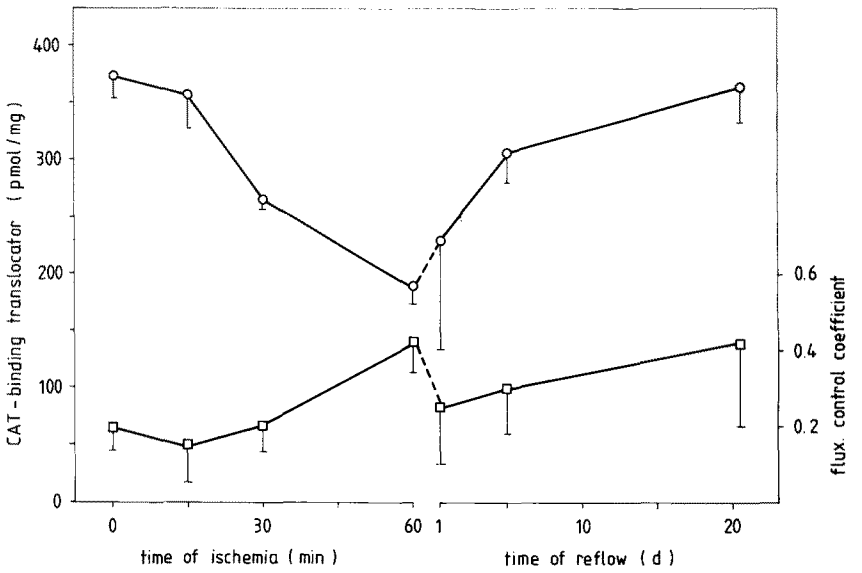

Fig. 2. Effect of ischemia and of blood reflow on the content and flux control coefficient of adenine nucleotide translocator. ○, Content of adenine nucleotide translocator; □, flux control coefficient. $n = 5$, ± S.D.

coefficient increased 2-fold (Figs. 1, 2). The rate of state 3 respiration was diminished to about 35% (Fig. 1) and that of state 4 respiration was unchanged within this time period (not shown).

During 21 days of blood reflow the translocator content was restored (Fig. 2). The state 4 respiration (not shown) and the flux control coefficient did not significantly differ from that of the nonischemic control at this time.

During 60 min of ischemia the mitochondrial content of ATP, ADP and AMP decreased progressively from 4.4 nmol/mg, 2.1 nmol/mg and 0.7 nmol/mg protein, respectively, to about 0.5–1 nmol/mg protein of the respective nucleotide (Fig. 3). At this time interval, the sum of adenine nucleotides and the ratio of ATP and ADP diminished to about 30% and 50%, respectively, in comparison with those of mitochondria from non-ischemic kidneys. However, both the sum of adenine nucleotides and the ATP to ADP ratio were restored after 21 days of blood reflow (Fig. 4).

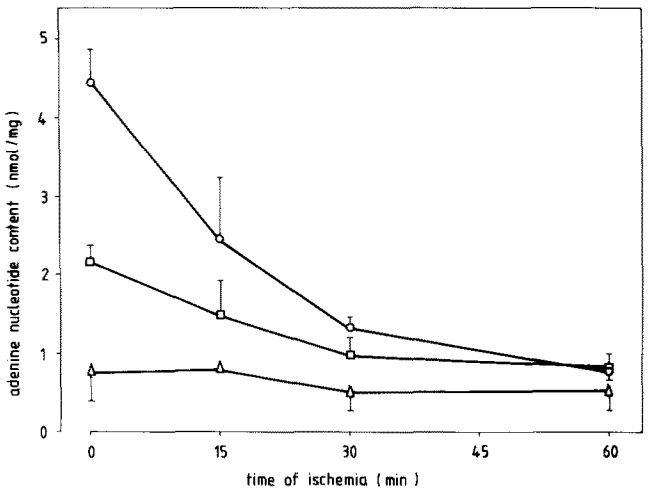

Fig. 3. Effect of ischemia on the adenine nucleotide content of mitochondria. ○, ATP; □, ADP: △, AMP. $n = 3$, ± S.D.

Discussion

There have been discussed three mechanisms of the ischemia-induced loss of translocator activity: inhibition by acyl-CoA esters accumulated, deenergization of mitochondrial membrane and decrease of intramitochondrial adenine nucleotides.

Acyl-CoA esters are well known as reversible inhibitors of the translocator [14,23–25]. During ischemia, activation of phospholipases and accumulation of acyl-CoA esters occur [26,27]. However, the physiological significance of their inhibitory effect has been doubtful [24,28,29].

There is a much evidence that the activity of the translocator depends on the membrane potential [4], but this view has also been questionned [30]. Nevertheless, ischemia is associated with a series of alterations in mitochondrial structure and function, including changes of the membrane potential [11,14].

Mitochondria lose adenine nucleotides with ischemia. It has been proposed that this loss impairs the translocator activity [11,24,31]. Concerning the decrease of nucleotides, this paper confirms the data published on heart [12,24,32] and liver [15,31] by using those from kidney cortex. This suggested mechanism of diminishing the translocator activity might be caused at ischemia by a phosphate-induced efflux [33,34] and/or an intramitochondrial catabolism of adenine nucleotides [31,35].

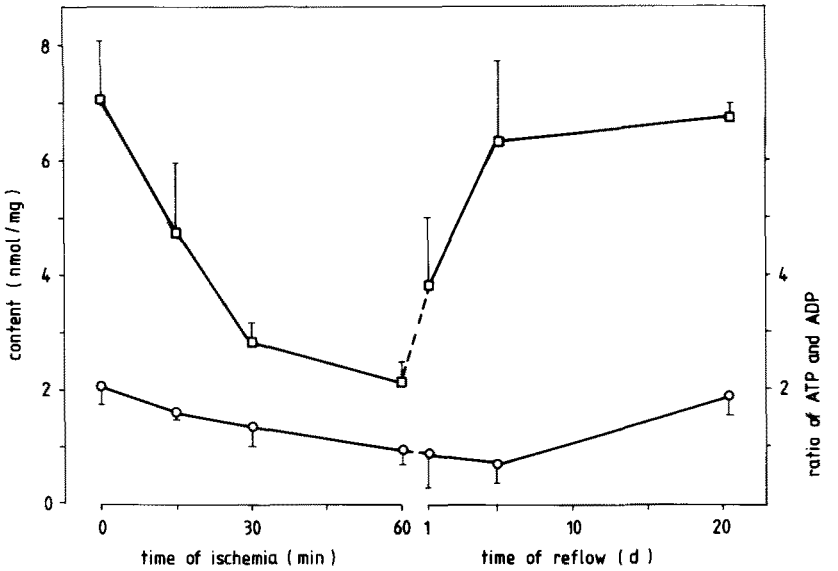

Fig. 4. Effect of ischemia and of blood reflow on the sum of adenine nucleotides. □, sum of ATP, ADP, and AMP; ○, ratio of ATP and ADP. $n = 3$, ±S.D.

In the present paper a fourth mechanism has been evolved to explain the ischemia-induced impairment of the translocator activity: the decrease of the mitochondrial content of the translocator protein. The content of the translocator was measured by titrating the state 3 respiration with CAT. CAT is a noncompetitive tight-binding inhibitor which is bound by an 1 : 1 stoichiometry to the translocator [4]. Tight-binding inhibitors are useful to estimate the amount of enzymes. Kinetic equations derived for noncompetitive tight-binding inhibitors illustrate that changes of the substrate concentration or the presence of a reversible inhibitor is without influence on the determination of the enzyme content [17,18]. In accordance with this theoretical based conclusion, no changes in the translocator content were measured by varying the respiratory rate via different extramitochondrial ATP/ADP ratios [7,8].

CAT is used for differentiating specific exchange and binding of adenine nucleotides [38,39], for measuring the content of translocator binding sites [40,41], for separating the active from the inactive translocator protein in the course of its purification [42] and for verifying the active portion after its incorporation into liposomes [43]. Inactivation of the translocator by *N*-ethylmaleimide as well as by phenylglyoxal [40] or by ultraviolet irradiation [44] deminishes the extent of CAT binding.

Summarizing these results, it is concluded that CAT is highly specifically bound by the translocator and, therefore, may be used to measure the portion of the active translocator protein in ischemic mitochondria by titrating the state 3 respiration. The method was successfully applied to estimate the translocator content, especially in the context of analyzing the controlling steps of mitochondrial respiration [7,8,18,20,21]. Data determined in this way are close to estimates obtained by measuring the CAT inhibition of the translocator [17] and uncoupler-activated ATPase activity [19], respectively, as well as the direct binding of CAT or bonkrekic acid, another specific translocator inhibitor [40,41,45].

The translocator content determined for nonischemic mitochondria of kidney cortex is similar to those published for liver and heart [4,7,8,17–20,40,41,45]. During ischemia the translocator content is decreased. Therefore, this decrease is considered as a fourth mechanism involved in the ischemia-induced loss of translocator activity. The observed increase in values of the flux control coefficient is in accord with this conclusion.

During ischemia an activation of phospholipases, an increased phospholipid peroxidation and a production of radicals occur [26,27,45]. Therefore, it is supposed that the translocator is inactivated by oxidative reactions. The degradation of the damaged protein by mitochondrial proteolytic systems [47,48] might be increased. The content of both translocator and adenine nucleotides were recovered by slow processes extending over several days. This behaviour is indicative for a slow synthesis of both.

Considering that the adenine nucleotide translocator is one of the rate-limiting steps of the oxidative phosphorylation [7,8,16] it is suggested that the decrease in the mitochondrial content of both translocator and adenine nucleotides contributes, at least partially, to the diminished respiratory rate observed with ischemic mitochondria and, therefore, should be taken into account as mechanisms of the decreased translocator activity.

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