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Increase in the adenine nucleotide translocase protein contributes to the perinatal maturation of respiration in rat liver mitochondria

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An assay based on the high-affinity binding of tritium-labelled atractyloside to the adenine nucleotide translocase (ANT) was developed for estimation of its content in samples of mitochondria, cells and tissue homogenate. The assay was used to study the developmental change of the ANT protein concentration in perinatal rat liver. Within the last 3 days before birth the content of the ANT protein per mg tissue protein increased from 29 to 45% of the maximum value found 2 days after birth. A similar developmental change of the ANT protein was found in isolated mitochondria, demonstrating that the perinatal increase in the ANT protein content was due mainly to a mitochondrial differentiation process and not the result of an increase in the number of mitochondria per cell. A close proportionality between the ANT protein and the ADP-stimulated respiration of liver homogenate was found in the perinatal period from 3 days before to 2 days after birth. This finding suggests that the developmental change in the ANT protein content plays an important role in the onset of oxidative phosphorylation after birth.

Introduction

Contrary to adult liver mitochondria, late fetal mitochondria have only a very low State 3 respiration. After birth the ADP-stimulated respiration inceases strongly within a few hours of extra-uterine life [1–4]. This finding has been explained initially by an accumulation of adenine nucleotides from cytoplasma into the mitochondrial matrix (see Refs. 1,5,6, for review, see Ref. 7). While the matrix adenine nucleotide content of newborns ranges from 3 to 6 nmol/mg mitochondrial protein, that of adults is within 10–15 nmol/mg mitochondrial protein [1,2,8,9]. In line with this interpretation is the observation that depletion of the matrix adenine nucleotide content below a threshold concentration of 5–7 nmol/mg mitochondrial protein decreased the State 3 respiration of adult rat liver mitochondria [6]. The fact that the threshold content is already achieved within 1 h after birth [7], while the development of full State 3 respiration needs about one day [2–4], reveals that accumulation of adenine nucleotides cannot be the only factor for development of ADP-stimulated respiration.

New results indicate that the biochemical basis of the postnatal development of the State 3 respiration is a complex process to which a developmental increase of enzymes of oxidative phosphorylation contributes [4,10]. So, it has been reported that the content of the F_1F_0-ATPase increased 2-fold within a period of 6 h after birth [4]. Moreover, it has been reported that the control of respiration by ANT is stronger in liver mitochondria from newborns than from adults [2]. This finding suggests that the ANT protein could exert an important role in the postnatal development of State 3 respiration. Therefore we studied the development of the ANT protein content in rat liver tissue in the perinatal period from 3 days before to 4 days after birth. For that purpose, an assay based on the specific binding of [^{3}H]atractyloside (ATR) to the ANT protein was developed. From the 2-fold increase of the ANT protein per mitochondrial protein found within 2 days after birth it is suggested that the ANT plays an important role in the postnatal onset of State 3 respiration.

Materials and Methods

Preparation of mitochondria, hepatocytes and homogenate

Mitochondria and a liver homogenate were prepared from newborn and adult Wistar rats. Fetuses were obtained from term-pregnant anesthetized rats by Cesarean section. Immediately after delivery of the

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Abbreviations: ANT, adenine nucleotide translocase; CAT, carboxyatractyloside; ATR, atractyloside; LH, rat liver homogenate; GlDH, glutamate dehydrogenase.

fetuses, mitochondria and homogenate were prepared from the removed liver. Mitochondria were isolated as described in Ref. 11. For the preparation of the homogenate, 1 g of liver tissue was added to 5 ml ice-cold 250 mM sucrose medium and disrupted by means of an Ultraturrax twice for 20 s. The protein content in the mitochondrial stock suspension and in the homogenate was determined by the biuret method. Hepatocytes from adult rats were prepared as described by Berry and Friend [12].

The activity of glutamate dehydrogenase was determined in aliquots of the stock suspensions of mitochondria, liver homogenate and isolated hepatocytes in the presence of 0.5% Triton X-100 [13].

Measurement of oxygen uptake

Oxygen uptake by the liver homogenate was measured polarographically in a water-jacketed chamber maintained at 30°C. Aliquots of 0.2 ml (25–35 mg protein/ml) were incubated in 2 ml medium containing 110 mM mannitol, 60 mM KCl, 60 mM Tris, 10 mM potassium phosphate, 10 mM succinate and 0.5 mM Na_2EDTA (pH 7.4).

Preparation of [^{3}H]atractyloside

[^{3}H]Atractyloside was prepared by Dr. Noll (from the former Zentralinstitut für Kernforschung, Rossendorf, Germany), essentially as described in Ref. 14. In brief, carboxyatractyloside (CAT) was decarboxylated to ATR and subsequently tritiated with HTO using palladium-barium sulfate as catalyst. [^{3}H]ATR was checked for radiochemical purity by thin-layer chromatography using n-butanol/glacial acetic acid/water (5 : 1 : 1). Titration of the State 3 respiration of adult rat liver mitochondria was used for the determination of the concentration of [^{3}H]ATR in the stock solution, in analogy to the procedure as described in Ref. 15. The concentration of atractyloside in the stock solution was 109 μM and the specific radioactivity was 348 000 dpm/nmol.

[^{3}H]ATR binding test

All steps of the binding test were done in a microcentrifuge tube (1.5 ml) at room temperature. Aliquots of 0.1 ml of the mitochondrial stock suspension (2–3 mg protein) or of the homogenate (3–5 mg protein) were added to 0.9 ml 250 mM sucrose medium containing 2 μM [^{3}H]ATR. After a 3-min incubation, the tubes were centrifuged, the supernatant aspirated and the pellet resuspended in 0.5 ml sucrose medium. Centrifugation and washing of the pellet with sucrose medium was repeated twice. Finally, the pellet was dissolved in 2% SDS solution. Radioactivity in the pellet was counted in 100 μl of the SDS sample by liquid scintillation (Tri-Carb 1600). The assay was performed as triplicate for each incubation. In order to correct the data for radioactivity due to adherent [^{3}H]ATR in the pellet (blank radioactivity), each incubation was repeated in the presence of 10 μM CAT.

The blank was subtracted from the total radioactivity measured in the absence of CAT. Binding of [^{3}H]ATR was expressed on the basis of the tissue protein content or glutamate dehydrogenase activity.

Results

To elucidate the role of the ANT in the maturation of the ADP-stimulated respiration, an assay was developed which can be applied for the estimation of the ANT protein content in tissue samples. This assay is based on the specific binding of [^{3}H]ATR to the ANT protein. In the first step, the suitability of the assay was checked with samples of adult isolated rat liver mitochondria. Fig. 1 shows the relation between the concentration of added [^{3}H]ATR and its binding to mitochondria. There was no saturation in the [^{3}H]ATR binding when the binding was calculated on the basis of the total radioactivity in the pellet. However, a typical saturation characteristic was obtained after correction for the radioactivity which is due to adherent [^{3}H]ATR in the pellet.

Next, it was examined whether the binding assay gives similar results when applied to so different samples as isolated mitochondria, hepatocytes and homogenate. Due to the well known poor permeability of the plasma membrane for atractyloside, the samples

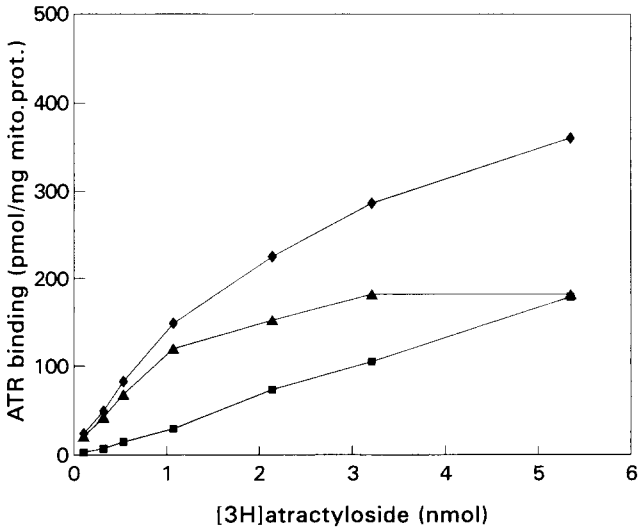

Fig. 1 Specific binding of [^{3}H]atractyloside to rat liver mitochondria. Mitochondria were incubated with [^{3}H]ATR alone (♦) or after a 2-min preincubation with 10 μM CAT (■) and treated as described in Materials and Methods. The specific binding of [^{3}H]ATR to the mitochondria (▲) was obtained by subtracting the blank radioactivity counted in the CAT containing samples from the total radioactivity counted in the CAT-free samples. The points are the means from three preparations.

containing hepatocytes were routinely supplemented with digitonin. With non-permeabilized hepatocytes, only one third of the ANT protein was accessible to the added ATR, even after 30 min of incubation with [^{3}H]ATR (not shown). Fig. 2 shows the specific binding of [^{3}H]ATR to mitochondria, hepatocytes and homogenate prepared from adult rat liver. For comparison, the ATR binding was expressed on the basis of glutamate dehydrogenase activity, which is exclusively localized in mitochondria [16]. As it can be seen, the same maximal specific binding of [^{3}H]ATR was found in the different preparations. Fig. 3 shows that in the range up to 7 mg liver protein the binding of [^{3}H]ATR was proportional to the amount of incubated homogenate protein.

The results of Figs. 1–3 demonstrate that the [^{3}H]ATR binding assay is a suitable tool to determine quantitatively the ANT content in liver tissue preparations. The assay was applied to the estimation of the ANT content in liver homogenates during the developmental age 3 days before to 4 days after birth. Fig. 4 shows the results. As can be seen, the ANT content increased in the late prenatal period only slightly in liver tissue. But after birth there was a 2-fold increase within 2 days of extra-uterine life. The level attained after 3–4 days was higher than in adult liver. At the 4th day of the extrauterine life the ANT content declined. In order to examine whether the observed increase in the ANT content was caused by proliferation of mitochondria within the cell or by enrichment of mitochondria with ANT protein, the ANT content was estimated in samples of isolated mitochondria during

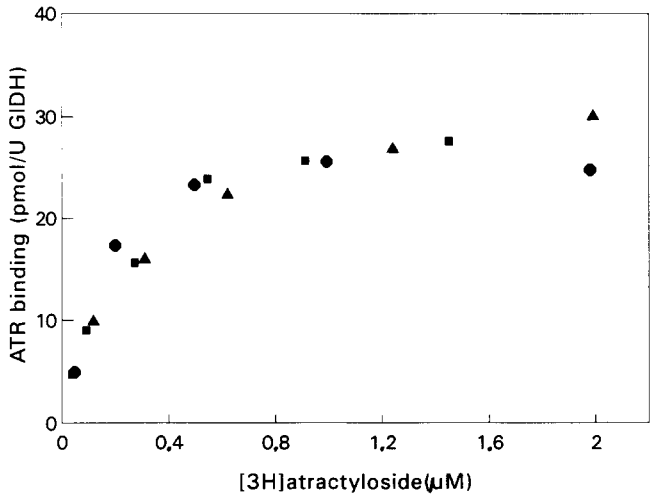

Fig. 2. Specific binding of [^{3}H]atractyloside to mitochondria, liver homogenate and hepatocytes. Aliquots of mitochondria (▲), hepatocytes (■) or liver homogenate (●) were treated as in Fig. 1. For hepatocytes the incubation mixture was additionally supplemented with 20 μg digitonin per mg dry weight for permeabilization of the cells. In order to allow a direct comparison, data for [^{3}H]ATR binding are given per U glutamate dehydrogenase.

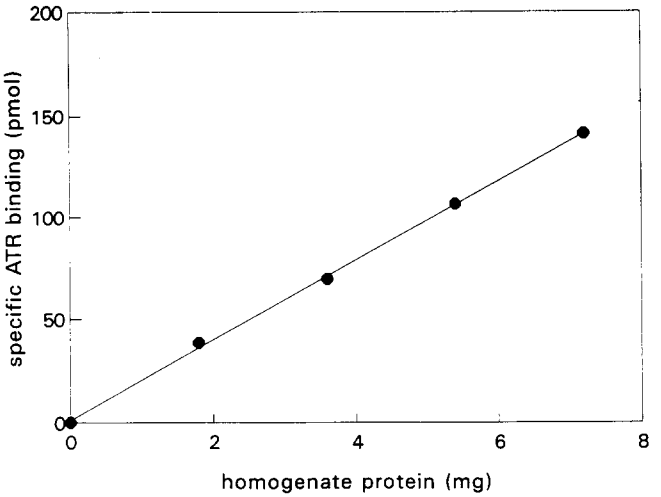

Fig. 3. Proportionality of specific [^{3}H]atractyloside binding to the liver tissue protein. Different amounts of liver homogenate were incubated in the presence of 4 μM [^{3}H]ATR in a total volume of 1 ml and treated as described in Materials and Methods.

the same period of developmental age. ANT content estimated in preparations from adults were included in Fig. 4. It can be seen that the ANT content in the homogenate and in mitochondria shows a similar developmental increase. This finding indicates that the perinatal increase in ANT content is the result of a mitochondrial maturation process, when the data were normalized by the ANT content of the corresponding adult samples (Fig. 4B). It becomes clear that the decline observed in the homogenate after 4th day is caused by a further proliferation of nonmitochondrial proteins.

In order to study the relationship between the developmental change of the ANT content and the change of the ADP-stimulated respiration the respiration was measured in homogenate samples shown in Fig. 5. It can be seen that the basal respiration increased strongly after addition of ADP as known from isolated mitochondria. This indicates that the mitochondria in the homogenate were well coupled. CAT inhibited the ADP-stimulated respiration slightly below the level before addition of ADP.

Fig. 6 summarizes the data of the ADP-stimulated respiration and, for reference, of the CAT-inhibited respiration. As can be seen, before birth there was only a very low State 3 respiration, but after birth the respiration increased within 4 days up to the adult level.

In Fig. 7 the ADP-stimulated respiration was plotted vs. the ANT content. There was a proportionality between respiration and ANT content within the perinatal period from 3 days before to 2 days after birth. The further increase of ADP-stimulated respiration after the second day of extrauterine life was not paralleled by an increase in the ANT content.

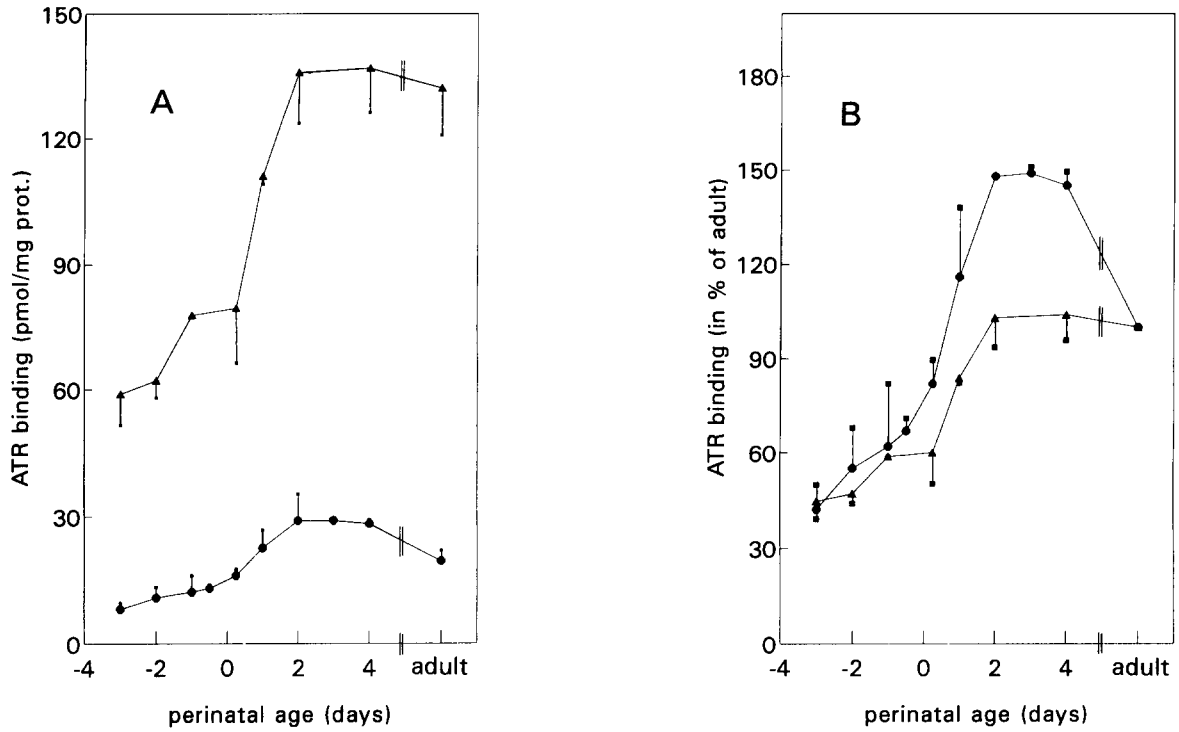

Fig. 4. Perinatal change of the ANT protein in mitochondria and liver tissue. The specific [^{3}H]ATR binding was determined for liver mitochondria (▲) or liver homogenate (●) prepared from fetal, newborn and adult rats. Shown are the absolute (A) and relative (B) specific binding as percentage of the adults. Data points are means of 3–5 separate experiments. Error bars indicate ±S.D. when S.D. is greater than the size of symbols.

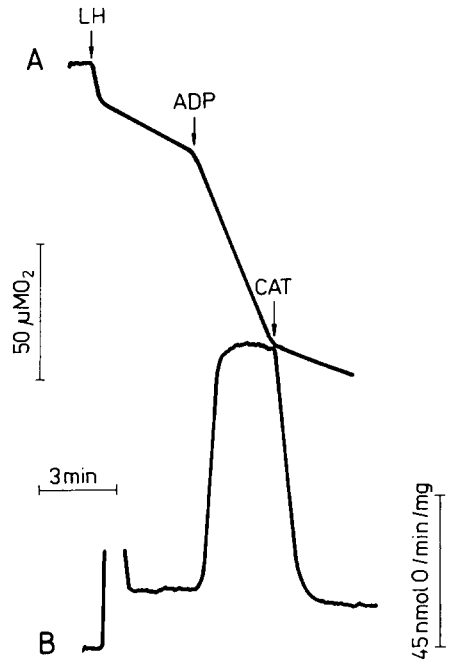

Fig. 5. ADP-stimulated respiration in adult rat liver homogenate. Oxygen consumption was measured as described in Materials and Methods. Additions were 4.5 mM ADP and 4.5 μM CAT. Shown are the traces of oxygen concentration (A) and its first derivative (B).

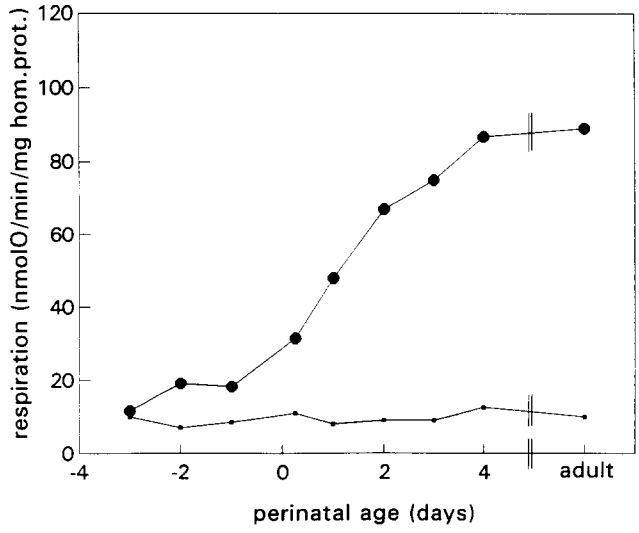

Fig. 6. Perinatal development of the ADP-stimulated respiration in homogenates of perinatal rat liver. Homogenates were prepared from rat liver of various perinatal ages. ADP-stimulated respiration (●) and the CAT-insensitive respiration (■) was measured as in Fig. 4. Data points are means of two preparations.

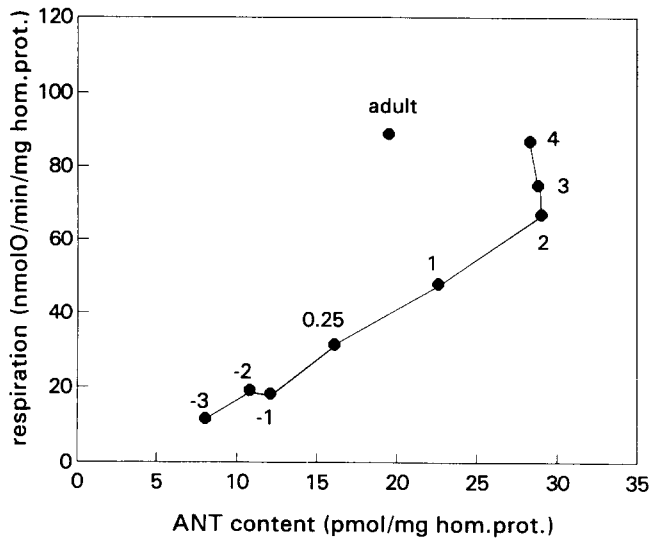

Fig. 7. Relation between ADP-stimulated respiration and ANT protein content in the homogenate. Data are from Fig. 4 and Fig. 6. The numbers at the data points indicate the perinatal age.

Discussion

In the present paper, we studied the developmental change in the content of the ANT protein in liver tissue using an assay based on the specific binding of [^{3}H]ATR to ANT protein. It was shown that no significant difference exits in the ligand-binding properties of the ANT protein of fetal, suckling and adult rat liver mitochondria [8]. The specific binding was taken as the difference of [^{3}H]ATR binding in absence and presence of the high-affinity ligand CAT. Other CAT-binding proteins in mitochondria are not known. Since we found the same maximal specific binding of [3H]ATR in relation to GlDH with isolated mitochondria, isolated hepatocytes and tissue homogenate, any extramitochondrial binding sites can be neglected. The ANT protein content of 125 pmol/mg protein found with our assay in adult rat liver mitochondria fits with available data in the literature [8,17]. The assay allows to estimate the content of ANT protein in tissue homogenates without prior isolation of mitochondria. Inhibitor titration of ADP-stimulated respiring mitochondria by CAT, a method often used for quantitative estimation of the ANT content in adult mitochondria [18–20], is not applicable to prenatal liver mitochondria due to their very low rates of respiration.

In the liver tissue the ANT protein content was found to increase 2-fold in the first two days of the extrauterine life. A similar increase of the ANT protein content was found in isolated rat liver mitochondria of the same developmental age. From this coincidence it is concluded that the doubling of the ANT protein is the result of a mitochondrial differentiation process, but not due to an increase of the number of mitochondria per cells. In line with this conclusion are stereometric data from electron micrographs [7]. Moreover, during the course of further development of the newborn to the adult the tissue ANT protein content declined. Since no such decrease was found in isolated mitochondria (but see Refs. 2,8), this decline must be caused by the syntheses of cytosolic proteins in the later developmental age.

Our results point to an involvement of postnatal synthesis of the ANT as factor in the developing of the State 3 respiration. During the first 2 days of extrauterine life the mitochondrial ANT protein content reached the adult level. Since the increase of respiration continued also after this period, the expression of additional functional proteins must contribute to the further maturation of mitochondria.

Interestingly, a 2-fold increase of the content of F_1F_0-ATPase protein occurred within a few hours after birth [4,21]. These findings suggest that the development of State 3 respiration is a bimodal process. In the first hour after birth the development of the State 3 respiration is mainly governed by accumulation of adenine nucleotides in the matrix [7]. After that period, the development of full State 3 respiration depends on the increase of ANT content in mitochondria and of F_1F_0-ATPase content as shown by Cuezva [4] and possibly further functional proteins.

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