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Impairment of mitochondrial 5-aminolevulinic acid synthase activity in Gunn rat liver

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Abstract—Gunn rats are characterized by hereditary hyperbilirubinemia and a decrease, when compared to Wistar rats, in hepatic heme pool size which could result from an alteration of mitochondrial functions. Unconjugated bilirubin present in Gunn rat liver did not modify either the ultrastructural morphology or oxidative metabolism of the mitochondria as compared to those in Wistar rat liver. However, 5-aminolevulinic acid synthase activity is reduced by nearly 40% in Gunn rat liver mitochondria, thus explaining the reduced size of the hepatic heme pool.

Gunn rats derive from Wistar rats and are known for their hereditary hyperbilirubinemia [1]. We showed previously that, in these rats, the hepatic heme pool was decreased when compared to Wistar rats [2], limiting the response of cytochrome(s) P450 to induction by polycyclic aromatic hydrocarbons [3]. Since bilirubin has been reported to be toxic for mitochondria, notably by increasing inner membrane conductance [4], our aim was to determine any effect of bilirubin chronically present in the livers of Gunn rats on mitochondrial functions and specifically on 5-aminolevulinic acid (ALA*) synthase activity.

ALA synthase (EC 2.3.1.37) catalyses the first step of heme synthesis by condensing succinyl CoA and glycine to form ALA, and is located in the mitochondrial matrix. This enzyme is a rate-limiting step in the pathway of hepatic heme synthesis and is the most sensitive enzyme to variations in the regulatory heme pool [5]. Consequently, we determined the activity of this key enzyme and we evaluated mitochondrial integrity in both Wistar and Gunn rat livers.

Materials and Methods

Animals. Two-month-old homozygous male Gunn rats (originated from CSEAL-CNRS, France, and inbred in our animal unit) and male Wistar rats (IFFA-CREDO, France) were starved for 24 hr before killing.

Mitochondrial functions. Liver mitochondria were isolated by differential centrifugation as described by Schnaitman and Greenawalt [6]. Measurement of oxygen consumption was carried out polarographically at 30° with a Clark-type oxygen electrode coupled to a Gilson oxygraph (Model 5/6). State 3 and state 4 respirations were defined as ADP-stimulated (after addition of 250 nmol ADP) and ADP-limited respiration, respectively. NAD^+-linked substrates, glutamate (5 mM) and pyruvate (5 mM), each added with malate (5 mM), and 10 mM succinate, a FAD^+-linked substrate, were used. Respiratory control ratio (RCR) (ratio of state 3 to state 4 rate of oxygen consumption) was calculated according to Estabrook [7].

Electron microscopy. Mitochondria were fixed immediately after isolation in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 for 2 hr at 0°. After washing in the same buffer plus 0.1 M sucrose, they were post-fixed in 2% OsO_4, dehydrated and embedded in Spurr's resin [8].

Enzymatic determinations. Citrate synthase activity was quantified in mitochondrial preparations by a spectrophotometric method [9]. Assay of ALA synthase was adapted from Strand *et al.* [10] using [2,3-^{14}C]succinic acid (1.8 GBq/mmol) (New England Nuclear, Boston, MA, U.S.A.) and non-radioactive succinic acid (10 mM). Coenzyme A (Sigma Chemical Co., St Louis, MO, U.S.A.) and succinate thiokinase (Boehringer Mannheim, Germany) were used to generate succinyl CoA. Protein concentrations were determined according to Lowry *et al.* [11].

Results and Discussion

To check the integrity of mitochondria, we first explored their respiration and coupling efficiency with several substrates. As shown in Table 1, no significant differences were found between rat strains whatever the substrate considered. Oxygen consumption and RCR were similar in Wistar and Gunn rats indicating that the mitochondria of the two strains preserved their phosphorylating capacities and were similarly coupled. Values observed were in agreement with those reported in the literature [12]. Succinate, which is also a substrate for ALA synthase, is similarly oxidized by Wistar and Gunn mitochondria suggesting that there is no leak of substrate out of mitochondria subsequent to damage or to a modification to the permeability of the mitochondrial membranes. Citrate synthase is located in the mitochondrial matrix and is considered a good marker for evaluating the quality of preparations. The activity measured in Wistar versus Gunn rats was 298 ± 135 versus 300 ± 116 pmol/min/mg protein, indicating no noticeable difference in the catalytic capacity of mitochondria prepared from the two strains. This is confirmed by morphological aspects, depicted in Fig. 1, which were similar in both strains. Mitochondrial membranes derived from either Wistar or Gunn rat livers preserved their ultrastructural integrity during isolation. Both samples exhibited a condensed configuration, as expected for mitochondria freshly isolated at 4°.

The first set of experiments was designed to validate the isolation procedure and the integrity of mitochondria. However, the original purpose of our study was to estimate ALA synthase activity in mitochondrial fractions. Results are shown in Table 2: Gunn rats presented a 40% decrease in ALA synthase activity when expressed either per milligram of protein or per unit of citrate synthase. Direct inhibition of *in vitro* ALA synthase activity by bilirubin cannot be ruled out but it is unlikely because of the multiple washings performed during the preparation of mitochondria. The decrease in ALA synthase activity parallels the decrease that we reported recently in the size of the heme pool available in the liver [1]. It has been established that a heme excess lowers the mRNA concentration of the enzyme [13] and completely blocks the maturation of the enzyme and its processing in the mitochondria [14]. The effects of heme depletion are less clear-cut: succinylacetone treatment decreases the heme

* Abbreviations: ALA, 5-aminolevulinic acid; RCR, respiratory control ratio.

Table 1. Oxidative metabolism in hepatic mitochondria from Wistar and Gunn rats

	Wistar (N = 6)	Gunn (N = 6)
Oxygen consumption (state 3 rate) (nano atoms O/min/mg protein)		
Substrate:		
Glutamate + malate	91 ± 32	63 ± 18
Pyruvate + malate	39 ± 12	36 ± 16
Succinate	93 ± 14	96 ± 38
RCR		
Substrate:		
Glutamate + malate	8.1 ± 2.4	10.0 ± 2.5
Pyruvate + malate	4.1 ± 1.8	4.5 ± 0.6
Succinate	5.0 ± 1.7	5.7 ± 1.1

Mitochondria were isolated from starved animals as described in the text. Substrate concentrations are given in Materials and Methods. For each substrate, determinations were performed in separate polarographic assays in triplicate.
Results are expressed as means ± SD.

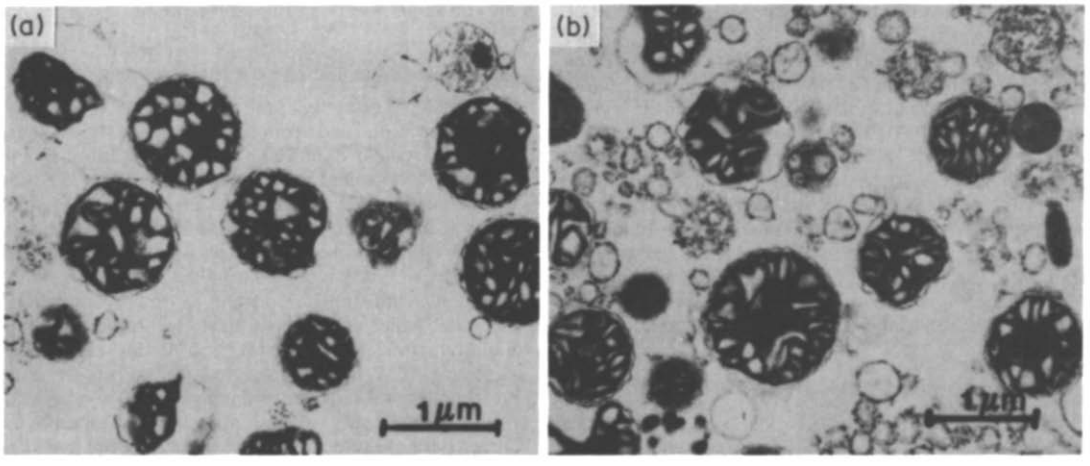

Fig. 1. Electron microscopy of hepatic mitochondria from Wistar and Gunn rats. Magnification: ×15,000 (bar equals 1 μm). Coloration: uranyl acetate and lead citrate. (a) Wistar liver; (b) Gunn liver.

Table 2. ALA synthase activity in hepatic mitochondria from Wistar and Gunn rats

	pmol/min/mg protein	pmol/unit citrate synthase
Wistar (N = 6)	10.5 ± 1.5	0.042 ± 0.018
Gunn (N = 6)	6.5 ± 2.5*	0.027 ± 0.012*

Assays were performed in duplicate for each mitochondrial preparation.
Results are expressed as means ± SD.
* $P < 0.01$.

pool and is associated with a transient de-repression of ALA synthase activity at early (3 hr) but not at later time points [15]. In Gunn rats, the chronic decrease in heme pool size does not result in a surge in ALA synthase activity. Hamilton *et al.* [16] have postulated in the chick embryo the existence of a labile repressor protein which is sensitive to heme and modulates ALA synthase mRNA expression. The existence of such a repressor and its sensitivity to bilirubin remain to be demonstrated in Gunn rat liver. An abnormal regulation of the expression of the gene, an impaired processing of the protein in the mitochondria or a mutation affecting the catalytic velocity or the stability of the protein could also be hypothesized.

In conclusion, our results demonstrate that the impairment of the heme pool that we observed in Gunn rats could result from a decrease in ALA synthase activity,

and exclude any deleterious effect on mitochondria of unconjugated bilirubin chronically present in the liver of Gunn rats.

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