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Cytochrome aa_3 depletion is the cause of the deficient mitochondrial respiration induced by chronic valproate administration

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Abstract—Liver mitochondria from rats fed 1% (w/w) valproic acid for 75 days displayed an approximate 30% decrease in coupled respiration rates with substrates entering the respiratory chain at complexes I and II. Uncoupling the respiration from proton-pumping, or measuring the respiration without complex IV removed this inhibition. The treatment induced a loss of activity of cytochrome oxidase consistent with a decrease in the mitochondrial content of cytochrome aa_3. The inhibition induced by long lasting administration of valproate is apparently located at the site of the proton-pumping activity of complex IV. Furthermore, the capacity of electron transport through complex IV, being far in excess of that required for normal functioning in coupled mitochondria, seems to be controlled by the coupling to proton-pumping in which cytochrome aa_3 appears to play a major role.

The efficacy of sodium valproate in the treatment of generalized epileptic seizures is well established [1]. Its use, however, occasionally causes severe and even fatal toxicity [2, 3]. Liver complications resembling that in Reye's syndrome have been reported in a small number of patients [4]. The appearance of dicarboxylic acids in urine during valproate therapy [5] suggests interference with the function of mitochondria.

Inhibitory effects of valproate on the mitochondrial respiratory chain *in vitro* have been reported [6] but not confirmed [7]. We have shown recently that the addition of valproate to a mitochondrial fraction isolated from the liver of control rats decreases the rate of oxygen consumption not by inhibiting the respiratory chain directly, but by impairing the oxidation of substrates in the mitochondrial matrix [8].

The purpose of the present work was to study the effects of chronic *in vivo* administration of valproate on the respiratory chain of rat liver mitochondria. We report that this treatment results in decreased rates of oxidative phosphorylation in the liver and that the site of the inhibtion lies within complex IV of the respiratory chain.

Materials and Methods

Valproic acid and rotenone were from Janssen Chimica (Beerse, Belgium); glutamic and succinic acids from Merck (Darmstadt, F.R.G.); and pyruvate kinase (EC 2.7.1.40), lactate dehydrogenase (EC 1.1.1.27) and oligomycin from Boehringer (Mannheim, F.R.G.). FCCP* and TMPD were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Other chemicals were of the best grades available from local suppliers.

Adult male Wistar rats (approx. 150 g) were fed a standard laboratory rat chow (A-03, UAR, France) with or without 1% (w/w) neutralized valproic acid. After 75 days, the animals were killed by decapitation and mitochondrial fractions prepared as described by Veitch and Van Hoof [8].

Oxidative activities were measured at 30° with a Clark-type oxygen electrode (Yellow Springs Inst., OH, U.S.A.) as described by Veitch and Van Hoof [8] with either 10 mM succinate or 10 mM glutamate/1 mM malate as substrates. Coupled conditions were obtained by addition of 0.33 mM ADP and uncoupled conditions with 1 μM FCCP. Succinate oxidation rates were also measured spectrophotometrically at 30° as the antimycin-sensitive reduction of ferricyanide [9]. Cytochrome *c* oxidase (complex IV, EC 1.9.3.1) was assayed both spectrophotometrically at 25° by the oxidation of cytochrome *c* at 550 nm [10] and polarographically at 30° with 10 mM ascorbate (+250 μM TMPD) and 50 μM cytochrome *c* [11].

The content of cytochromes aa_3, *b*, *c* and c_1 was calculated using the appropriate wavelength pairs [12] from dithionite reduced–oxidized difference spectra obtained with an Aminco DW-2 spectrophotometer [13].

ATPase (EC 3.6.1.3) activity was measured at 30° in mitochondrial fractions stored previously at −80° with an ATP regenerating system in a medium containing 33 mM Tris–acetate, 83 mM sucrose, 20 mM $MgCl_2$, 1 mM EDTA, 4 mM ATP, 1.5 mM phosphoenolpyruvate, 0.17 mM NADH, 6 U pyruvate kinase, 12 U lactate dehydrogenase and 2.5 μg/mL rotenone, as described by Pullman *et al.* [14] with slight modifications. ATPase activity was calculated from the difference in the rates of NADH oxidation followed at 340 nm in the presence and in the absence of 5 μM oligomycin. Mitochondrial enzymes citrate synthase (EC 4.1.3.7) [15] and succinate dehydrogenase (EC 1.3.99.1) [16] were assayed as described.

Results and Discussion

After 75 days of valproate treatment, the animals displayed no modification in behaviour or body weight gain

* Abbreviation: FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; TMPD, tetramethyl-*p*-phenylenediamine.

Table 1. Effect of 75 days of valproate on liver weight and enzyme activities

	Controls	Valproate
Liver weight (g)	8.9 ± 0.5	11.9 ± 0.4*
Liver as % of body weight	3.22 ± 0.16	3.88 ± 0.12*
Liver protein (mg/g)	214 ± 6	256 ± 14*
Citrate synthase†	16.6 ± 0.6	29.3 ± 1.2*
Succinate dehydrogenase†	6.3 ± 0.5	11.0 ± 0.9*
Citrate synthase‡	192 ± 14	179 ± 8
Succinate dehydrogenase‡	58 ± 6	59 ± 4

Results are expressed as means ± SEM for 4–5 animals in each group.

* $P \leq 0.05$ vs controls.

† Enzymatic activities in homogenates expressed in U/g liver.

‡ Enzymatic activities in liver mitochondrial fractions expressed in mU/mg protein.

as compared to controls, although there was a significant hepatomegaly (Table 1) consistent with previous reports [17]. The 34% increase in liver weight was accompanied by a significant elevation in the hepatic content of both citrate synthase and succinate dehydrogenase which increased by approximately 75% in the homogenates, without modification of specific activities in the mitochondrial fractions (Table 1). This strongly suggests an increase in the protein content in the mitochondrial fractions of valproate-treated animals. As shown in Table 2, coupled oxidation rates in state 3 were decreased in mitochondria from valproate-treated rats with both succinate and glutamate/malate, indicating an effect at or subsequent to complex III. However, in uncoupled mitochondria, there was no difference in the succinate oxidation rate between control and valproate-treated animals. As shown in Table 2, ADP:O ratios were slightly decreased after valproate administration.

Succinate oxidation was also measured at 420 nm as the antimycin-sensitive reduction of ferricyanide [9] in which the electrons are "tapped-off" from cytochrome *c* to ferricyanide on the outer face of the inner mitochondrial membrane. Hence, the activity of cytochrome oxidase (complex IV) is not involved in this assay. As shown in Table 3, the succinate oxidation rates measured under these conditions did not differ between control and valproate samples, whether coupled or uncoupled, indicating that the inhibition was not located in the span of complex II to cytochrome *c*. It is worth pointing out that the rate of reduction of ferricyanide by rotenone-blocked rat liver mitochondria in the presence of succinate is comparable to the rate of oxygen uptake (assuming one electron per molecule of ferricyanide and two electrons per atom oxygen).

The activity of cytochrome oxidase was then measured polarographically in liver mitochondrial fractions and spectrophometrically in liver homogenates and mitochondria. It was decreased in liver homogenates after 75 days of valproate administration (38.1 ± 0.9 and 9.3 ± 0.6 U/g liver in control and valproate-treated animals, respectively). This decrease was also present in mitochondrial fractions measured by either method (Table 4), there being an excellent correlation between the two methods ($r = 0.98$). In order to determine whether the decrease in cytochrome oxidase activity was due to a loss of cytochrome aa_3, the concentrations of the cytochromes in liver mitochondria were determined. The amount of cytochrome aa_3 decreased by 57% in valproate-treated mitochondria while cytochromes *c*, *b* and c_1 were not significantly affected. This effect was confirmed in liver homogenates in which cytochrome aa_3 content was decreased by 53% (not shown) and cytochrome *c* oxidase activity was 24% of control values. Oligomycin-sensitive ATPase activity was not affected by administration of valproate (272 ± 24 and 287 ± 19 mU/mg protein in control and valproate-treated rats, respectively).

The mechanism of the inhibition induced by chronic administration of valproate on complex IV was further investigated. There was no direct effect of valproate itself on the enzyme (not shown). However, a direct effect of a metabolite of the drug on cytochrome *c* oxidase cannot be ruled out. A more likely explanation is the inhibition of the synthesis of a subunit of the enzyme.

Table 3. Succinate oxidation measured in intact rat liver mitochondria as the antimycin-sensitive reduction of ferricyanide

	+ADP	+FCCP
Controls	388 ± 18	364 ± 10
Valproate	398 ± 26	352 ± 12

Succinate oxidation was measured as the antimycin-sensitive reduction of ferricyanide. Coupled conditions were obtained by the addition of 1 mM ADP and uncoupled conditions by the addition of 1 μM FCCP.

Results are expressed as nmol ferricyanide/min/mg protein (mean ± SEM, N = 4–5).

Table 2. Effect of chronic administration of valproate (1% w/w in the diet for 75 days) on the oxidation rates of either 10 mM succinate or 10 mM glutamate in the presence of 1 mM malate

	State 3	State 4	RCR	+FCCP	ADP:O
Succinate					
Controls	148.0 ± 7.1	27.6 ± 1.0	5.30 ± 0.35	174.2 ± 11.7	1.66 ± 0.05
Valproate	104.1 ± 5.2‡	26.9 ± 2.7	4.10 ± 0.61	187.6 ± 12.5	1.52 ± 0.03*
Glutamate/malate					
Controls	105.2 ± 7.8	14.6 ± 1.6	7.51 ± 0.89	80.3 ± 7.8	2.47 ± 0.06
Valproate	70.2 ± 3.1†	18.7 ± 1.1	3.75 ± 0.27†	77.6 ± 7.6	2.10 ± 0.07†

Experimental conditions are described in Materials and Methods. Respiration rates in states 3 and 4 are expressed as ng atoms oxygen/min/mg protein. RCR, state 3 respiration/state 4 respiration. Statistical difference between groups was determined with a *t*-test.

Results are given as means ± SEM for five animals.

* $P \leq 0.05$; † $P < 0.01$; ‡ $P \leq 0.001$.

Table 4. Effect of chronic administration of valproate on cytochrome oxidase and cytochrome aa_3 in liver mitochondria

	Cytochrome oxidase		
	Spectrophotometric	Polarographic	Cytochrome aa_3
Controls	279 ± 26	1.07 ± 0.07	169 ± 11
Valproate	50 ± 3†	0.21 ± 0.01†	72 ± 19*

Cytochrome oxidase was measured as nmol cytochrome *c*/min/mg protein (spectrophotometric method) or μg atoms oxygen/min/mg protein (polarographic method); cytochrome aa_3, as pmol/mg protein (mean ± SEM; N = 6).
* $P \leq 0.01$ and † $P \leq 0.001$.

The valproate-treated animal itself is of interest for the study of the role of complex IV in mitochondrial respiration. It is striking that in spite of the significant loss of the activity of complex IV (82%), there was an approximate 30% inhibition only of coupled respiration with succinate and glutamate (+malate), and a normal rate of respiration in mitochondria uncoupled with 1 μM FCCP. This indicates that the capacity for electron transfer across complex IV is far in excess of that required for normal functioning in coupled mitochondria. Furthermore, the fact that an uncoupling agent relieves the inhibition due to a marked loss of cytochrome aa_3 suggests that, in complex IV, this cytochrome is more involved in the proton-pumping activity than in electron transfer. This conclusion is supported by the observation in this study that the rate of electron transfer from succinate to oxygen is faster in uncoupled mitochondria (348 ± 24 nmol electrons/min/mg protein, assuming two electrons per atom of oxygen) than in coupled mitochondria (296 ± 14 nmol electrons/min/mg protein). This apparent control by coupling to ATP production appears to be located within complex IV. Indeed, omitting this step as in the ferricyanide reduction assay gives similar rates under both coupled and uncoupled conditions (388 ± 18 and 364 ± 10 nmol electrons/min/mg protein, respectively assuming one electron per molecule of ferricyanide).

In summary, liver mitochondria from rats fed 1% (w/w) valproic acid for 75 days displayed an approximate 30% decrease in coupled respiration rates with succinate and glutamate/malate but respiration rates in uncoupled mitochondria were not affected. Cytochrome *c* oxidase activity was decreased by 82% in treated animals, associated with a decrease in cytochrome aa_3 content. Thus, valproate inhibits coupled respiration in hepatic mitochondria due to loss of cytochrome aa_3. Furthermore, the capacity for electron transport through complex IV, being far in excess of that required for normal functioning in coupled mitochondria, appears to be controlled by coupling to proton-pumping in which cytochrome aa_3 appears to play a major role. The valproate-treated animal is, thus, a useful tool for studying this effect and defining further the mechanism of action of complex IV. The mechanism by which valproate decreases cytochrome aa_3 content remains to be determined.

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Correlation between *trans*-stilbene oxide–glutathione conjugation activity and the deletion mutation in the glutathione *S*-transferase class Mu gene detected by polymerase chain reaction

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Abstract—Glutathione *S*-transferase (GST) class Mu activity was determined in 145 unrelated hospital patients in Berlin by measuring their conjugation activity towards the specific substrate *trans*-stilbene oxide (TSO) with two substrate concentrations (50 and 250 μM) in homogenates prepared from lymphocytes. Eighty individuals (55.2%) had an activity lower than 10 pmol/min/10^6 lymphocytes and were classified as GST class Mu deficient. In 142 of 145 cases, phenotype was confirmed by the results of a genotyping procedure using the polymerase chain reaction technique. Two fragments of 273 and about 650 bp including one and two introns, respectively, could always be amplified from genomic DNA in individuals with high GST class Mu activity and could not be amplified in persons with impaired glutathione–TSO conjugation activity. This indicates that persons with low activity carry a large deletion mutation within the GST class Mu gene. The enzymatically determined antimode between low and high activity determined as 10 pmol/min/1 million lymphocytes in the assay with 50 μM TSO could be clearly confirmed by genotyping.

The glutathione *S*-transferases (GSTs*) take a prominent place among phase II drug metabolizing and detoxifying enzymes [1–3]. Among the three different classes of cytosolic GSTs (Alpha, Mu and Pi), the near-neutral isoenzymes μ and ψ of class Mu are of special interest in molecular-epidemiological studies since, in Caucasian populations, GST Mu activity can only be detected in about half of the individuals tested. GST isoenzyme μ was originally purified from human liver by virtue of its high conjugation activity towards benzo(*a*)pyrene-4,5-oxide [4]. Subsequently, it was discovered that these enzymes, also present in high activity in human lymphocytes, can be specifically assayed by their unique conjugation activity towards the substrate TSO [5].

Since GST class Mu isoenzymes are capable of detoxifying mutagenic electrophilic environmental toxins and their reactive intermediates generated by metabolic activation via cytochrome P450 isoenzymes, such as the benzo(*a*)pyrene-4,5-oxide mentioned above, persons with heritably high activity of these enzymes are assumed to be better protected against certain carcinogens than persons lacking these enzymes. A recent epidemiological study [6] revealed an over-representation of phenotypically GST class Mu deficient individuals among lung cancer patients, as tested *ex vivo* with TSO.

Isolation of the cDNA of a human GST class Mu has allowed the performance of restriction fragment length polymorphism analysis [7]. Sequence analysis of this cDNA and addition of knowledge about gene structure of the highly homologous rat genes [8] enabled the design of primers for PCR diagnosis. Using the PCR technique, a complete correlation between immunologically detectable GST class Mu protein and the genetically detected deletion mutation has been described in a limited number of individuals [9]. Thus, to date, carriers of the deficiency of GST Mu can be identified by three different means, namely enzymatically, genetically, and by specific radioimmunoassays [10]. Although the correspondence between GST class Mu specific immunoreactivity and the detection of a deletion mutation by PCR has been shown casuistically, a strict correlation between the PCR results and enzyme activity requires experimental confirmation in a large population. Such a sample size is necessary to detect rare mutations.

This study describes the distribution of the TSO conjugation activity among hospital patients recruited in Berlin and its correlation with the diagnosis of a deletion mutation obtained by use of the PCR.

Materials and Methods

Reagents. Non-radioactive TSO and 2-chloro-1,2-diphenylethanone (desylchloride)were from Aldrich (Steinheim, F.R.G.). GSH was from Boehringer (Mannheim, F.R.G.). Tritiated sodium borohydride was from Amersham (Amersham, U.K.). All other chemicals were analytical grade from Merck (Darmstadt, F.R.G.). All syntheses were performed in a closed vial-system in an appropriate fumehood. The tritiated substrate TSO was synthesized [11] by [^{3}H]sodium borohydride (100 mCi, 9.76 Ci/mmol) reduction of 2-chloro-1,2-diphenylethanone (55.9 μmol) to chlorohydrin and subsequent alkaline epoxide formation. The resulting mixture of *cis*- and *trans*-

* Abbreviations: GST, glutathione *S*-transferase; TSO, *trans*-stilbene oxide; PCR, polymerase chain reaction; GSH, reduced glutathione.