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CHRONIC ADMINISTRATION OF THE ORAL HYPOGLYCAEMIC AGENT DIPHENYLENEIODONIUM TO RATS

AN ANIMAL MODEL OF IMPAIRED OXIDATIVE PHOSPHORYLATION (MITOCHONDRIAL MYOPATHY)

J. MARK COOPER,*† RICHARD K. H. PETTY,† DAVID J. HAYES,‡§ JOHN A. MORGAN-HUGHES† and JOHN B. CLARK*‖

* Department of Biochemistry, The Medical College of St. Bartholomew's Hospital, University of London, Charterhouse Square, London EC1M 6BQ; † Department of Clinical Neurology, Institute of Neurology, University of London, Queen Square, London WC1N 2NS; and ‡ Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

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Abstract—Daily subcutaneous administration of the oral hypoglycaemic agent, diphenyleneiodonium at a low dose (1.5 mg/kg body weight) over a 4–5 week period resulted in a normoglycaemic stable animal model of impaired oxidative phosphorylation in the rat. Diphenyleneiodonium specifically inhibits NAD-linked mitochondrial oxidation [Bloxham, *Biochem. Soc. Trans.* **7**, 103 (1979)], and in isolated mitochondrial preparations from heart, soleus and gastrocnemius muscle and liver from treated animals NAD-linked respiration was reduced by 40% or more of mean control values. Brain and kidney mitochondria isolated from the treated group had similar rates of NAD-linked respiration to their respective control values. The activity of NADH–ferricyanide reductase was significantly reduced in all tissues tested, even in the isolated brain and kidney mitochondria where the activity in these tissues was 60–75% of control values. This suggests that at least 40% of Complex I activity must be inhibited before there is a decline in NAD-linked mitochondrial respiration. This paper discusses the use of diphenyleneiodonium as a means of establishing an animal model of the human disease state, termed mitochondrial myopathy.

The administration of the oral hypoglycaemic agent, diphenyleneiodonium [1] to rats has been demonstrated to be cumulatively toxic [2, 3]. The site of inhibition of diphenyleneiodonium was shown to be within Complex I (NADH–ubiquinone reductase) of the mitochondrial respiratory chain [4] where a 23.5 kD polypeptide was covalently modified with a stoichiometry of 1 mol of diphenyleneiodonium per mol of FMN [5], thereby inhibiting mitochondrial NAD-linked respiration [5]. The hypoglycaemic consequence of diphenyleneiodonium administration was suggested to be due to a reduced rate of ATP supply for liver gluconeogenesis [3, 4].

Subcutaneous injection of diphenyleneiodonium (2–3 doses at 3 mg/kg body weight) into rats has been used previously to establish an acute animal model [6] of the human disease state termed mitochondrial myopathy [7, 8]. Typically patients suffering from this disease present with muscle weakness and exercise-induced lactic acidosis, where the underlying biochemical defect is thought to be due to a primary lesion in the mitochondrial respiratory chain [7, 8]. A relatively high proportion of these patients have defects localised within Complex I [9]. In the acute animal model, the animals sometimes became hypoglycaemic and the recovery of phosphocreatine and pH was incomplete following muscle stimulation [6]. Thus, a chronic model at a lower daily dose has been established and using this protocol the potential hypoglycaemic action was negated. In this paper the animal model has been characterised and some adaptations to a reduced oxidative capacity have been demonstrated.

MATERIALS AND METHODS

Diphenyleneiodonium (2,-2 bis biphenylene iodonium sulphate) was prepared from 2-iodobiphenyl (K & K Rare Chemicals, Kodak Ltd, Kirby, Liverpool, U.K.) by the method of Collette *et al.* [10] as described by H. S. A. Sherratt (personal communication; Dept. of Pharmacological Sciences, University of Newcastle upon Tyne). All other reagents were obtained from suppliers previously detailed [11].

Animals. Male Wistar rats (local inbred strain) were used (100–120 g body weight). Control and experimental animals were housed together in groups of 6–8 and food was always freely available. The drinking water was fortified with 5% w/v glucose [11] to minimise the potential hypoglycaemic action. The diphenyleneiodonium was dissolved in warm 5% w/v glucose and was injected subcutaneously

§ Present address, Department of Biochemical Microbiology, The Wellcome Research Laboratories, Langley Court, Beckenham, Kent, BR3 3BS, U.K.

‖ To whom all correspondence should be addressed.

at 1.5 mg/kg body weight for 4–5 weeks. Control animals received similar volumes of 5% w/v glucose. The dose was given between 1800 and 1900 hours, i.e. just prior to the animals nightly intake of food.

Mitochondrial isolation. The rats were either killed by cervical dislocation or by guillotine (for brain studies), the relevant tissue(s) were rapidly dissected and placed in ice cold medium (see below). Liver mitochondria were isolated from a single liver [6]. Skeletal muscle mitochondria were prepared from 4 gastrocnemius muscles and six to eight soleus muscles by a trypsin digestion method [12]. Brain mitochondria were isolated from six animals [13]. Kidney mitochondria were prepared from two animals [6]. In all cases the final mitochondrial pellet was resuspended in ice-cold medium (225 mM mannitol; 75 mM sucrose; 10 mM Tris; 500 μM EDTA; pH 7.4). All respiratory activities were measured at 25° [14] and state 3 respiration was induced by the addition of 100–250 nmoles ADP. Protein was measured [15] using bovine serum albumin to construct standard curves.

Metabolite assays. The relevant tissue was freeze clamped between two aluminium plates at 77°K and quenched in liquid N_2. The frozen samples were stored in liquid N_2. Nucleotides were extracted in 10 vol. of 10% w/v trichloroacetic acid containing 20% v/v methanol [16] and quantified by reverse phase HPLC [11, 17]. Phosphocreatine and creatine were measured following extraction in 6 vol. of 4% w/v perchloric acid [18]. Lactate and glycogen were measured as described in [19].

Cytochrome content. The concentration of mitochondrial cytochromes were determined at 77 K [20, 21] as described previously [14]. Separate cytochrome *b* spectra were always obtained by incubating the isolated mitochondrial fraction with succinate (10 mM), rotenone (10 μM) and antimycin A (40 μg/ml).

Maximal enzyme activities. Maximal enzyme activities were measured at 25° using either freshly prepared mitochondrial fractions or freshly homogenised tissue samples [22]. Where necessary Triton-X-100 was added to release full activity (0.05–0.5% v/v). The activities of hexokinase, 6-phosphofructokinase, lactate dehydrogenase, citrate synthase and 2-oxoglutarate dehydrogenase were measured as described in [22]. Pyruvate dehydrogenase was measured in isolated mitochondria by the method of Reed and Williams [23], activation was induced by incubation with Mg^{2+} (8 mM) and Ca^{2+} (0.8 mM) [14]. The activity of pyruvate dehydrogenase was also measured in freeze-clamped liver samples [24] and full activity was induced by incubation with purified bovine heart pyruvate dehydrogenase phosphatase [25]. NADH ferricyanide reductase [26], NADH menadione reductase [4], malate dehydrogenase [27], glutamate–oxaloacetate transaminase [28], malate–aspartate-shuttle [29], mitochondrial ATPase (+24 μM FCCP) [30], glutamate-pyruvate transaminase [31], NADH cytochrome *c* reductase [32], and succinate cytochrome *c* reductase [33] were measured as previously described.

Presentation of data and statistics. All results are presented as the mean ± SD. Statistical significance was assessed with a two tailed Student's *t*-test.

RESULTS

In vivo *observations*

The diphenyleneiodonium injected animals were as active as their co-housed litter mates. The injected group remained healthy and groomed normally. Random sampling of blood from the tail vein revealed that the injected animals were always normoglycaemic (glucose $\geq$ 5 mM). The growth rate curve for the injected animals dropped below the control rate after 21 days ($P \leq 0.05$) and was 75% of the mean control value at 5 weeks (Table 1). The wet weight of the plantaris muscle was significantly lower than the mean respective control value (Table 1). The wet weight of the injected group's heart was similar to control values but was significantly increased when compared as a fraction of total body weight (Table 1).

Isolated mitochondrial studies

Mitochondria were isolated from the following tissues, gastrocnemius and soleus muscle, heart, liver, kidney and brain and the rates of oxygen uptake are shown in Table 2. The respiratory activities of NAD-linked substrates (pyruvate or glutamate) were significantly lower in the diphenyleneiodonium injected group except those observed in the isolated brain and kidney mitochondria. In general all muscle tissues displayed a reduction of about 60% in their pyruvate or glutamate respiration when compared to their mean control values respectively. Liver mitochondria from injected animals showed a 40% decrease in glutamate respiration (Table 2). Palmitoylcarnitine oxidation was also significantly inhibited (by approx. 60%) in mitochondria from gastrocnemius and heart muscle, whereas the rate of succinate respiration was not affected in any tissue, thereby confirming the site of inhibition was within Complex I [2–5]. Moreover the activity of succinate cytochrome *c* reductase was also unaffected (data not shown). The rate of duroquinol oxidation (this artificial substrate feeds reducing equivalents directly to cytochrome *b* [34]) was elevated almost twofold in isolated liver mitochondria in the injected animal group (Table 2). The oxidation of ascorbate + TMPD was essentially unaffected

Table 1. Whole body, heart and skeletal muscle weights of rats which had been chronically injected with 1.5 mg/kg diphenyleneiodonium (DPI)/day

	Control	DPI
Bodyweight (g)	274 ± 32	204 ± 27**
Gastrocnemius (g/kg)	5.44 ± 0.37	5.10 ± 0.61
Plantaris (g/kg)	0.83 ± 0.05	0.63 ± 0.10**
Soleus (g/kg)	0.42 ± 0.07	0.37 ± 0.10
Heart (g/kg)	3.77 ± 0.37	4.35 ± 0.39*

Animals were injected with diphenyleneiodonium (1.5 mg/kg/day in 5% w/v glucose) for 5 weeks. Controls were aged matched and were injected with an equal volume of 5% w/v glucose. The results are the mean ± SD of 6 animals. The tissue mass is expressed as tissue weight/body weight in g/kg. * $P \leq 0.05$, ** $P \leq 0.02$. DPI = animals injected chronically with diphenyleneiodonium (see Methods).

except in the case of liver mitochondria where the experimental group showed marginally higher respiratory activities (Table 2).

Maximal enzyme activities

The functional activity of the mitochondrial Complex I system may be assessed by the use of artificial dyes [2, 26, 35] or by adding exogenous cytochrome *c* [33]. The rates of NADH oxidation supported by ferricyanide and cytochrome *c* were significantly reduced in all the tissues studied from the treated rats (gastrocnemius muscle, heart, liver, kidney and brain—Table 3). The NADH–menadione reductase was also reduced except in the case of brain (Table 3). Another NADH oxidizing system, the malate–aspartate shuttle [29, 36], was reduced in mitochondria from the gastrocnemius muscle, heart and liver (Table 4). This diminished activity was not due to a loss of activity of the component enzymes of the shuttle, e.g. malate dehydrogenase or glutamate oxaloacetate transaminase (see Table 4) but may be explained on the basis of the reduced Complex I activity. The number of mitochondria present in the heart of the diphenyleneiodonium injected group was significantly increased as judged by the elevated citrate synthase activity measured (Table 4). The activity of the pyruvate dehydrogenase complex in all the mitochondrial fractions tested was similar to their mean respective control values, except in the case of the liver (Table 4) where it was significantly reduced. The lower basal pyruvate dehydrogenase activity observed in the isolated liver mitochondria from the injected group was enhanced to the full activity by pre-incubating the mitochondrial preparations with Ca^{2+} and Mg^{2+} ions (see Methods [39]; data not shown). Under these activating conditions the rates recorded in the experimental group were similar to control values. Therefore, the phosphorylated (inactive) conformation of the pyruvate dehydrogenase complex was the dominant form. It is known that during the isolation of mitochondrial fractions the ratio of active/inactive pyruvate dehydrogenase may shift (Kerbey, personal communication; Hayes, unpublished data). The activity of the pyruvate dehydrogenase complex *in vivo* was therefore measured using an *in vitro* assay [24] on freeze clamped tissue samples. The dephosphorylated enzyme activity in control and diphenyleneiodonium injected animals' livers, was 0.74 ± 0.38 (N = 5) and DPI 0.72 ± 0.07 (N = 5) μmoles/min/g wet weight respectively. Furthermore, the maximal activity was measured by incubating an aliquot of each preparation with purified bovine heart pyruvate dehydrogenase phosphatase [25] and the activated rates were control 2.55 ± 0.29 (N = 5) and DPI 2.56 ± 0.33 (N =5) μmoles/min/g wet weight. Thus under conditions *in vivo* there was no reduction in basal or fully activated pyruvate dehydrogenase complex activity.

Mitochondrial cytochrome content

The cytochrome content of isolated mitochondria from the gastrocnemius muscle, heart, liver and brain was determined at 77K. There were no differences recorded between control values and those from the diphenyleneiodonium injected group except in the

Table 2. Respiratory activities of rat gastrocnemius muscle, soleus muscle, heart, liver, brain and kidney mitochondria isolated from animals which had been chronically injected with diphenyleneiodonium (DPI)

Tissue	Gastrocnemius muscle		Soleus muscle		Heart		Liver		Brain		Kidney	
Substrate	Control (N = 6)	DPI (N = 6)	Control (N = 3)	DPI (N = 3)	Control (N = 3)	DPI (N = 3)	Control (N = 6)	DPI (N = 6)	Control (N = 3)	DPI (N = 3)	Control (N = 3)	DPI (N = 3)
Pyruvate + malate	152 ± 19	60 ± 12**	145 ± 4	67 ± 13**	296 ± 42	99 ± 14**	ND	ND	113 ± 6	107 ± 7	72 ± 12	56 ± 4
Glutamate + malate	183 ± 29	66 ± 10**	149 ± 14	58 ± 15**	307 ± 42	134 ± 13**	47 ± 4	28 ± 3**	91 ± 7	92 ± 22	89 ± 9	75 ± 8
Palmitoylcarnitine + malate	91 ± 10	30 ± 8**	ND	ND	228 ± 57	93 ± 25**	ND	ND	ND	ND	ND	ND
Succinate + rotenone	183 ± 11	181 ± 15	214 ± 39	195 ± 34	307 ± 19	255 ± 33	65 ± 10	74 ± 13	150 ± 13	148 ± 36	154 ± 19	155 ± 10
Duroquinol	284 ± 30	327 ± 34	ND	ND	ND	ND	48 ± 2	89 ± 14**	ND	ND	ND	ND
Ascorbate + TMPD	339 ± 38	347 ± 31	471 ± 39	476 ± 51	421 ± 14	377 ± 18	111 ± 4	132 ± 8*	249 ± 63	235 ± 68	220 ± 41	228 ± 29

Respiration studies were performed at 25° in respiration buffer (100 mM KCl, 75 mM mannitol, 25 mM sucrose, 10 mM Tris, 5 mM phosphate, 50 μM EDTA, pH 7.4 see Methods [14]) and are expressed as natmO/min/mg mitochondrial protein. The results are shown as the mean state 3 rate ± SD with the number of preparations in parenthesis. The respiratory control ratio was assessed in all tissues and was ⩾ 4.0, 2.7, 6.4, 4.5, 3.6 and 3.1 in both control and injected groups in the gastrocnemius, soleus, heart, liver, brain and kidney mitochondria respectively in the case of glutamate (+ malate) respiration. ND = not determined. * $P \leqslant 0.05$, ** $P \leqslant 0.02$. DPI = animals injected chronically with diphenyleneiodonium (see Methods).

liver and kidney. The concentrations of cytochrome *b* in the liver was twofold higher in the injected group and the concentration of cytochrome *c* was significantly increased in the kidney and heart (Table 5).

Resting muscle metabolite levels

Various metabolites were measured in neutralised perchloric acid or trichloroacetic acid extracts (see Methods) from gastrocnemius and soleus muscle, which had been freeze clamped at rest under urethane anaesthesia (Table 6). The concentration of IMP and lactate was significantly elevated in the muscles from animals treated with diphenyleneiodonium, whereas ATP was essentially unchanged in the gastrocnemius muscle but significantly lowered in the case of the treated soleus muscle. Phosphocreatine was decreased in the diphenyleneiodonium treated muscle but only significantly in the case of the gastrocnemius (Table 6). *In vivo* measurements of phosphocreatine concentrations in the gastrocnemius muscle were also made using ^{31}P-NMR [6, 22] (Table 7). Those studies confirmed the HPLC data indicating the significant fall in phosphocreatine in the treated muscle and the lack of change in ATP concentrations. However, the calculated free ADP concentration was almost doubled in the treated muscle whereas the intracellular pH was unchanged (Table 7).

DISCUSSION

The bio-availability of diphenyleneiodonium has been studied [2]. The tissue distribution was affected by the route of administration, the major site of uptake being the liver if blood drainage was predominantly through this organ, i.e. intraperitoneal injection or via the hepatic portal vein [2]. The amount deposited in the liver could be reduced by 50% if the drug was delivered via the femoral vein [2]. In the latter case the quantity of diphenyleneiodonium trapped in skeletal muscle and heart was 50% greater than by the portal vein route. The distribution to brain tissue did not appear to be dependent on the route of administration. The data in Table 2 demonstrated that diphenyleneiodonium inhibited isolated heart and skeletal muscle mitochondrial NAD-linked respiration by about 60%, and by about 40% in the liver (Table 2). Although diphenyleneiodonium crosses the blood–brain barrier [2] no effect on brain mitochondrial respiration was noted (Table 2). However, the activity of NADH–ferricyanide reductase in the diphenyleneiodonium injected brain mitochondria was 75% of the control value and 60% of the control value in the kidney. This may be explained on the basis that between 25 and 40% of the Complex I activity must be inhibited before a reduction in NAD-linked respiration may be experimentally observed (Tables 2 and 3).

In the human disease state, examination of skeletal muscle biopsies in some patients has shown increased numbers of mitochondria [7, 8] and the activity of the mitochondrially located enzyme citrate synthase was greater than the mean control value (Cooper, unpublished data; [37]). Such changes were not evident in the gastrocnemius muscle (see Table 4) (Petty, unpublished observations; [38]). However, the activity of citrate synthase in the heart in the

Table 3. Effect of chronic diphenyleneiodonium (DPI) administration on mitochondrial NADH oxidising enzymes

Mitochondrial source	NADH–menadione reductase	NADH–ferricyanide reductase	NADH–cytochrome *c* reductase
Gastrocnemius muscle:			
DPI	36 ± 6**	725 ± 96**	41 ± 4**
Control	78 ± 15	2898 ± 810	150 ± 24
Soleus muscle:			
DPI	62 ± 4*	ND	ND
Control	75 ± 8	ND	ND
Heart:			
DPI	29 ± 6**	320 ± 72**	26 ± 8**
Control	60 ± 19	1320 ± 100	106 ± 18
Liver:			
DPI	18 ± 5**	800 ± 120*	ND
Control	59 ± 9	1226 ± 202	ND
Brain:			
DPI	44 ± 4	1901 ± 80**	ND
Control	43 ± 8	2514 ± 37	ND
Kidney:			
DPI	ND	1181 ± 90**	ND
Control	ND	2015 ± 170	ND

Activities were determined at 25° as detailed in the Methods section. The results are the means ± SD of between 3 and 7 preparations and are expressed in nmoles·min^{-1} mg mitochondrial protein $^{-1}$. ND =not determined. * $P \leq 0.05$; ** $P \leq 0.02$. DPI = animals injected chronically with diphenyleneiodonium (see Methods).

Table 4. Effect of chronic diphenyleneiodonium (DPI) administration on enzymes of energy metabolism

Enzyme	Gastrocnemius muscle		Heart		Liver	
	DPI	Control	DPI	Control	DPI	Control
Units μmole·min^{-1}·g·wet wt^{-1}						
Hexokinase	1.2 ± 0.1	1.18 ± 0.12	6.2 ± 0.4	6.7 ± 0.2	ND	ND
6-Phosphofructokinase	40.9 ± 7.0	43.4 ± 1.6	8.3 ± 0.7	9.1 ± 1.1	ND	ND
Phosphorylase	38.5 ± 2.2**	44.4 ± 1.6	9.3 ± 0.3	8.3 ± 1.1	ND	ND
Lactate dehydrogenase	423 ± 18**	522 ± 27	340 ± 80	275 ± 36	280 ± 48	241 ± 21
Citrate synthase	12.8 ± 1	13.3 ± 2.1	115 ± 12**	82 ± 12	10.2 ± 0.9	10.5 ± 1.8
nmol·min·mg·mit·prot^{-1}						
Citrate synthase	1465 ± 189	1760 ± 209	1207 ± 112	1097 ± 120	250 ± 48	230 ± 34
Pyruvate dehydrogenase (basal)	97 ± 7	117 ± 14	68 ± 16	70 ± 20	0.4 ± 0.2**	7 ± 1.9
2-Oxoglutarate dehydrogenase	191 ± 28	199 ± 9	94 ± 8	108 ± 10	34 ± 6	34 ± 5
Malate dehydrogenase	26689 ± 690	25951 ± 2440	ND	ND	7828 ± 1070	6880 ± 960
Glutamate-oxaloacetate transaminase	3369 ± 316	2785 ± 364	ND	ND	1511 ± 20	1546 ± 78
Glutamate-pyruvate transaminase	480 ± 89	510 ± 109	ND	ND	87 ± 5	67 ± 3
ATPase (+FCCP)	484 ± 89	385 ± 57	355 ± 58	416 ± 10	ND	ND
Malate-aspartate shuttle (+ADP)	15 ± 8**	78 ± 15	8 ± 1.6**	39 ± 4	1.2 ± 0.1**	15 ± 3

Activities determined as indicated in the Methods section and Table 3. ND = not determined. * $P \leq 0.05$, ** $P \leq 0.02$. DPI = animals injected chronically with diphenyleneiodonium (see Methods).

Table 5. Cytochrome content of gastrocnemius muscle, heart, liver and brain mitochondria isolated from animals which had been chronically injected with diphenyleneiodonium (DPI)

	Cytochrome (nmol/mg mitochondrial protein)			
	b	c_1	*c*	a/a_3
Gastrocnemius				
Control (N = 4)	0.41 ± 0.03	0.51 ± 0.02	0.81 ± 0.03	0.43 ± 0.10
DPI (N = 3)	0.37 ± 0.09	0.49 ± 0.09	0.86 ± 0.05	0.45 ± 0.05
Heart				
Control (N = 3)	0.39 ± 0.05	0.43 ± 0.02	0.53 ± 0.03	0.38 ± 0.06
DPI (N = 3)	0.38 ± 0.05	0.43 ± 0.01	0.84 ± 0.01**	0.41 ± 0.03
Liver				
Control (N = 6)	0.04 ± 0.01	0.29 ± 0.05	0.30 ± 0.05	0.09 ± 0.02
DPI (N = 6)	0.08 ± 0.01**	0.30 ± 0.09	0.34 ± 0.08	0.14 ± 0.04
Brain				
Control (N = 3)	0.27 ± 0.08	0.39 ± 0.06	0.55 ± 0.06	0.31 ± 0.05
DPI (N = 3)	0.24 ± 0.01	0.34 ± 0.06	0.52 ± 0.06	0.29 ± 0.01*
Kidney				
Control (N = 3)	0.15 ± 0.03	0.43 ± 0.03	0.76 ± 0.04	0.28 ± 0.01
DPI (N = 3)	0.17 ± 0.03	0.44 ± 0.06	1.13 ± 0.14**	0.37 ± 0.02

The cytochrome content of isolated mitochondria was determined at 77°K (see Methods) and are shown as the mean ± SD with the number of preparations in parenthesis. * $P \leq 0.05$, ** $P \leq 0.02$. DPI = animals injected chronically with diphenyleneiodonium (see Methods).

injected group was significantly higher than the control value (Table 4). The isolated liver mitochondria from diphenyleneiodonium injected animals revealed a twofold higher concentration of cytochrome *b* (Table 5). This apparent adaptation might explain why the oxygen uptake supported by duroquinol was significantly greater than the control value (Table 2), since this substrate feeds reducing equivalents directly to cytochrome *b* [34]. Such a change might enable the oxidation of non-NAD-linked substrates, e.g. certain amino acids/succinate to be accelerated, hence maintaining ATP levels in the liver, thereby maintaining the capability for hepatic gluconeogenesis and glucose homeostasis (cf. Ref. 3).

The activity of the pyruvate dehydrogenase complex in the isolated liver mitochondria from diphenyleneiodonium injected animal was less than 6% of the control value (Table 4). This enzyme is regulated by a phosphorylation/dephosphorylation cycle [39] and can be fully activated by incubation with Ca^{2+} and Mg^{2+} ions [25]. The full activity of the pyruvate

Table 6. Resting metabolite concentrations in rat gastrocnemius and soleus muscles from animals which had been injected chronically with diphenyleneiodonium (DPI)

	Gastrocnemius		Soleus	
Metabolite	Control (N = 14)	DPI (N = 14)	Control (N = 5)	DPI (N = 5)
Inosine	0.15 ± 0.10	0.21 ± 0.09	0.05 ± 0.01	0.03 ± 0.02
IMP	0.10 ± 0.07	0.22 ± 0.12**	0.12 ± 0.04	0.25 ± 0.07**
NAD	0.48 ± 0.06	0.49 ± 0.04	0.43 ± 0.02	0.38 ± 0.04
AMP	0.03 ± 0.01	0.03 ± 0.01	0.07 ± 0.03	0.08 ± 0.10
ADP	0.81 ± 0.05	0.85 ± 0.06	0.70 ± 0.07	0.61 ± 0.09
ATP	6.68 ± 0.25	6.77 ± 0.23	4.46 ± 0.32	3.66 ± 0.33**
ATP/ADP	8.31 ± 0.50	8.02 ± 0.82	6.41 ± 0.85	6.06 ± 0.61
ANP	7.52 ± 0.27	7.64 ± 0.20	5.22 ± 0.31	4.36 ± 0.38**
AEC	0.94 ± 0.01	0.94 ± 0.01	0.92 ± 0.01	0.91 ± 0.01
Phosphocreatine	18.9 ± 1.8	16.8 ± 1.3**	12.9 ± 0.7	11.0 ± 1.2
Total creatine	27.7 ± 2.9	27.3 ± 2.0	18.3 ± 1.0	17.3 ± 1.2
Lactate	2.2 ± 0.4	3.7 ± 0.7**	3.5 ± 1.3	5.6 ± 1.2**
Glycogen	20.9 ± 3.7	19.3 ± 5.2	ND	ND

The animals were anaesthetised with urethane [6] and the muscle was freeze clamped (see Methods section). Nucleotides and creatine + phosphocreatine were measured on neutralised tissue extracts by HPLC [11, 17, 18]. Lactate and glycogen were determined enzymatically [19]. The results are the mean ± SD with the number of animals in parenthesis and are expressed as μmoles/g wet weight. ANP total adenine nucleotides. AEC is the adenylate energy charge = [ATP] + ½[ADP]/[ATP] + [ADP] + [AMP], ND = not determined, * $P \leq 0.05$, ** $P \leq 0.02$. DPI = animals injected chronically with diphenyleneiodonium (see Methods).

Table 7. The resting *in vivo* metabolite concentrations in rat gastrocnemius muscle from animals which had been injected chronically with diphenyleneiodonium (DPI)

Metabolite	Control	DPI
Total creatine (mM)	41.4 ± 1.2	40.7 ± 1.1
Phosphocreatine (mM)	36.1 ± 0.7	32.1 ± 0.7**
ATP (mM)	10.0 ± 0.1	10.1 ± 0.1
ADP (free, μM)	9.0 ± 1.0	17.0 ± 2.0**
pH	7.03 ± 0.02	7.03 ± 0.02

The results are expressed as the mean ± SD from 5 animals in each group. The mean [ATP] and [Total creatine] from Table 6 were used to quantitate ^{31}P NMR spectra. The NMR studies were performed at 73.84 MHz (see Refs 6, 22 for details). The results are expressed as molar concentrations, assuming a tissue water content of 0.73 ml/g wet weight (see Ref. 22) and free ADP was calculated from the creatine kinase equilibrium (see Ref. 22). ** $P \leq 0.02$. DPI = animals injected chronically with diphenyleneiodonium (see Methods).

dehydrogenase complex was not significantly different from control values but the basal activity in liver mitochondria was reduced. Therefore, we measured the activity of this complex in the liver *in vivo* using *in vitro* assay system (see Methods), thereby obtaining the dephosphorylated (active form)/phosphorylated (non-active form) ratio for both controls and diphenyleneiodonium injected animals. The measured ratio was not significantly different from control values. Thus the low pyruvate dehydrogenase activity present in the isolated liver mitochondrial fraction (Table 3) was an artefact due to fractionation. Liver mitochondria are usually isolated with more endogenous substrate (Hayes, unpublished data) than mitochondria isolated from other tissues and since the rate of glutamate respiration was reduced in the diphenyleneiodonium injected animals; the ratio of NADH/NAD and/or AcCoA/CoA might be shifted hence providing the required conditions for inactivating the pyruvate dehydrogenase complex [39].

Several patients with mitochondrial disorders have been investigated by phosphorous magnetic resonance spectroscopy [40, 41] and some general trends established [40]. The concentration of phosphocreatinine was lower than controls and the level of free ADP was abnormally high in many patients [40, 41]. The gastrocnemius muscle was studied in the rat by phosphorus magnetic resonance spectroscopy and the concentration of phosphocreatine was significantly lower than controls and free ADP was elevated (Table 7), therefore closely reflecting the human situation.

The purpose of the investigation reported in this paper was to establish a stable small animal model of the human disease state termed mitochondrial myopathy [7, 8]. Previous models were limited to acute drug induced myopathy [11, 42, 43] and our earlier study had the added potential complication of transient periods of hypoglycaemia [6]. The model established in this paper related to animals after 4–5 weeks of daily injection of diphenyleneiodonium. The animals appeared healthy and were as active as co-housed control litter mates, although the injected animals failed to maintain the growth rate observed in the control group (Table 1). The model closely mimics the human disease state in terms of impaired NAD-linked oxidation (Table 3) and the typical phosphorus metabolite changes (Table 6) [40, 41]. The patients also show muscle weakness exercise intolerance and exercise induced lactic acidosis [7, 8, 41], and these phenomenon have been investigated in our animal model and the results are reported elsewhere [44].

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