

## Hyperthermia, Muscle Rigidity, and Uncoupling in Skeletal Muscle Mitochondria in Rats Treated with Halothane and 2,4-Dinitrophenol

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Lethal and abrupt hyperthermia can be produced in rats when a sublethal dose of 2,4-dinitrophenol (10  $\mu\text{g/g}$ , ip) is administered during anesthesia with 2 per cent halothane in a chamber kept at 25 C, but not at 20 C. Dinitrophenol injection, after about an hour, triples the metabolic rate, increases the rectal temperature to above 44 C, and produces skeletal muscle rigor at the time of death. Muscle mitochondria from rats treated with dinitrophenol + halothane show uncoupled oxidative phosphorylation but no acceleration of respiration. Treatment with halothane or dinitrophenol separately does not produce lethal hyperthermia or uncoupling in skeletal muscle mitochondria. Liver mitochondria from lethally hyperthermic rats show normal oxidative phosphorylation. Pretreatment with L-thyroxine does not sensitize rats to halothane, nor do peripheral anti-thyroxine agents prevent or reverse the dinitrophenol + halothane syndrome;  $\text{Mg}^{++}$  salts do prevent the muscle rigor. Lethal fulminant hyperthermia can be produced in laboratory animals not chosen for genetic susceptibility by administering an uncoupling agent during halothane anesthesia. (Key words: Fulminant hyperthermia; Halothane; 2,4-Dinitrophenol; Oxidative phosphorylation; Skeletal muscle mitochondria; Muscle rigor.)

THE SYNDROME of fulminant hyperthermia that occurs occasionally in patients undergoing general anesthesia is characterized by extreme production of heat, hypermetabolism, and muscular rigidity. These symptoms are reminiscent of the effects of agents that uncouple mitochondrial oxidative phosphorylation, like 2,4-dinitrophenol (DNP), as pointed out by Wilson.<sup>1</sup> Halothane, a widely used anesthetic,

can under *in-vitro* conditions uncouple oxidative phosphorylation in isolated rat liver mitochondria,<sup>2</sup> as can a number of general and local anesthetics, probably because they are soluble in the lipids of biologic membranes.<sup>3</sup> However, anesthetic agents do not produce demonstrable mitochondrial uncoupling *in vivo* or hyperthermia under either experimental or clinical conditions. Clinical fulminant hyperthermia has been attributed to individual sensitivity to certain anesthetic agents, apparently genetically linked.<sup>4-6</sup>

To study clinical fulminant hyperthermia, it would be of obvious advantage to be able to produce an experimental counterpart in laboratory animals. Certain strains of pigs become hyperthermic and rigid during halothane anesthesia,<sup>7</sup> confirming a genetic basis. However, it does not appear to be necessary to select susceptible animals if combinations of an anesthetic and an uncoupling agent are used; halothane and DNP given together to dogs<sup>8</sup> produce a lethal hyperthermia with most of the features of the clinical syndrome. We demonstrate here some of the conditions necessary for producing hyperthermia in rats treated with both halothane and DNP, and show that their skeletal muscle mitochondria phosphorylate inefficiently.

### Materials and Methods

Male Sprague-Dawley rats, obtained from Hormone Assay Company, were maintained on a diet of Rockland Rat/Mouse food and water *ad lib.*, and fasted overnight prior to each measurement. Each weighed 120-300 g at time of use.

Metabolic rates and rectal temperatures were measured during diazepam sedation in a temperature-controlled closed system.<sup>9</sup> Ten minutes after subcutaneous injection of diaze-

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pam, a rectal probe (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio) was inserted and temperature was measured. Four rats were used in each experimental group. Each animal was confined in a restraining cage (Nuclear Associates Inc., Westbury, N.Y.), and placed in a separate, closed, water-jacketed chamber. The temperature of the chambers was kept constant at 20 or 25 C by a coupled heating and cooling system. Water evolved by each rat was absorbed by fresh, packaged silica gel (Protek-Sorb, Frank Paper Products, Detroit, Mich.), and CO<sub>2</sub> was absorbed by 30- to 50-mesh Mallcosorb (Mallinckrodt Chemical Works, St. Louis, Mo.). Each chamber was attached to a volume meter (Med-Science Electronics, St. Louis, Mo.), and oxygen uptake was measured. Metabolic rates were calculated as kilocalories per hour per kilogram<sup>3/4</sup>, assuming 4.7 calories per ml O<sub>2</sub> consumed in fasted rats.<sup>10, 11</sup>

Anesthesia was induced with halothane (Fluothane, a generous gift from Ayerst Laboratories, New York, N. Y.) introduced into the chamber at concentrations of 1.5 and 2.0 per cent for 9 min by placing a Fluotec vaporizer (Fraser Sweatman, Inc., Lancaster, N. Y.) into the O<sub>2</sub>-inlet line of the chamber. The chambers were purged with U.S.P. therapy-grade oxygen at flows of 3 to 5½ l/min, such that when only two of the four rats received halothane, flow through the Fluotec was still about 3 l/min. Anesthesia was maintained by subsequent 1.5-min purges with halothane-O<sub>2</sub> every 30 min. Oxygen consumption and rectal temperature were monitored continuously and recorded at 5-minute intervals.

After 40 to 60 minutes of anesthesia, the rats were removed from the chambers, injected intraperitoneally with 10 µg/g neutralized 2,4-dinitrophenol (DNP, Eastman Organic Chemicals, Rochester, N.Y.), and returned to the chambers. They were then treated with halothane-O<sub>2</sub> for 8 min, followed as before by 1.5-min purges every 30 min until termination of the experiment. Purge times and flow rates through the Fluotec were kept constant throughout, and as much as possible the rats were positioned identically in the restraining cages and chambers. This was considered necessary because of the lack of facilities for monitoring chamber or tissue halothane concentrations.

Chemical reagents were obtained commercially. Solutions of L-thyroxine sodium pentahydrate (LT<sub>4</sub> from Sigma Chemical Company, St. Louis, Mo.) were prepared just prior to injection.<sup>12</sup> Solutions of 4-methyl-2-thiouracil from Sigma Chemical Co. were adjusted to pH 9.1-9.4. Dactinomycin (MSD-injection Lyovac Cosmegen from Merck, Sharp and Dohme, West Point, Pa.) in 0.5-mg vials and magnesium sulfate in sterile 500 mg/ml solution for injection from The University of Michigan Hospital Pharmacy were appropriately diluted with sterile water.

Skeletal muscle mitochondria were prepared from about 10 g of minced leg and back muscle, as described previously<sup>13</sup>; the procedure uses a Chappell-Perry medium<sup>14</sup> in conjunction with digestion with *B. subtilis* proteinase from Nagarse and Co., Ltd. (Osaka, Japan). Liver mitochondria were prepared in 0.25 M sucrose. Protein was measured by a rapid biuret method.<sup>15</sup> Respiration of mitochondrial suspensions was measured with a Clark electrode and a Gilson oxygraph. For skeletal muscle mitochondria and liver mitochondria, the 3.0-ml reaction mixture at pH 7.4, room temperature, contained (final concentrations): 0.25 M sucrose; 0.2 mM EDTA; 10 mM KCl; 10 mM Tris buffer; 4 mM Pi; 4.5 mg protein from a suspension of muscle or liver mitochondria in 0.25 M sucrose. Respiration is expressed as µg atoms of oxygen × hr<sup>-1</sup> × mg<sup>-1</sup> protein. The substrate was added first: 1.67 mM glutamate for skeletal muscle, 2.67 mM succinate for liver mitochondria. Oxidative phosphorylation was measured, after the addition of 116 µM ADP, from the subsequent recorded State 3 respiration.<sup>16</sup> State 4 respiration was measured after phosphorylation of the added ADP; State 3u was estimated after the subsequent addition of 0.7 mM DNP. Respiratory control is the ratio State 3:State 4. Phosphorylating respiration was determined by adding first 174 µM ADP, then 2.7 µM oligomycin; the subsequent degree of inhibition was expressed as per cent change from State 3 respiration.

## Results

Figure 1 illustrates the effects of DNP and of 2 per cent halothane, singly and in combination, on the mean rectal temperatures and

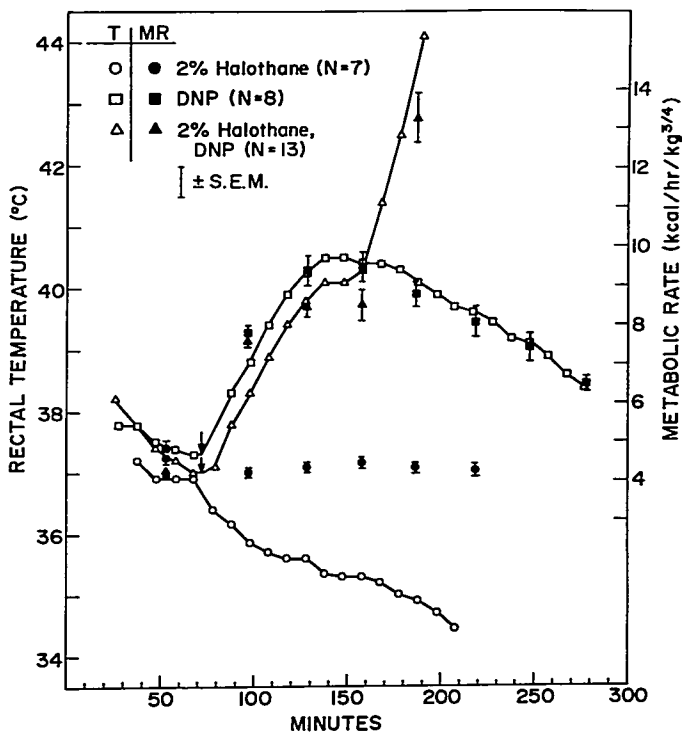


FIG. 1. Responses of rectal temperature and metabolic rate to halothane (2.0 per cent) and 2,4-dinitrophenol. Solid symbols indicate metabolic rate; open symbols indicate rectal temperature. Arrows show intraperitoneal injection of DNP (10  $\mu$ g/g). Each point is the mean of N rats. Chamber temperature was 25 C for all measurements.

metabolic rates of rats. The chamber temperature was kept at 25 C during measurements. All rats were sedated with diazepam, which caused mild hypothermia ( $-1.7 \pm 0.9$  C in 220 min, N=8), and a slight decrease in metabolic rate. Halothane further depressed the body temperature progressively, but not the metabolic rate. DNP injection promptly elevated metabolic rate by about 100 per cent and body temperature by about 10 per cent. Thereafter, slow decreases began,

and the animals survived. The combination of halothane and DNP, when halothane was started one hour prior to DNP treatment, produced an elevation similar to normal DNP calorigenesis, followed by a rapid marked additional temperature increase of about 4.0 C and a tripling of the metabolic rate. Within 2-7 min following maximal calorigenesis, oxygen consumption fell to zero and the animals became muscularly rigid and died. When the chamber temperature was 20 C, DNP did not

cause lethal hyperthermia or rigidity. When diazepam pretreatment was omitted and the halothane dosage (concentration or exposure time) was increased to compensate, the animals became even more hypothermic than those shown in figure 1; subsequent injection of DNP did not cause fulminant hyperthermia. DNP-induced calorigenesis is depressed by similar changes in environmental temperature,<sup>17</sup> and active cooling is reported to reverse malignant hyperthermia in patients.<sup>18</sup>

Oxidative phosphorylation was measured in the skeletal muscle and liver mitochondria of rats treated as in figure 1, but sacrificed either 2 hours after the start of the experiment or, in the halothane-DNP group, as soon as their temperatures and rates of O<sub>2</sub> consumption started their penultimate decline. Muscle mitochondria obtained from rats in premature rigor after the injection of lethal doses of uncoupling agents showed normal oxidative phosphorylation,<sup>19</sup> probably due to the removal of the agents during the isolation of the mitochondria. We did not measure mitochondrial DNP or halothane in the present studies. The rigor in our rats indicates depletion of muscle ATP.

In skeletal muscle mitochondria (table 1), the combination of halothane and DNP de-

pressed respiratory control, the ADP:O ratio, State 3u respiration, and oligomycin sensitivity, that is, uncoupled phosphorylation linked to glutamate oxidation ( $\alpha$ -ketoglutarate oxidation behaves similarly). The combination of halothane and DNP produced a much greater effect than the sum of the effects of the agents themselves; DNP produced no change at all, and halothane accelerated State 3u respiration slightly but not significantly, but did not depress oligomycin sensitivity. The uncoupling observed was not of the usual type, in which State 4 respiration is accelerated; here, State 4 was not changed, and State 3 was markedly depressed. Thus, the great calorigenesis observed *in vivo* after combined treatment was not reflected in increased muscle mitochondrial respiration in the resting state (State 4). Since the presence of early rigor mortis indicates a depletion of muscle ATP *in vivo*, and since the State 4 respiration in the mitochondria from DNP-injected rats was normal, it appears that respiration in these mitochondria *in vitro* did not completely reflect their respiration *in vivo*. The depressed ADP:O ratio and the decreased sensitivity to inhibition by oligomycin indicate, however, that the combined treatment does depress oxidative phosphorylation in a manner that remains de-

TABLE 1. The Rates of Oxygen Uptake, ADP:O Ratios, and Oligomycin Sensitivities of Liver and Muscle Mitochondria from Rats Treated with Halothane (2 Per Cent), and DNP (10  $\mu$ g/g, ip) (Means  $\pm$  SE)

Mitochondria	Treatment	Number of Rats	Respiration ( $\mu$ atoms O/hr/mg protein)			ADP:O	Per Cent Inhibition by 2.7 $\mu$ g Oligomycin
			State 3	State 4	State 3u		
Muscle	Control*	11	3.0 $\pm$ 0.4	0.9 $\pm$ 0.1	3.9 $\pm$ 0.5	2.8 $\pm$ 0.1	-75 $\pm$ 4
	Diazepam	5	3.1 $\pm$ 0.5	1.0 $\pm$ 0.1	3.8 $\pm$ 0.6	3.0 $\pm$ 0.1	-74 $\pm$ 10
	Halothane	4	3.1 $\pm$ 0.4	1.4 $\pm$ 0.3	4.5 $\pm$ 0.4	1.9 $\pm$ 0.6	-77 $\pm$ 8
	DNP	5	2.8 $\pm$ 0.4	1.0 $\pm$ 0.1	3.3 $\pm$ 0.5	2.8 $\pm$ 0.2	-65 $\pm$ 6
	Halothane/DNP	5	1.3 $\pm$ 0.2†	0.9 $\pm$ 0.1	2.1 $\pm$ 0.5	1.4 $\pm$ 0.7†	-33 $\pm$ 5‡
Liver	Control	5	2.8 $\pm$ 0.4	0.9 $\pm$ 0.1	4.5 $\pm$ 0.5	1.8 $\pm$ 0.2	-68 $\pm$ 6
	DNP	2	2.7	1.0	4.8	2.3	-60
	Halothane/DNP	2	2.6	1.1	5.1	2.2	-57

\* Controls were given no injections or other treatments, but were handled in the metabolic chambers like the other groups. In each day's experiment, an untreated animal was included with the treated rat. Significant differences were calculated by a method of paired comparisons. Diazepam was used in all treated groups.

† Significant difference from control,  $P < 0.025$ .

‡ Significant difference,  $P < 0.001$ .

TABLE 2. The Effects of LT<sub>4</sub> Pretreatment and Chamber Temperature on the Response of Metabolic Rate to Halothane

Chamber Temperature (C)	Control Metabolic Rate*	Effect of Halothane		LT <sub>4</sub> Treatment		Effect of Halothane on LT <sub>4</sub> Metabolic Rate*
		Per Cent Concentration	Metabolic Rate*	μg/K × Days	Metabolic Rate*	
20	4.7	1.5	4.4	0	4.3	4.3
20	4.4	1.5	4.0†	5.2 × 1	5.0†	5.1
20	4.5	1.5	4.0†	2.5 × 3	5.9†	5.7
25	4.3	1.5	4.4	2.5 × 3	5.7†	6.7†
25	4.3	2.0	4.2	2.5 × 3	5.6†	5.5

\* Metabolic rates are in units of kilocalories per hour per kilogram<sup>2/3</sup>, assuming 4.7 calories per ml O<sub>2</sub> consumed in fasted rats.<sup>10,11</sup> Values given are the means for S-16 rats. Standard errors are all ±0.1 or ±0.2.

† Significant difference from control,  $P < 0.05$ .

‡ Significant difference from control or from LT<sub>4</sub> metabolic rate,  $P < 0.001$ .

tectable *in vitro*. In contrast, mitochondria isolated from the livers of rats in these experimental groups all oxidized succinate normally, and showed no uncoupling (table 1). The synergism between halothane and DNP was not seen in the liver mitochondria.

Because of the similarity between halothane-DNP synergism and LT<sub>4</sub>-DNP synergism,<sup>12</sup> the combined effects of halothane and L-thyroxine were studied (table 2). When the metabolic rate was measured at 20 C, pretreatment with LT<sub>4</sub> was calorogenic, but subsequent introduction of 1.5 per cent halothane for 9 min had little or no effect. Measurements at 25 C appeared to elicit slight calorigenesis after anesthesia with 1.5 per cent halothane, but these animals did not become rigid or die. They were more restless after the LT<sub>4</sub> treatment, however, and increasing the concentration of halothane to 2 per cent for 9 min eliminated both the increased muscular activity and the apparent calorigenesis. Under these conditions, LT<sub>4</sub> did not sensitize rats to a calorogenic action of halothane.

Attempts were made to reverse the lethal hypermetabolism seen in combined halothane-DNP treatment (table 3). Because the thyroid state controls the *in-vivo* calorogenic responses of animals to DNP and other agents, antagonists of the peripheral actions of thyroid hormones were administered either at the same time as the DNP, or after DNP injection when the rectal temperature rose to 41.0 C. Methylthiouracil<sup>20</sup> and actinomycin D<sup>21</sup> were used in the doses shown; neither improved the survival rate, the terminal metabolic rate or

temperature, the lethal time, or the occurrence of rigidity. Mg<sup>++</sup> ions also antagonize thyroxine actions,<sup>3</sup> although not those of DNP<sup>22</sup>; increasing doses of MgSO<sub>4</sub> did not alter the survival rate, but did eliminate muscle rigidity and prevented any marked rise in temperature or metabolic rate. The rats in this group died with rapidly decreasing rates of O<sub>2</sub> consumption, as if there were central respiratory failure; assisted respiration was not possible under our conditions of experimentation, but might be of interest to study if hyperthermia can be induced in larger animals through endotracheal administration of halothane.

### Discussion

A malignant hyperthermia, with hypermetabolism of O<sub>2</sub> and with muscle rigidity, is produced in rats treated with both an anesthetic, 2 per cent halothane, and an uncoupling agent, DNP, each in a dose that separately is not fatal, when the animals are kept in a sufficiently warm environment. Modest changes in environmental temperature, such as those that here produced marked differences in animal response, are known to affect calorigenesis after administration of uncoupling agents.<sup>17</sup> It is not clear whether the higher ambient temperature facilitates penetration of the agents to their mitochondrial sites of action or sensitizes the mitochondria. The syndrome does not appear to require preselection of genetically susceptible animals, in contrast to that seen in certain strains of pigs<sup>7, 23</sup> and in human families.<sup>5</sup>

TABLE 3. The Effects of Three Antithyroid Agents on Halothane-DNP-induced Hyperthermia (Means  $\pm$  SE)\*

Agent	When Administered	Dose ( $\mu$ g/kg)	Terminal Metabolic Rate* (kcal/hr/kg <sup>0.75</sup> )	Terminal Temperature (C)	Lethal Time* (Minutes after DNP Treatment)	N Died/N Total
Methylthiouracil	$\bar{c}$ DNP at 41 C†	5-100	13.6 $\pm$ 0.7	44.2 $\pm$ 0.3	107 $\pm$ 6	39/50
			13.9 $\pm$ 0.7	44.1 $\pm$ 0.3	116 $\pm$ 4	11/11
Actinomycin D	$\bar{c}$ DNP	0.05-0.5	12.9 $\pm$ 0.6	44.6 $\pm$ 0.3	108 $\pm$ 7	44/48
MgSO <sub>4</sub>	$\bar{c}$ DNP	200	14.8 $\pm$ 0.4	44.7 $\pm$ 0.3	137 $\pm$ 7	6/8
		400	12.2	44.1	145	5/8
		600	10.0	43.6	114	5/8
		800	11.1	41.8	89	7/8
		1000	—	39.8 $\pm$ 0.6	29 $\pm$ 7	8/8 ‡
	at 41 C	1200	—	37.5 $\pm$ 0.3	54 $\pm$ 9	8/8 ‡
		200	10.5 $\pm$ 0.9	43.8 $\pm$ 0.5	69 $\pm$ 2	5/5
		600	8.8 $\pm$ 1.2	42.4 $\pm$ 0.2	90 $\pm$ 9	5/5
		1200	—	41.0 $\pm$ 0.3	81 $\pm$ 2	4/4 ‡

\* Where no SE is given, the value is the average for only two animals dying with high temperature, high metabolic rate, and muscle rigidity. Others died shortly after combined treatment, and had none of the symptoms of malignant hyperpyrexia.

† Agent administered when the rectal temperature reached 41 C.

‡ There was no muscle rigidity in these rats.

The fatal syndrome, as produced here, is accompanied by a relative uncoupling of oxidative phosphorylation in skeletal muscle mitochondria. No significant mitochondrial changes occurred in the groups treated with either halothane or DNP. The 2 per cent halothane that, in conjunction with DNP, was necessary to produce uncoupling in skeletal muscle mitochondria is somewhat higher than the 0.7-1 per cent concentration expected in tissues during clinical anesthesia.<sup>24</sup> The 2 per cent halothane concentration did not affect liver mitochondria when administered *in vivo*, in agreement with the findings of others.<sup>2, 24, 25</sup> Liver mitochondria are sensitive to halothane *in vitro*.<sup>2</sup> So the halothane administered *in vivo* either does not reach the liver mitochondria, or does reach them but is washed out during the isolation of the mitochondria.

Uncoupling of skeletal muscle mitochondria should produce rigidity, hyperthermia, and an increased metabolic rate. However, the hypermetabolism *in vivo* is not reflected by increased State 4 respiration *in vitro*. It may be that halothane + DNP does not primarily stimulate the utilization rate of O<sub>2</sub>, but only depresses the efficiency of phosphorylation; the ensuing increase in heat production and tem-

perature of tissues might account for increased O<sub>2</sub> consumption secondarily. However, the rate of O<sub>2</sub> consumption is the chief factor in heat production.<sup>26</sup> It seems more likely that the respiration of the skeletal muscle mitochondria *in vitro* does not reflect that *in vivo*. We think, therefore, that the malignant hyperthermia seen here is the result of uncoupling in skeletal muscle mitochondria. Whether this experimental hyperthermia corresponds to the clinical catastrophe in humans, it is not possible to say from our data. Other investigators have suggested muscle as the target tissue in clinical and experimental fulminant hyperthermia.<sup>7, 27-29</sup>

Our starting hypothesis was that malignant hyperthermia in rats might resemble the lethal synergism we reported between LT<sub>4</sub> and a known uncoupling agent, DNP,<sup>12</sup> because halothane can uncouple.<sup>2, 30</sup> Pretreatment with LT<sub>4</sub> in doses that sensitize to DNP does not make halothane produce hyperthermia, rigidity or death, however; and the administration of antithyroid agents does not reverse the lethal actions of halothane plus DNP. There seems to be no relationship between hyperthyroidism and this hyperthermia. The use of Mg<sup>++</sup> salts deserves further study.

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