

Mitochondrial Complex I Function Affects Halothane Sensitivity in *Caenorhabditis elegans*

Ernst-Bernhard Kayser, Ph.D.,* Phil G. Morgan, M.D.,† Margaret M. Sedensky, M.D.†

Background: The gene *gas-1* encodes a subunit of complex I of the mitochondrial electron transport chain in *Caenorhabditis elegans*. A mutation in *gas-1* profoundly increases sensitivity of *C. elegans* to volatile anesthetics. It is unclear which aspects of mitochondrial function account for the hypersensitivity of the mutant.

Methods: Oxidative phosphorylation was determined by measuring mitochondrial oxygen consumption using electron donors specific for either complex I or complex II. Adenosine triphosphate concentrations were determined by measuring luciferase activity. Oxidative damage to mitochondrial proteins was identified using specific antibodies.

Results: Halothane inhibited oxidative phosphorylation in isolated wild-type mitochondria within a concentration range that immobilizes intact worms. At equal halothane concentrations, complex I activity but not complex II activity was lower in mitochondria from mutant (*gas-1*) animals than from wild-type (N2) animals. The halothane concentrations needed to immobilize 50% of N2 or *gas-1* animals, respectively, did not reduce oxidative phosphorylation to identical rates in the two strains. In air, adenosine triphosphate concentrations were similar for N2 and *gas-1* but were decreased in the presence of halothane only in *gas-1* animals. Oxygen tension changed the sensitivity of both strains to halothane. When nematodes were raised in room air, oxidative damage to mitochondrial proteins was increased in the mutant animal compared with the wild type.

Conclusions: Rates of oxidative phosphorylation and changes in adenosine triphosphate concentrations by themselves do not control anesthetic-induced immobility of wild-type *C. elegans*. However, they may contribute to the increased sensitivity to volatile anesthetics of the *gas-1* mutant. Oxidative damage to proteins may be an important contributor to sensitivity to volatile anesthetics in *C. elegans*.

THE mechanisms for the anesthetic effects of volatile anesthetics are incompletely understood.^{1,2} Using immobility as an endpoint, we established that the interactions of multiple genes are crucial in controlling the sensitivity of *Caenorhabditis elegans* to halothane.³ The mutation *fc21* in one gene, *gas-1* (general anesthetic sensitive), causes hypersensitivity to all straight chain alcohols up to C12 and to volatile anesthetics.^{3,4} Nematodes with the *gas-1(fc21)* mutation move normally in air, indicating a functional neuromuscular system. However, they have a

reduced lifespan, slow growth rates, and an increased sensitivity to the deleterious effects of paraquat and hyperoxia.⁵

Gas-1 encodes a homolog of the bovine 49-kd subunit of nicotinamide adenine dinucleotide (NADH) ubiquinone oxidoreductase (complex I), the first complex of the mitochondrial electron transport chain.⁶ GAS-1 protein expression is consistent with mitochondrial localization. In isolated mitochondria, complex I-dependent oxidative phosphorylation and electron transport are specifically depressed by the *gas-1(fc21)* mutation, confirming that *gas-1* encodes a functional subunit of complex I in *C. elegans*.⁷

In all organisms studied, complex I catalyzes the first step of electron transport, the transfer of electrons from NADH to a quinone.⁸⁻¹⁰ The energy released is harnessed to establish a proton gradient across the inner mitochondrial membrane. In mitochondria, the proton gradient ultimately drives the production of adenosine triphosphate (ATP). The exact composition of complex I varies widely across species, but all forms of complex I contain homologs of the 15 subunits that constitute the enzyme in *Paracoccus*. One of these core subunits is the 49-kd protein, whose function is incompletely defined. A knockout mutant of the "49-kd gene" in *Neurospora* completely lacked complex I activity because the matrix arm of the enzyme complex failed to assemble.¹¹ The matrix arm of complex I contains the binding site for NADH as well as all but one of the redox centers. The 49-kd subunit from *Rhodobacter* is thought to bind the head group of quinones such as the electron acceptor, ubiquinone,¹² and may contribute to the movement of ubiquinone from the membrane to the matrix arm of complex I.¹³ Recent data indicate that this subunit interacts with another subunit of complex I to bind ubiquinone.¹⁴ Lastly, our previous studies indicated that mutations in the 49-kd subunit of *C. elegans* specifically decreased complex I-dependent metabolism.⁷ Therefore, 49-kd proteins seem to be essential for the structure and function of complex I.

Electron transport to oxygen (aerobic respiration) in intact mitochondria is ordinarily tightly coupled to ATP production (phosphorylation). Measurement of oxygen consumption and adenosine diphosphate (ADP) utilization by isolated mitochondria provide an assessment of the ability of mitochondria to generate high-energy phosphate bonds for cellular metabolism. Individual complexes of the electron transport chain can then be assayed for their effects on oxidative phosphorylation.

The volatile anesthetic halothane accumulates in mito-

* Research Associate, Department of Anesthesiology, † Professor, Departments of Anesthesiology and Genetics, University Hospitals and Case Western Reserve University.

Received from the Department of Anesthesiology, University Hospitals and Case Western Reserve University, Cleveland, Ohio. Submitted for publication October 31, 2003. Accepted for publication March 26, 2004. Drs. Sedensky and Morgan were supported in part by grant Nos. GM51881 and GM45402 from the National Institutes of Health, Bethesda, Maryland. Dr. Kayser was supported in part by a grant from the Muscular Dystrophy Association, Tucson, Arizona, and by grant No. GM51881 from the National Institutes of Health, Bethesda, Maryland.

Address reprint requests to Dr. Morgan: Department of Anesthesiology, University Hospitals, 11100 Euclid Avenue, Cleveland, Ohio 44106. Address electronic mail to: philip.morgan@uhhs.com. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

chondria in *C. elegans* (Roderick Eckenhoff, M.D., Professor, Department of Anesthesiology, University of Pennsylvania, Philadelphia, Pennsylvania, written personal communication, February 2004) and in mammals.¹⁵ Previous studies from other investigators indicated that complex I in mammalian mitochondria is the most sensitive complex of the electron transport chain to inhibition by volatile anesthetics.^{16,17} In contrast, the remaining mitochondrial complexes (II-V) are less sensitive to these compounds. In addition, children with certain complex I deficiencies are very sensitive to induction with the volatile anesthetic sevoflurane.¹⁸ However, it remains unclear whether volatile anesthetics actually cause anesthesia by inhibition of mitochondrial function. Because patients with mitochondrial disease may show altered sensitivities to volatile anesthetics, it is important to understand the mechanisms by which such changes may arise.

Gas-1 animals are hypersensitive to halothane, and their mitochondria have profoundly decreased rates of oxidative phosphorylation *via* complex I-specific substrates, suggesting that complex I-dependent oxidative phosphorylation may directly control the anesthetic response in worms. We hypothesize that the same minimum absolute value of complex I-dependent oxidative phosphorylation is necessary in both wild-type and *gas-1* animals to sustain movement. This threshold value of complex I activity would occur at the EC₅₀s for each strain, the concentrations of halothane that immobilize each strain of animals. We therefore compared the doses of halothane required to decrease oxidative phosphorylation in mitochondria to those required to immobilize both wild-type and *gas-1* animals. We measured the effects of halothane on oxidative phosphorylation with electron donors specific for complexes I or II. We also compared the effects of halothane on state 3 respiration (high ADP availability and thus close to maximal respiration rates) and state 4 respiration (ADP-limiting conditions and thus respiration is constrained by the inability to efficiently transport electrons to generate ATP). By comparing the effects of halothane on state 3 and state 4, we determined whether decreases in respiration were the result of direct inhibition of the electron transport chain or inhibition of coupling of electron transport to the generation of ATP.

Other facets of mitochondrial function may contribute to the behavior of *C. elegans* in anesthetics. Energy stores may be a major determinant of mobility in the face of anesthetics, if anesthetics decrease rates of oxidative phosphorylation. Therefore, we measured the ratio of ATP to ADP and adenosine monophosphate in wild-type and *gas-1* animals in air and in halothane. In addition, we have noted that *gas-1* is very sensitive to increased oxygen tensions and paraquat.⁵ Therefore, we examined whether oxidative damage correlated with changes in anesthetic sensitivity.

Materials and Methods

Nematode Strains

The conventions for *C. elegans* nomenclature are followed throughout.¹⁹ The wild-type *C. elegans*, N2, was obtained from the *Caenorhabditis* Genetics Center (Minneapolis, MN). *Gas-1(fc21)* was isolated in a screen for immobile worms in 3.5% enflurane after mutagenesis of N2 with ethylmethanesulfonate (N2 is immobilized by 6.5% enflurane).²⁰ Standard techniques were used for growing and maintaining cultures of *C. elegans*.^{20,21}

Preparation of Mitochondria

All preparations were performed on ice. Two to three grams of worms cleaned of *Escherichia coli* were suspended in MSM-E (220 mM mannitol, 70 mM sucrose, 5 mM MOPS, 2 mM EDTA, pH to 7.4 with KOH). A polytron (Brinkman Instruments, Westbury, NY) was used for initial rupture: 20 s at 14,000 rpm. To the homogenate, 5 mg/(g worms) proteinase type XXVII (Sigma, St. Louis, MO) was added, and the resulting mixture was stirred for 10 min. Immediately afterward, the slurry was homogenized in a glass Potter/Elvehjem tissue grinder with a Teflon pestle (Dupont, Wilmington, DE). After adding 1 volume MSM-E containing 0.4% bovine serum albumin, the homogenate was centrifuged (300g, 10 min, 4°C). The supernatant was filtered through three layers of gauze and recentrifuged (7,000g, 10 min, 4°C). The mitochondrial pellet was resuspended in MSM-E and washed twice by centrifugation (7,000g, 10 min, 4°C). The final mitochondria pellet was resuspended in 100 μ l MSM-E. Total protein was determined by the Lowry assay with bovine serum albumin as a standard.²²

Oxidative Phosphorylation Assay

Polarographic measurement of oxidative phosphorylation was performed as previously described.²³ Briefly, mitochondrial oxygen uptake was followed with a Clark type electrode (Yellow Springs Instruments, Yellow Springs, OH) connected to a chart recorder *via* a YSI Oxygen monitor. The electrode chamber was loaded with 500 μ l incubation medium (100 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM potassium phosphate, 1 mg/ml defatted bovine serum albumin) and equilibrated at 30°C before the oxygen monitor was set to 100% oxygen saturation (222.5 natom O) (fig. 1). The following components were then added sequentially: mitochondria (maximum 500 μ g); 25–50 nmol ADP, allowing the mitochondria to consume endogenous substrates; either 10 mM L-malate or 20 mM succinate, providing a defined electron donor specific for complex I or II, respectively; 25–100 nmol ADP to determine state 3 (phosphorylating conditions) and state 4 (nonphosphorylating conditions) respiration rates; 1,000 nmol ADP to determine “high ADP” rate (maximally phosphorylating); and 100 nmol

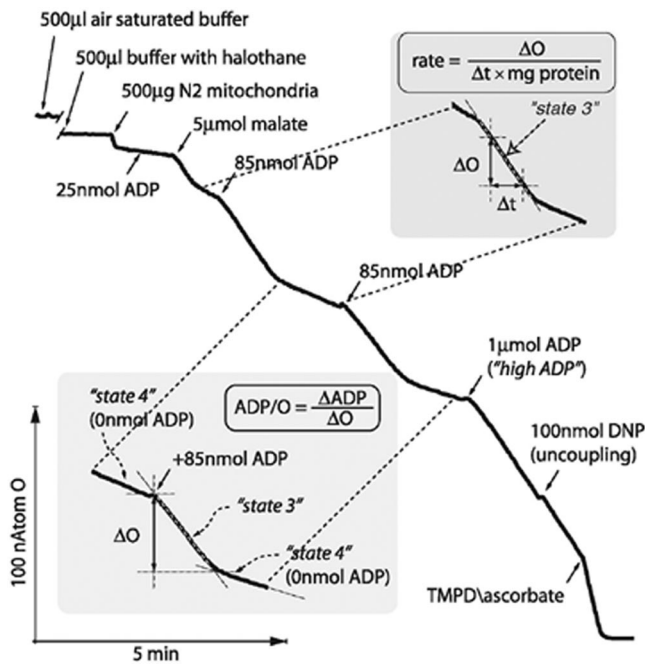


Fig. 1. A typical oxidative phosphorylation showing oxygen consumption (down y-axis) over time (x-axis). To start, the chamber is filled with 500 μ l halothane-treated buffer. Then, 500 μ g mitochondria and a small amount of adenosine diphosphate (ADP) (to allow metabolizing of internal substrates if they are present—here, not the case) are added. Next, the complex I-dependent substrate malate is added. (In other experiments, succinate is added as a substrate for complex II.) Finally, 85 nmol ADP is added to allow oxidative phosphorylation to occur. State 3 is the fast respiration in response to ADP. When the ADP is all phosphorylated and becomes limiting, respiration slows to state 4. As an example for all rate determinations, the top inset depicts the calculation of a state 3 respiration rate. The lower inset shows the ADP/O ratio, calculated as the consumption of ADP added divided by the amount of oxygen consumed during state 3 respiration. These measurements are repeated a second time. A much higher amount of ADP is then added to ensure that ADP was not limiting in the state 3 measurements. The uncoupler 2,4-dinitrophenol (DNP) is added to determine whether phosphorylation of ADP was limiting for respiration. At the end of the experiment, N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD)-ascorbate is added as a direct electron donor to cytochrome c. The ensuing respiration rate depends only on the amount of intact mitochondria present and is therefore used as an internal loading control.

2,4-dinitrophenol (2,4 dinitrophenylhydrazine) to uncouple the mitochondria. Finally, N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD)-ascorbate (a complex intravenous substrate) is added as an internal control for mitochondrial quality.

For measurements in the presence of halothane, halothane-saturated incubation medium was created as follows. A three-phase system of liquid halothane, incubation medium, and air in a closed test tube was shaken and incubated at 30°C overnight, allowing the phases to separate and the concentrations to come to an equilibrium in all phases. Halothane concentration of the air over the saturated solution was determined to be 34% by gas chromatography. Dilutions of the saturated solution were made in a sealed chamber before mitochondria

were added. The final concentration of halothane was calculated from the dilution. If the mitochondria acted as pure lipid, we estimated that this would decrease the concentration of halothane in solution by approximately 1% only (*i.e.*, from 34% to 33%). Therefore, further corrective calculations were unnecessary. Experimental data reported are the results from three independent experiments for N2 and four separate experiments for *gas-1(fc21)*.

Calculated Values

Respiration (oxygen uptake) rates and ADP/O were calculated as previously described.^{7,24,25} The rate of phosphorylation was calculated by multiplying the rate of state 3 oxygen consumption ($\text{natom O} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$) by ADP/O to obtain $\text{nmol ADP phosphorylated} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$. Values for calculating the mean and SD were the results from four separate preparations from each strain.

ATP Measurements

Worms were synchronized by allowing adult worms to lay eggs for 2 h on agar plates with bacterial lawns. The adults were then removed, and the resulting eggs were grown to young adults. The resulting adults were cleaned of *E. coli* by moving them to an agar plate devoid of bacteria. After 10 min, the animals were moved to a second agar plate without bacteria for 90 min and then placed into 20 μ l S-basal solution, frozen in liquid nitrogen overnight. The frozen animals were lyophilized and stored at -80°C until use. Animals exposed to halothane were also synchronized young adults cleaned of *E. coli* and then placed on an agar plate for exposure to halothane for 2 h. These worms were then immediately placed in S-basal, transferred to a dry ice-acetone bath, and then transferred into liquid nitrogen and lyophilized. *Gas-1* worms, which lag in development compared with the wild type, were synchronized as for the wild type but required an additional 24 h to reach young adulthood. Lyophilized animals were sonicated in 100 μ l trichloroacetic acid, 2.5%, on ice and extracted for 10 min in room air. Five microliters of extract was added to 95 μ l of a 20-mM HEPES buffer to measure ATP.²⁶ ATP was measured by light emission in a standard luciferin-luciferase assay. ADP was measured after adding phosphoenol pyruvate and phosphoenol pyruvate kinase to the same assay. Adenosine monophosphate was measured by further adding myokinase (Sigma) to the mixture with cytosine triphosphate to drive the reaction. In each case, the enzymes were allowed to convert their substrates to ATP for 30 min at room temperature. ATP was expressed as a ratio of ATP to the total adenosine species. All values reported are the results of 11 separate measurements.

Table 1. Oxidative Phosphorylation in *Caenorhabditis elegans*

	N2	<i>gas-1(fc21)</i>
State 3 (malate), natom O · min ⁻¹ · mg protein ⁻¹	102.9 (10)	29.1 (3.2)*
State 3 (succinate), natom O · min ⁻¹ · mg protein ⁻¹	99.7 (9.2)	145.5 (16)*
ADP/O (malate), nmol ADP/nA O	2.69 (0.20)	2.26 (0.26)
ADP/O (succinate), nmol ADP/nA O	1.30 (0.3)	1.10 (0.08)
2,4-Dinitrophenol (malate), natom O · min ⁻¹ · mg protein ⁻¹	108.0 (11.7)	23.1 (3.3)*
2,4-Dinitrophenol (succinate), natom O · min ⁻¹ · mg protein ⁻¹	59.0 (13.3)	115.0 (9.3)*
Phosphorylation (malate), nmol ADP · min ⁻¹ · mg protein ⁻¹	280 (10)	66 (3)*
Phosphorylation (succinate), nmol ADP · min ⁻¹ · mg protein ⁻¹	130 (9.3)	160 (16)

Baseline values for respiration and phosphorylation in *Caenorhabditis elegans*. After 2,4-dinitrophenol-uncoupled rates were determined, as a control the mitochondria were stimulated with TMPD-ascorbate to demonstrate the presence of functional mitochondria. Values in this table and the following tables and figures were calculated from at least three independent preparations from *gas-1* and N2, respectively, and are given as means with SDs in parentheses. Results for N2 and *gas-1(fc21)* were compared using the Student *t* test.

All values are the result of five separate measurements. ADP/O refers to the ratio of adenosine diphosphate consumed to oxygen consumed and is a measure of coupling of oxidation to phosphorylation.

Different from value for N2, *P* < 0.01.

Protein Oxidation

We used two antibody preparations for identifying oxidative damage to protein. The first is the OxyBlot protein oxidation detection kit (InterGen, Burlington, MA). As a consequence of protein oxidation, carbonyl groups are introduced into protein side chains.²⁷ The carbonyl groups are derivatized to 2,4-dinitrophenylhydrazones by a reaction with 2,4 dinitrophenylhydrazine. The derivatized protein samples are then run on polyacrylamide gels and Western blotted. The blots are exposed to the primary antibody, specific to the 2,4 dinitrophenylhydrazine moiety of the proteins, and followed by a horseradish peroxidase antibody secondary antibody.

The second procedure does not require a derivatization step. Instead, mitochondrial proteins are transferred to a Western blot and incubated with an antibody specific for 4-hydroxy-2-nonenal (HNE) Michael adducts.²⁸ HNE is a common reactive intermediate released by reactive oxygen species and interacts with cysteine, lysine, or histidine. Probing with the antibody uses common immunohistochemistry techniques.

Statistical Analysis

EC₅₀s were calculated using the methods described by Waud.²⁹ Values for oxidative phosphorylation rates and for EC₅₀s were compared using analysis of variance for multiple comparisons.³⁰ Mean values in tables 1 and 2 were compared by a Student *t* test. Significance was defined as *P* < 0.05.

Results

Oxidative Phosphorylation

Mitochondria from wild-type nematodes can metabolize malate or succinate.^{7,31} Malate is a complex I-dependent electron donor, whereas electrons enter complex II specifically *via* succinate. Complex I-dependent oxidative phosphorylation was decreased in mitochondria from *gas-1* animals compared with those from N2.

Complex II-dependent oxidative phosphorylation rates were increased in *gas-1* animals compared with those from N2 (table 1). These results were previously published⁷ and are presented here to serve as controls for the effects of halothane.

Halothane and Oxidative Phosphorylation

To address the question of whether halothane directly affects respiration, we used malate or succinate to specifically isolate the effects of halothane on complex I or II, respectively. Halothane inhibited complex I-dependent oxidative phosphorylation in mitochondria from both N2 and *gas-1* animals (fig. 2A) with IC₅₀s (IC₅₀ is that concentration causing 50% inhibition of activity) of 4.1% and 4.3% halothane, respectively (table 2). At all concentrations of halothane (excepting the highest one, 6.4% halothane) the rates of state 3 respiration were significantly lower in *gas-1* than they were in N2. In contrast, rates of state 4 (ADP-limited) respiration were not strongly affected by halothane (fig. 2B). In uncoupled mitochondria, rates were again inhibited by halothane (IC₅₀s of 4.7% for N2 and 4.8% for *gas-1*), indicating that phosphorylation of ADP to ATP was not limiting in the presence of halothane (fig. 2C).

Fifty percent of N2 animals are reversibly immobilized

Table 2. Effect of Halothane on Mitochondria from *Caenorhabditis elegans*

	N2	<i>gas-1(fc21)</i>
State 3 IC ₅₀ (malate)	4.1% (0.3)	4.3% (0.3)
State 3 IC ₅₀ (succinate)	6.2% (0.4)	6.2% (0.5)

Inhibitory concentrations causing a 50% decrease in oxygen consumption (IC₅₀s) for halothane inhibition of state 3 respiration with electron donors specific for complexes I and II. Note that while the rates for *gas-1* are consistently lower than those for N2 at all halothane concentrations (see fig. 1), the IC₅₀s are the same for both mitochondria from both strains. Values for IC₅₀s and SDs (in parentheses) were calculated by an exponential best fit using the plotting program Sigmaplot (Jandel Corp., San Rafael, CA). Values were compared using the Student *t* test. Significance was defined as *P* < 0.05, which neither comparison reached.

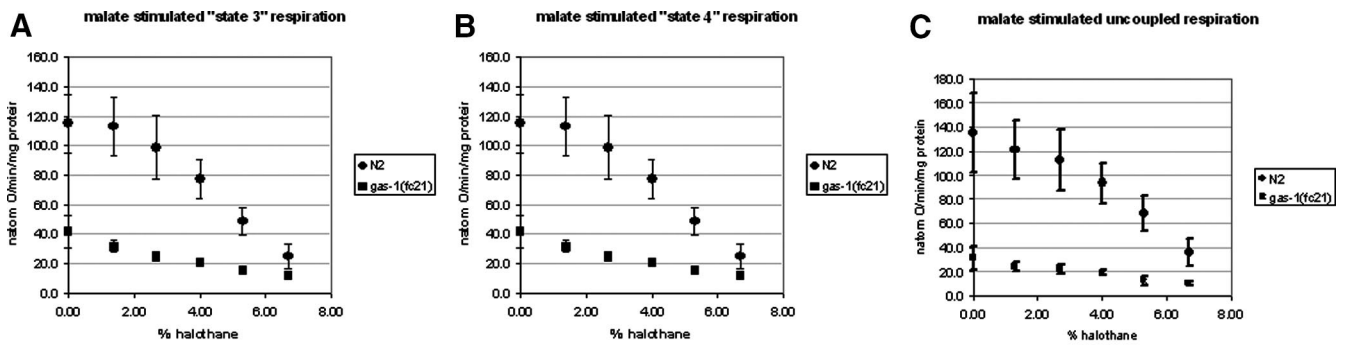


Fig. 2. Malate-dependent respiration at increasing halothane concentrations. (A) The effect of halothane on state 3 respiration *via* complex I. The *dashed line* denotes the respiratory rate of N2 mitochondria at the EC_{50} for immobility of the whole animal. This value, approximately $80 \text{ natom O} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, is higher than the respiratory rate for *gas-1(fc21)* mitochondria in the absence of anesthetic. (B) The effect of halothane on state 4 respiration *via* complex I. State 4 respiration was not significantly increased by halothane, even at high doses, ruling out that halothane acts as an uncoupler. (C) 2,4-dinitrophenol uncoupled respiration of malate in the presence of halothane. Note that the inhibition of complex I-dependent function is not removed when the mitochondria are uncoupled, indicating that halothane does not inhibit respiration by blocking adenosine triphosphate synthesis. *Bars and error bars* represent means and SDs, respectively. *n* = number of repeats for each experiment (*n* = 3 for N2; *n* = 4 for *gas-1*).

at 3.2% halothane.⁴ At this concentration, mitochondria isolated from N2 consume $80 \text{ natom O} \cdot \text{min}^{-1} \cdot \text{mg mitochondrial protein}^{-1}$ in malate-dependent state 3 respiration (fig. 2A). *Gas-1(fc21)* animals are hypersensitive to halothane; the EC_{50} of halothane is 1.2% for this mutant. When exposed to 1.2% halothane, mitochondria from *gas-1(fc21)* animals respired at $24 \text{ natom O} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ in malate-dependent state 3 respiration (fig. 2A). At concentrations of halothane that immobilized N2 animals, complex I-dependent rates were higher than those seen in *gas-1* mitochondria in air. Therefore, no minimum threshold value of oxygen consumption in mitochondria correlated with the concentrations of halothane that immobilized animals in the two strains.

Halothane also inhibited complex II-dependent oxidative phosphorylation in mitochondria from N2 and *gas-1* animals (fig. 3). At all concentrations of halothane tested, the rates for state 3 respiration and 2,4 dinitrophenylhydrazine uncoupled respiration were *higher* in *gas-1* than in N2 (figs. 2A and C). State 4 respiration was not

significantly different between the two strains (fig. 3B). Notably, the rates in mitochondria from N2 in room air (when the animal moved well) were considerably lower than the rates in mitochondria from *gas-1* animals at concentrations that anesthetize the mutant animal (1% halothane). At concentrations of halothane causing immobility in *gas-1*, no significant change in complex II-dependent rates was observed. As reported earlier, when an uncoupler (2,4 dinitrophenylhydrazine) of respiration to ATP synthesis was added, there was no increase in respiration with any substrate compared with respiration without 2,4 dinitrophenylhydrazine.⁷ The addition of 2,4 dinitrophenylhydrazine also did not increase respiration at any halothane concentration (data not shown). These results again demonstrate that phosphorylation is not limiting in the presence of halothane.

Halothane and ATP Concentrations

The percent of adenosine species present as ATP was measured in the two strains. In the absence of halothane, we found no significant difference between N2 animals

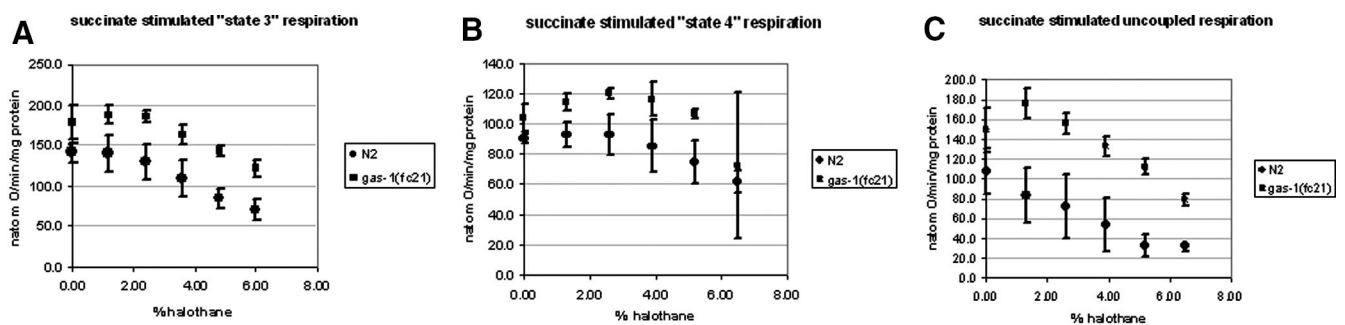


Fig. 3. Succinate-dependent respiration at increasing halothane concentrations. (A) The effect of halothane on state 3 respiration *via* complex II. Respiration is consistently higher in *gas-1(fc21)* mitochondria than in N2 mitochondria and is relatively resistant to halothane. (B) The effects of halothane on state 4 respiration with N2 and *gas-1(fc21)* mitochondria. State 4 respiration was not significantly increased by halothane, even at high doses, ruling out halothane functioning as an uncoupler. (C) Phosphorylation in N2 and *gas-1(fc21)* mitochondria in increasing halothane concentrations. As with state 3 respiration, phosphorylation is consistently higher in *gas-1(fc21)* mitochondria than in N2 mitochondria. *Bars and error bars* represent means and SDs, respectively. *n* = number of repeats for each experiment (*n* = 3 for N2; *n* = 4 for *gas-1*).

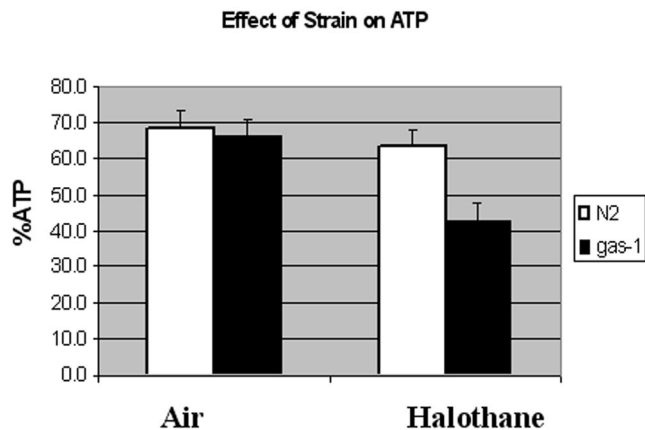


Fig. 4. Adenosine triphosphate (ATP) concentrations in N2 and *gas-1*. ATP as the percent of total adenylates (adenosine phosphates) (ATP, ADP, AMP) for N2 (white) and *gas-1* (black) in the absence (left) and presence (right) of halothane. Note that in air (left), both N2 and *gas-1* have approximately 67% of their adenylates present as ATP. In the presence of 1.5 EC₅₀ of halothane, the concentration in N2 remains constant, but that of *gas-1* decreases to 43%. Bars and error bars represent means and SDs, respectively. All values are the results of 11 separate measurements.

and *gas-1* animals (fig. 4). The percent adenine present as ATP was 68% for N2 and 66% for *gas-1*, in both cases consistent with values found in other species.³² N2 animals exposed to approximately 150% of their EC₅₀ (4.9% halothane) showed a slight decrease in their ratio of ATP to total adenine nucleotides (68% to 63%). In contrast, *gas-1* animals showed a more pronounced decrease in their ATP levels (66% to 43%) when exposed to 150% of their EC₅₀ concentration of halothane (1.9% halothane).

Oxidative Damage

To identify whether oxidative damage to anesthetic targets or effectors play a role in anesthetic sensitivity, we grew N2 and *gas-1* animals in low and high concentrations of oxygen. *Gas-1* animals grown in 3% oxygen had a significant decrease in their sensitivity to halothane (EC₅₀ of 1.6%) (fig. 5) compared with animals grown in room air (EC₅₀ of 0.9%). N2 animals grown in 60% oxygen had a significant increase in their sensitivity to halothane (EC₅₀ of 2.2%) compared with those grown in room air (EC₅₀ of 3.2%) (fig. 5). *Gas-1* animals did not survive exposure to 60% oxygen for a sufficient time to test the anesthetic effect. N2 animals grown in 3% oxygen had no significant change in their halothane sensitivity (data not shown).

Exposure to oxygen is expected to increase oxidative damage to proteins. We examined whether a difference existed in oxidative damage to mitochondrial proteins from N2 and *gas-1* when grown in room air. When an antibody that detects oxidative damage to proteins was used as a probe to mitochondrial proteins (OxyBlot), increased oxidation of specific proteins from *gas-1* mitochondria was seen compared with mitochondria from

Effect of Oxygen on Halothane Sensitivity

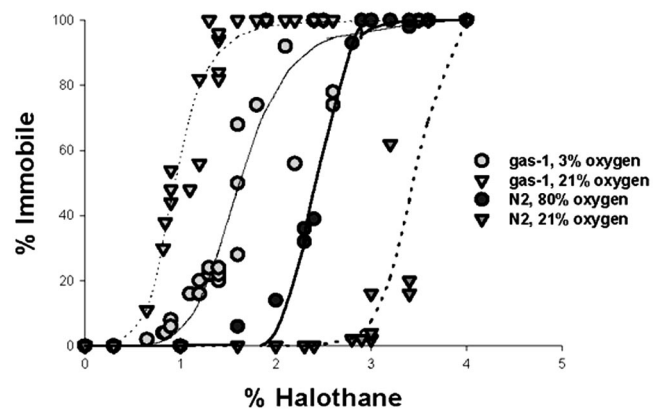


Fig. 5. Dose-response curves for halothane of N2 and *gas-1* grown under different concentrations of oxygen. Nematodes were grown in 3%, 21% (room air), and 60% oxygen, and the dose-response curves in halothane were determined for young adult animals. *Gas-1* animals did not survive exposure to 60% oxygen. The results show that hypoxia moves the *gas-1* response to the right (EC₅₀ = 2.0%), whereas hyperoxia moves the response of N2 to the left (EC₅₀ = 2.2%).

N2 (fig. 6A). When an antibody specific for the free radical damage inflicted by 4-hydroxynonenol, a product of lipid peroxidation, was used, one protein of approximately 26 kd was noted to be more damaged in *gas-1* than in N2 (fig. 6B). This is the same size as a major band seen on OxyBlot (fig. 6A). Therefore, the free radical damage resulting from the *gas-1* mutation is increased from that in N2 and specific to certain proteins. In addition, we previously showed that a mutation in a complex II subunit, known as *mev-1*,³³ did not alter anesthetic sensitivity.⁵ We found that increased oxidative damage was also seen in mitochondrial protein from *mev-1* but that the distribution of damaged proteins was different than that seen in *gas-1* (data not shown).

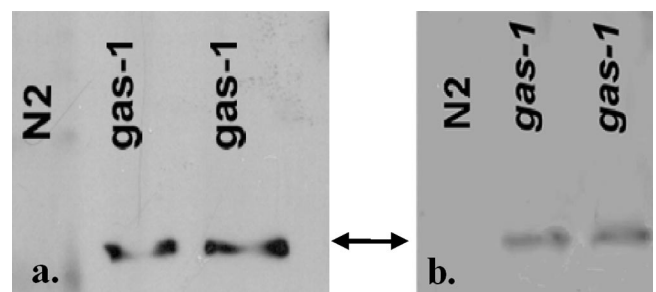


Fig. 6. Protein oxidation in N2 and *gas-1*. Western blot of mitochondrial proteins probed with an antibody to derivatized carbonyl groups (A) or with an antibody specific for 4-hydroxy-2-nonenol Michael adducts (B). A similar band of approximately 26 kd is identified by both techniques (arrow). Each lane of *gas-1* represents a separate isolation of mitochondria, indicating that the increased labeling of the 26-kd protein is consistent. Similar results were obtained in three different experiments.

Discussion

We have noted that changes in mitochondrial function may alter sensitivity to volatile anesthetics in nematodes and in man.^{6,18} Alterations in mitochondrial function could affect the phenotype of an animal in any of several ways. We studied three such possible mechanisms by which mitochondrial function could alter anesthetic sensitivity.

A straightforward mechanism may be that an acute decrease in metabolism would lead to inadequate energy supplies to the cell. In turn, this would lead to a decrease in neuromuscular function, causing the anesthetized state. If such a mechanism were solely responsible for immobility in the nematode, an identical rate of mitochondrial oxygen consumption should be reached at the concentration of halothane that produces immobility in each strain of worms. We found that the rate of oxidative phosphorylation was greatly decreased in *gas-1* mitochondria at all halothane concentrations when compared with those from N2. However, a common threshold value for oxygen consumption at halothane concentrations causing immobility was not seen. Notably, the rate of oxygen consumption seen in N2 mitochondria at its EC₅₀ was greater than that seen in *gas-1* mitochondria in room air. Because *gas-1* animals move well in room air (and their respiration must be adequate for movement), the rate of complex I-dependent oxidative phosphorylation cannot completely account for halothane-induced immobility in *C. elegans*. This is in contrast to our reported results for sensitivity to ethanol in these strains.^{3,4} In ethanol, both *gas-1* and N2 mitochondria reached a similar threshold rate for respiration at the concentrations of drug causing immobility. We interpreted these data as consistent with the possibility that the decrease in respiration was directly causative of the ethanol induced immobility. Currently, we do not know whether other volatile anesthetics, such as isoflurane, will demonstrate patterns similar to halothane or to ethanol. Because *gas-1* is hypersensitive to all volatile anesthetics and to ethanol, such data are of interest.

At all concentrations of halothane studied, the rates of complex II-dependent respiration were *higher* in *gas-1* than in N2. This is a significant disparity compared with the hypersensitivity of *gas-1(fc21)* animals to halothane^{3,4}; therefore, an absolute rate of succinate-dependent respiration could not be linked to halothane concentrations that cause immobility of whole animals.

It is possible that the combined rate of complex I and complex II oxidative phosphorylation controls the anesthetic response. Because complex II function actually increases in *gas-1* mitochondria at the halothane concentration causing immobility in the corresponding animals, this is an interesting possibility. However, because we do not know the relative contributions of complexes I and II to the nematode, we are unable to rule this in or

out. Our measurements indicate that ATP ratios do not decrease in wild-type animals on exposure to an anesthetizing concentration of halothane. Therefore, an acute lack of ATP is not necessary for immobilization of N2. However, a striking decrease is seen in *gas-1* ATP concentrations when they are exposed to halothane. In the case of *gas-1* animals, ATP production may be rate limiting in causing immobility. Such a decrease in ATP production may explain why some patients with mitochondrial disease have an increased sensitivity to sevoflurane.¹⁸ We have not ruled out that both ATP production and utilization are both decreased by volatile anesthetics in a congruent manner, leaving ATP stores intact before and after exposure to anesthetics in the normal worm. Our results also do not allow us to measure absolute ATP concentrations. Therefore, if the total pool of adenylates were decreased in *gas-1* compared with N2, the amount of ATP would also be decreased.

However, even if ATP production is rate limiting in the mutant, it does not seem to represent the only reason these animals are hypersensitive to halothane. We can make the mitochondrial mutant relatively resistant to halothane by growing it in a low concentration of oxygen; we can make wild-type animals hypersensitive to halothane by growing them in high concentrations of oxygen. In fact, the sensitivities of N2 and the mitochondrial mutant *gas-1* were almost identical after growing N2 in high oxygen concentrations and *gas-1* in low oxygen concentrations. However, we do not know whether these effects are reversible. Mitochondria are known to be a major source of free radical production with resulting oxidative damage to proteins.³⁵ It seems, then, that free radical damage may also mediate anesthetic sensitivity. By staining Western blots with antibodies specific for free radical damage, we found that, when grown in room air, oxidative damage was increased in *gas-1* compared with N2. Surprisingly, this damage was fairly specific, with the primary effect being seen in a 26-kd mitochondrial protein. Because a second mitochondrial mutant with increased oxidative damage, *mev-1*, had no changes in anesthetic sensitivity, nonspecific oxidative damage does not seem to alter anesthetic sensitivity. Instead, it may be that certain specific proteins are damaged in *gas-1* mutants and that the accumulation of these damaged molecules is responsible for the increase in anesthetic sensitivity. This is consistent with our observation that *gas-1* larvae are less sensitive to volatile anesthetics than adults. We are in the process of identifying the 26-kd protein as a first candidate for such a protein.

Oxidative damage resulting from mitochondrial function is currently thought to be one of the major causes of aging in all animals, including humans.³⁵ Because it is also known that anesthetic sensitivity increases with advancing age,^{36,37} the current results may also indicate that hypersensitivity of the aged might be the result of deterior-

rating mitochondrial function. The results in children with mitochondrial myopathies indicate that mitochondrial changes can cause anesthetic hypersensitivity.¹⁸

Our results are most consistent with mitochondrial function in *gas-1* affecting anesthetic sensitivity *via* at least two mechanisms. Acute changes in respiration contribute to immobility as indicated by decreased oxidative phosphorylation and changes in ATP concentrations in the mutant. Overlaid on the changes in respiration are long-term changes in oxidative damage by reactive oxygen species. Future studies will be directed at identification of the proteins damaged by oxidation and understanding whether free radical production is increased in *gas-1* compared with wild-type animals. These results should further our understanding of how a mutation in a mitochondrial protein can profoundly change behavior in volatile anesthetics.

The authors are indebted to Charles L. Hoppel, M.D. (Professor, Departments of Pharmacology and Medicine, Department of Veterans Affairs Medical Center, Case Western Reserve University, Cleveland, Ohio), for his ongoing support and discussions. The authors thank Judy Preston (Research Assistant, Department of Anesthesiology, Case Western Reserve University, Cleveland, Ohio) for excellent technical assistance. They also thank Pam Szweda (Research Associate, Department of Physiology and Biophysics, Case Western Reserve University) and Luke Szweda (Associate Professor, Department of Physiology and Biophysics, Case Western Reserve University) for the generous sharing of their antibodies and continued advice and support in these studies.

References

- Dickinson R, White I, Lieb WR, Franks NP: Stereoselective loss of righting reflex in rats by isoflurane. *ANESTHESIOLOGY* 2000; 93:837-43
- Rajaram S, Sedensky MM, Morgan PG: A stomatin homologue controls sensitivity to volatile anesthetics in *C. elegans*. *Proc Natl Acad Sci U S A* 1998; 95:8761-6
- Morgan PG, Sedensky MM, Meneely PM: Multiple sites of action of volatile anesthetics in *C. elegans*. *Proc Natl Acad Sci U S A* 1990; 87:2965-9
- Morgan PG, Sedensky MM: Mutations affecting sensitivity to ethanol in the nematode *Caenorhabditis elegans*. *Alcohol Clin Exp Res* 1995; 19:1423-9
- Hartman PS, Ishii N, Kayser E-B, Morgan PG, Sedensky MM: Varied phenotypes caused by mutations altering mitochondrial complex I or complex II subunits in *Caenorhabditis elegans*. *Mech Ageing Dev* 2001; 122:1187-201
- Kayser E-B, Morgan PG, Sedensky MM: A mitochondrial protein controls sensitivity to volatile anesthetics in *C. elegans*. *ANESTHESIOLOGY* 1999; 90:545-54
- Kayser E-B, Morgan PG, Hoppel CL, Sedensky MM: Mitochondrial expression and function of GAS-1 in *Caenorhabditis elegans*. *J Biol Chem* 2001; 276:20551-8
- Ragan CI: Structure of NADH-ubiquinone reductase (complex I), Current Topics in Bioenergetics. Vol 15. Edited by Lee CP. San Diego, Academic Press, 1987, pp 1-36
- Walker JE: The NADH: ubiquinone oxidoreductase (complex I) of respiratory chains. *Q Rev Biophys* 1992; 25:253-324
- Xu X, Matsuno-Yagi A, Yagi T: Gene cluster of the energy-transducing NADH-quinone oxidoreductase of *Paracoccus denitrificans*: Characterization of four structural gene products. *Biochemistry* 1992; 31:6925-32
- Preis D, van der Pas JC, Nehls U, Rohlen D-A, Sackmann U, Jahnke U, Weiss H: The 49 K subunit of NADH: ubiquinone reductase (complex I) from *Neurospora crassa* mitochondria: Primary structure of the gene and the protein. *Curr Genet* 1990; 18:59-64
- Darroutzet E, Issartel J-P, Lunardi J, Dupuis A: The 49-kDa subunit of NADH-ubiquinone oxidoreductase (complex I) is involved in the binding of piericidin and rotenone two quinone-related inhibitors. *FEBS Lett* 1998; 431:34-8
- Zickermann V, Bostina M, Hunte C, Ruiz T, Radermacher M, Brandt U: Functional implications from an unexpected position of the 49-kDa subunit of NADH:ubiquinone oxidoreductase. *J Biol Chem* 2003; 278:29072-8
- Kashani-Poor N, Zwicker K, Kerscher S, Brandt U: A central functional role for the 49-kDa subunit within the catalytic core of mitochondrial complex I. *J Biol Chem* 2001; 276:24082-7
- Eckenhoff RG, Shuman H: Subcellular distribution of an inhalational anesthetic in situ. *Proc Natl Acad Sci U S A* 1990; 87:454-7
- Cohen PJ: Effects of anesthetics on mitochondrial function. *ANESTHESIOLOGY* 1973; 39:153-64
- Hanley PJ, Ray J, Brandt U, Daut J: Halothane, isoflurane and sevoflurane inhibit NADH:ubiquinone oxidoreductase (complex I) of cardiac mitochondria. *J Physiol* 2002; 544:687-93
- Morgan PG, Hoppel CL, Sedensky MM: Mitochondrial defects and anesthetic sensitivity. *ANESTHESIOLOGY* 2002; 96:1268-70
- Horvitz HR, Brenner S, Hodgkin J, Herman RK: A uniform genetic nomenclature for the nematode *Caenorhabditis elegans*. *Mol Gen Genet* 1979; 175:129-33
- Brenner S: The genetics of *Caenorhabditis elegans*. *Genetics* 1974; 77:71-94
- Herman RK: Genetics of *C. elegans*, The Nematode *Caenorhabditis elegans*. Edited by Wood WB. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press, 1988, pp 22-33
- Lowry OH, Rosebrough NJ, Farr AL, Rose JR: Protein measurement with the folin phenol reagent. *J Biol Chem* 1951; 193:265-75
- Gudz TI, Tserng KY, Hoppel CL: Direct inhibition of mitochondrial respiratory chain complex III by cell-permeable ceramide. *J Biol Chem* 1997; 272:24154-8
- Chance B, Williams GR: Respiratory enzymes in oxidative phosphorylation. *J Biol Chem* 1955; 217:409-27
- Estabrook RW: Mitochondrial respiratory control and the polarographic measurement of ADP:O ratios. *Methods Enzymol* 1967; 10:41-7
- Lundin A, Hasenson M, Persson J, Pousette A: Estimation of biomass in growing cell lines by adenosine triphosphate assay. *Methods Enzymol* 1986; 133:27-42
- Stadtman ER: Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. *Annu Rev Biochem* 1993; 62:797-821
- Tsai L, Szweda PA, Vinogradova O, Szweda LI: Structural characterization and immunochemical detection of a fluorophore derived from 4-hydroxy-2-nonenal and lysine. *Proc Natl Acad Sci U S A* 1998; 95:7975-80
- Waud DR: On biological assays involving quantal responses. *J Pharmacol Exp Ther* 1972; 183:577-607
- Snedecor GW, Cochran WG: Analysis of variance, Statistical Methods, 6th edition. Edited by Snedecor GW, Cochran WG. Ames, Iowa, Iowa State University Press, 1968, pp 271-5
- Murfit RR, Vogel K, Sanadi DR: Characterization of the mitochondria of the free living nematode *Caenorhabditis elegans*. *Comp Biochem Physiol* 1976; 53B:423-30
- Hochachka PW, Lutz PL: Mechanism, origin, and evolution of anoxia tolerance in animals. *Comp Biochem Physiol B Biochem Mol Biol* 2001; 130:435-59
- Ishii N, Fujii M, Hartman PS, Tsuda M, Yasuda K, Senoo-Matsuda N, Yanase S, Ayusawa D, Suzuki K: A mutation in succinate dehydrogenase cytochrome b causes oxidative stress and ageing in nematodes. *Nature* 1998; 394:694-7
- Kayser EB, Morgan PG, Hoppel CL, Sedensky MM: A mutation in mitochondrial complex I increases ethanol sensitivity in *Caenorhabditis elegans*. *Alcohol Clin Exp Res* 2003; 27:584-92
- Lenaz G, Bovina C, D'Aurelio M, Fato R, Formiggini G, Genova ML, Giuliano G, Pich MM, Paolucci U, Castelli GP, Ventura B: Role of mitochondria in oxidative stress and aging. *Ann N Y Acad Sci* 2002; 959:199-213
- Gregory GA, Eger EI, Munson ES: The relationship between age and halothane requirement in man. *ANESTHESIOLOGY* 1964; 30:488-91
- Stevens WD, Dolan WM, Gibbons RT, White A, Eger EI, Miller RD, DeJong RH, Elashoff RM: Minimum alveolar concentrations (MAC) of isoflurane with and without nitrous oxide in patients of various ages. *ANESTHESIOLOGY* 1975; 42:197-200