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Report

Mitochondrial Complex I Function Modulates Volatile Anesthetic Sensitivity in *C. elegans*

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Summary

Despite the widespread clinical use of volatile anesthetics, their mechanisms of action remain unknown [1–6]. An unbiased genetic screen in the nematode *C. elegans* for animals with altered volatile anesthetic sensitivity identified a mutant in a nuclear-encoded subunit of mitochondrial complex I [7, 8]. This raised the question of whether mitochondrial dysfunction might be the primary mechanism by which volatile anesthetics act, rather than an untoward secondary effect [9, 10]. We report here analysis of additional *C. elegans* mutations in orthologs of human genes that contribute to the formation of complex I, complex II, complex III, and coenzyme Q [11–14]. To further characterize the specific contribution of complex I, we generated four hypomorphic *C. elegans* mutants encoding different complex I subunits [15]. Our main finding is the identification of a clear correlation between complex I-dependent oxidative phosphorylation capacity and volatile anesthetic sensitivity. These extended data link a physiologic determinant of anesthetic action in a tractable animal model to similar clinical observations in children with mitochondrial myopathies [16]. This work is the first to specifically implicate complex I-dependent oxidative phosphorylation function as a primary mediator of volatile anesthetic effect.

Results

We previously observed that two mutations that decreased mitochondrial complex I function also increased sensitivity to volatile anesthetics. Conversely, a mutation that decreased complex II function did not alter anesthetic sensitivity. These results correlated with limited clinical findings in children with mitochondrial defects. This raised the question of whether the dependence of anesthetic sensitivity on complex I function is of general significance. We present here our determination of anesthetic sensitivity in a variety of *C. elegans* mitochondrial mutants generated either by classical means or by RNAi (Table 1).

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Both classical gene mutants and RNAi-induced hypomorphs inhibited mitochondrial respiration in a specific manner appropriate to the individual complex involved as well as to the site of electron entry into the mitochondrial respiratory chain (MRC). Inhibition of complex I-dependent oxidative phosphorylation capacity was observed in the complex I mutant *gas-1*, in *isp-1* (complex III mutant), and in *clk-1* (defect in coenzyme Q synthesis) (Figure 1). This capacity was measured as state 3 respiration rates in the presence of malate and internally normalized to complex IV-dependent rates measured with tetramethyl-p-phenylenediamine (TMPD) and ascorbate as combined electron donors. The effects of RNAi were studied first in *K09A9.5* (the gene locus of the *gas-1* allele) to demonstrate that an RNAi knockdown could mimic the phenotype of the corresponding missense mutant. Impairment in complex I-dependent oxidative phosphorylation capacity was seen, although to a lesser degree than is typical of *gas-1*. All of the other mutants with RNAi-induced complex I defects similarly demonstrated complex I-dependent oxidative phosphorylation capacity impairment (Figure 1A). However, complex I-dependent oxidative phosphorylation capacity was normal in the complex II mutant (*mev-1*), in agreement with previous reports from our laboratory [10]. Complex II-dependent oxidative phosphorylation capacity, measured as state 3 respiration rates in the presence of succinate, was significantly impaired in the complex II (*mev-1*) mutant and somewhat diminished in the complex III (*isp-1*) mutant, but appeared increased in all mutants with primary complex I defects (Figure 1B). No significant variation in complex IV-dependent oxidative phosphorylation, as measured in the presence of TMPD and ascorbate, was observed in any of the strains (Figure 1C).

All mutants that inhibited complex I function were hypersensitive to halothane. Complex I classical mutants had increased sensitivity to halothane as measured by a lower EC_{50}, the effective concentration required to produce immobility in fifty percent of animals. However, their degree of hypersensitivity varied (Figure 2A). Anesthetic hypersensitivity was most pronounced for *gas-1*, whereas *clk-1*, *seg-1;gas-1*, and *seg-2;gas-1* displayed more moderate increases in sensitivity. The complex II and III classical mutants, *mev-1* and *isp-1*, respectively, had normal sensitivities to halothane. *daf-2* was resistant to halothane. RNAi-induced hypomorphs of specific complex I subunits were all sensitive to halothane (Figure 2B).

When only complex I mutants were considered, the correlation between complex I-dependent oxidative phosphorylation capacity and anesthetic sensitivity was strongly positive ($r = 0.69$, $p = 0.056$) (Figure 3). The magnitude of the correlation diminished when the non-complex I respiratory-chain mutants (*clk-1*, *isp-1*, *mev-1*) were also taken into consideration ($r = 0.58$, $p = 0.063$). Two of these mutants had decreased complex I-dependent rates (*clk-1*, *isp-1*), but no defects within

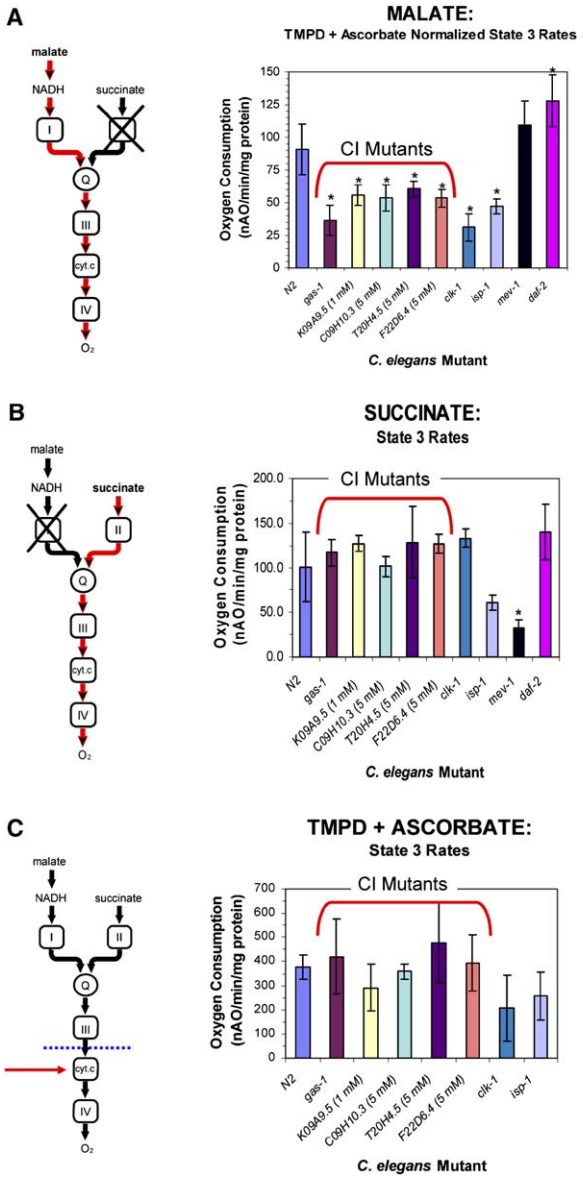


Figure 1. Integrated Oxidative Phosphorylation Capacity of *C. elegans* Mitochondria

State 3 rates of oxidative phosphorylation represent mean ADP-stimulated, near-maximal oxygen consumption capacity of freshly isolated mitochondria for each mutant. Error bars represent the standard deviation of the means. * indicates significantly different than wild-type (N2) with nonoverlapping standard deviations. IPTG concentration used to induce each RNAi gene-knockdown mutant is indicated in parentheses following mutant labels. All single mutations are listed in Table 1. Two suppressors of the *gas-1* phenotype were studied as double mutations, *seg-1;gas-1* and *seg-2;gas-1*. These suppressors have not yet been cloned [10]. In all figures, K09A9.5, C09H10.3, T20H4.5, and F22D6.4 are RNAi-induced mutants. N2 is the wild-type strain and *gas-1*, *clk-1*, *isp-1*, *mev-1*, and *daf-2* are classical genomic mutants.

(A) Complex I-dependent oxidative phosphorylation is *decreased* in all complex I, complex III, and coenzyme Q biosynthesis mutants but *increased* in the insulin receptor mutant *daf-2*. State 3 rates are normalized to TMPD plus ascorbate rates for each mutant compared to N2 (Supplemental Data).

(B) Complex II-dependent oxidative phosphorylation is moderately *increased* in all complex I mutants and significantly *decreased*, as expected, in the complex II mutant.

Table 1. Mutations in Mitochondrial Subunits of *C. elegans*

Gene Name[a]	MRC Complex	Subunit	Mutation Type
N2	Wild-type	n/a	n/a
gas-1(fc21)	I	49 kDa	Missense
K09A9.5	I	49 kDa	RNAi
C09H10.3	I	51 kDa	RNAi
T20H4.5	I	23 kDa	RNAi
F22D6.4	I	13 kDa	RNAi
clk-1(qm30)	Coenzyme Q	n/a	Deletion
isp-1(qm150)	III	ISP	Missense
mev-1(kn1)	II	Cytochrome b	Missense
daf-2(e1368)	n/a	Insulin receptor	Missense

Single mutations studied were either known defects within the MRC [11, 13, 14, 22] or RNAi-generated hypomorphs corresponding to highly conserved orthologs implicated in humans mitochondrial disease [23]. RNAi protocols were performed with worms grown for two generations in culture containing bacteria induced to produce a dsRNA corresponding to a gene encoding a specific complex I subunit [15, 24]. A nonmitochondrial mutant displaying volatile anesthetic resistance, *daf-2*, was also studied [25]. Integrated oxidative phosphorylation capacity was measured by polarography in intact, coupled mitochondria from each mutant. See Supplemental Data for greater detail.

[a] Strain names follow *Caenorhabditis* Genetics Center (CGC) gene names for genomic mutants and CGC sequence names for RNAi knockdown mutants.

complex I itself. These results implicate complex I specifically as a mediator of anesthetic sensitivity; the data imply that directly inhibiting complex I function increases anesthetic sensitivity.

Interestingly, an increased maximal capacity of complex I-dependent oxidative phosphorylation was noted in the nonmitochondrial mutant with a defect in the insulin-like receptor (*daf-2*) (165 nAO/min/mg protein versus 112 nAO/min/mg protein for N2). In general, high ADP rates are similar to state 3 rates for a given strain (see the Supplemental Data available online). In *daf-2* mitochondria, however, use of a higher concentration of ADP further stimulated the mitochondria over what is typical of their state 3 rates. Their degree of increased complex-I dependent oxidative phosphorylation capacity correlated with their degree of anesthetic *resistance* (Figure 3). Inclusion of *daf-2* with the complex I mutants strengthened the magnitude of the overall correlation between capacity of complex I-dependent respiration and sensitivity to the volatile anesthetic halothane ($r = 0.87$, $p = 0.002$). This implies that in a simple linear regression model, 76% of the variation in anesthetic sensitivity can be accounted for solely by the state 3 rate of complex I-dependent oxidative phosphorylation.

To further characterize this correlation, we determined whether a graded variation in the degree of RNAi in one and the same gene would incrementally decrease respiratory capacity and increase anesthetic sensitivity. RNAi was induced to varying extents for the complex I subunit gene *nuo-1* (*C09H10.3*) by using three different concentrations of IPTG. Increasing concentrations of IPTG led to increasing sensitivity to halothane (Figure 4A) and increasing inhibition of complex I-dependent oxidative phosphorylation capacity (Figure 4B), although no

(C) Mutants show no significant differences in state 3 oxidative phosphorylation respiration rates when electrons are donated directly to cytochrome C.

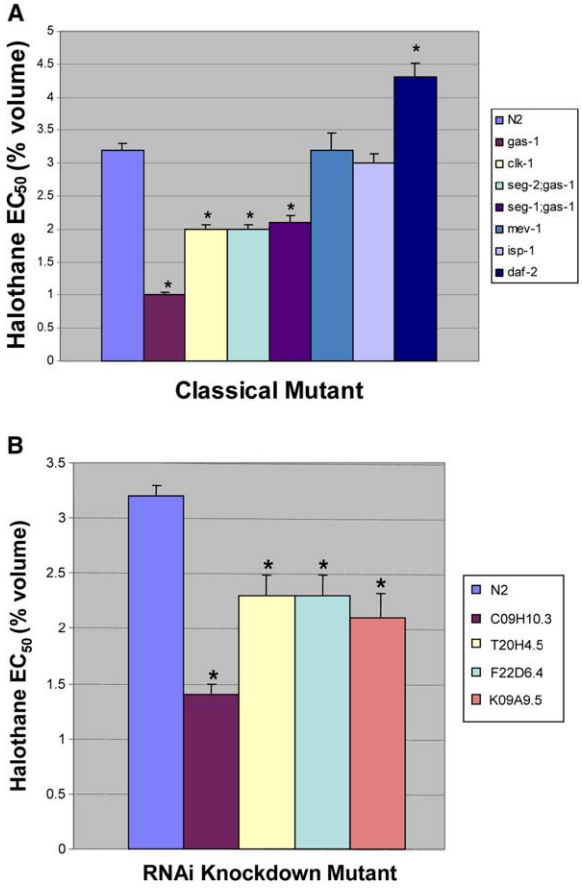


Figure 2. Anesthetic Sensitivity of *C. elegans*

The mean halothane concentrations necessary to immobilize 50% of worms, EC_{50}, is plotted for wild-type (N2) compared to the following: (A) worm strains with mutated nuclear genes expressing defective components of the MRC, and (B) worm strains where the expression of wild-type nuclear genes coding for complex I proteins has been inhibited by RNAi. Anesthetic sensitivity was measured in adults worms taken from the same cultures used for isolation of mitochondria in Figure 1 (Supplemental Data). Error bars represent standard deviations. * indicates difference from N2, $p < 0.05$. All means were compared by using ANOVA.

difference in oxidative phosphorylation capacity or anesthetic sensitivity was seen between the 5 mM and 10 mM IPTG RNAi mutants.

Previous studies demonstrated that the increased sensitivity to halothane of the complex I mutant *gas-1(fc21)* can be reverted by expressing the wild-type *gas-1* gene under control of its own promoter (P*gas-1*) from an extrachromosomal array. P*gas-1* is active in the nervous system and muscle [7]. Here, expression of the wild-type *gas-1* gene was placed under the control of either a neuronal-specific promoter (*ric-19*) or a muscle-specific promoter (*myo-3*). Both new constructs, introduced into the *gas-1(fc21)* mutant background, also partially restored halothane EC_{50}s toward normal (*gas-1(fc21)*, 1.0% ± 0.05%; *Pric-19::gas-1(+)*, 1.5% ± 0.2%*; *Pmyo-3::gas-1(+)*, 1.8% ± 0.2%*; *Pgas-1::gas-1(+)*, 2.1% ± 0.1%*; and N2, 3.2% ± 0.02%*. EC_{50}s + standard deviation; * indicates different than value for *gas-1*, $p < 0.05$ with a Bonferroni correction). None of the stable lines carrying these constructs were integrated into the genome; generally, the muscle-specific promoter showed stronger overall expression. Thus, the increased anesthetic sensitivity seen in *gas-1* mutants results from changes in both muscle and neuronal tissues. The relative contribution of these tissues to anesthetic sensitivity in the RNAi-induced mutant strains is not known.

Conclusions

A clear correlation exists between mitochondrial complex I oxidative phosphorylation capacity and volatile anesthetic sensitivity in *C. elegans*. In particular, the extent of complex I oxidative phosphorylation dysfunction is directly proportional to the degree of volatile anesthetic sensitivity (Figure 3). Some impairment of complex I-dependent oxidative phosphorylation function is seen in coenzyme Q biosynthesis and complex III structural-subunit mutants. This is not unexpected, given that these downstream components are assayed when testing integrated oxidative phosphorylation capacity with substrates that donate electrons through complex I. However, secondary complex I dysfunction (due to mutations affecting downstream respiratory-chain components, i.e., *clk-1* and *isp-1*) does not correlate with increased volatile anesthetic sensitivity as strongly as does primary complex I dysfunction (due to mutations affecting complex I itself). Furthermore, despite having impaired complex II-dependent oxidative phosphorylation capacity, the complex II mutant *mev-1* has normal complex I-dependent oxidative phosphorylation capacity and normal anesthetic behavior. This demonstrates that only those defects that directly impair complex I strongly increase anesthetic sensitivity.

Complex I-dependent oxidative phosphorylation capacity is thus implicated as a mediator of volatile anesthetic effect. Additional support for this role comes from the observation that an insulin receptor defect, *daf-2*, displays both increased complex I-dependent oxidative phosphorylation capacity and volatile anesthetic resistance. Because complex I is the rate-limiting component of the MRC [17], the discrepancy between high ADP and state 3 rates observed in *daf-2* likely reflects a change in complex I proper, rather than in downstream components of the MRC. This justifies the inclusion of *daf-2* in the analysis of complex I mutants.

This new insight into volatile anesthetic mechanism is significant for multiple reasons. Foremost, complex I oxidative phosphorylation capacity is a previously unrecognized determinant of anesthetic sensitivity. As such, it represents a novel mechanism by which anesthetics may mediate their effects. Previous work has shown that the mitochondrion is a preferred and saturable site for halothane localization [18]. More recently, analysis of rat-brain membrane proteins has shown selective binding of halothane to individual protein subunits of the MRC, including complex I [19]. Indeed, in both worms and mammals, complex I-dependent respiration is the most sensitive to inhibition by volatile anesthetics [9, 20]. Because complex I rates of electron transfer are rate determining for the MRC [17], inhibition of complex I may have a relatively immediate effect on oxidative phosphorylation and, in turn, on synaptic transmission within energetically demanding tissues such as the nervous system.

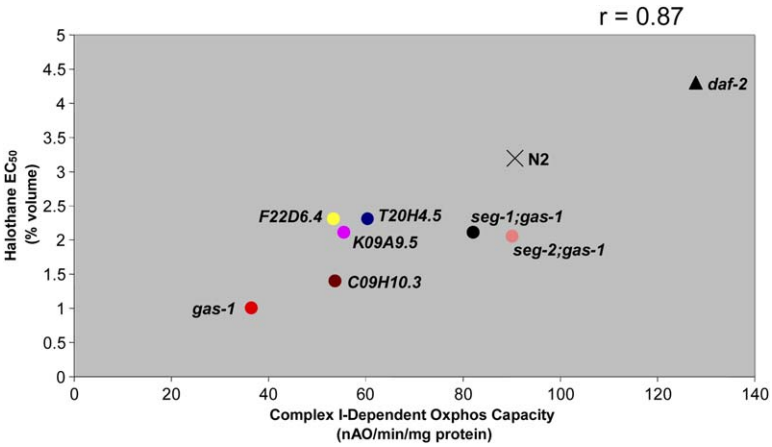
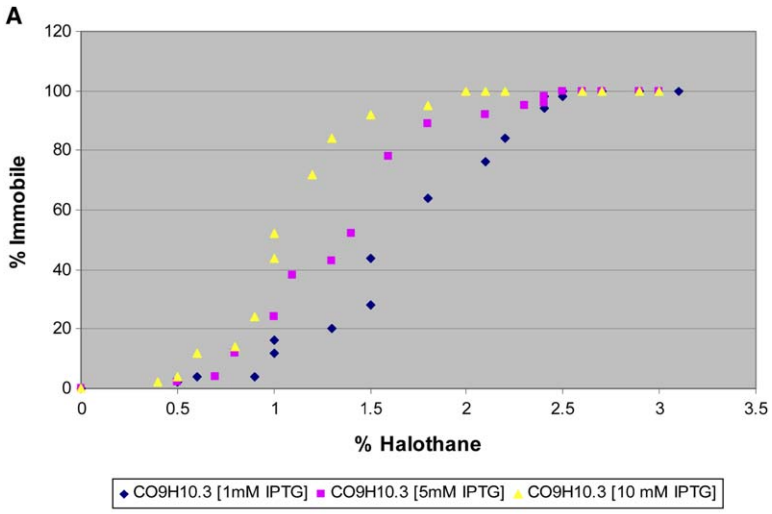


Figure 3. Halothane Sensitivity Is a Function of Complex I-Dependent Respiration

Complex I-dependent respiration rates of isolated mitochondria actively phosphorylating exogenous ADP (state 3 respiration) are plotted against the sensitivity to the anesthetic halothane (EC_{50}) of the live worms. Wild-type *C. elegans* (N2) is denoted with an "X." Mutants for genes encoding complex I proteins are denoted with a circle. A nonmitochondrial mutant is denoted with a triangle (*daf-2* is an insulin receptor mutant). Correlation value shown represents only complex I and insulin receptor mutants ($p = 0.002$); when other respiratory chain mutants are included, r decreases to 0.75 ($p = 0.005$).

Additionally, this new understanding holds promising implications for the care of patients with mitochondrial disease. There is a long-standing clinical belief that volatile anesthetic exposure results in high morbidity in an unpredictable subset of patients with putative mitochondrial disease [21]. Our recent clinical experience sheds light on these case reports by reinforcing the observations seen in *C. elegans*. We observed that children with mitochondrial diseases impairing complex I oxidative phosphorylation capacity appear to be uniquely sensitive to the volatile anesthetic sevoflurane [16]. Thus, the dependence of volatile anesthetic sensitivity on complex I function crosses phylogenetic boundaries from nematodes to humans.

We have previously shown that *gas-1* does not have an increased sensitivity to either aldicarb (a presynaptic



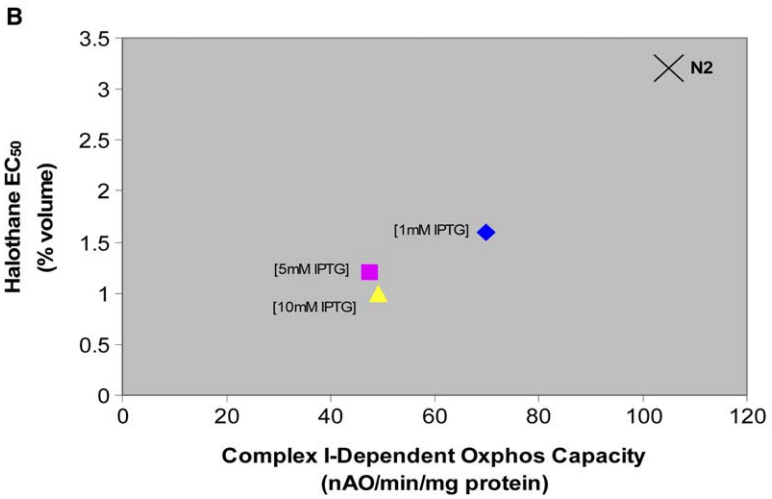


Figure 4. Graded RNAi Inhibition of Expression of a Complex I Subunit Leads to Graded Anesthetic Sensitivity

Expression of the wild-type allele of *C09H10.3*, encoding the 51 kDa subunit of complex I, was variably knocked down by RNAi.

(A) Increasing the concentration of the inductor (IPTG) for RNAi led to increasing sensitivity of live worms to the anesthetic halothane.

(B) Both complex I-dependent oxidative phosphorylation and anesthetic sensitivity were altered in a dose-related fashion.

paralytic) or levamisol (a postsynaptic paralytic). In fact, *gas-1* is resistant to aldicarb, indicating a clear neuronal function of this gene [14]. Results from our present study indicate that the increased sensitivity of *gas-1* animals to halothane is dependent on both muscle and neuronal expression. However, complex I activity correlates with a purely neuronal response to anesthesia in at least some human patients with mitochondrial dysfunction [16]. High-energy-requiring tissues, which are most dependent on mitochondrial function, are those most likely to be affected by complex I dysfunction. Although it is of some interest as to what tissue might be causative for anesthetic-induced immobility in nematodes (probably reflecting the most energy-demanding tissue), the response causing loss of consciousness in humans clearly resides in the nervous system.

Recognition that anesthetic hypersensitivity is dependent on complex I function may alert clinicians to the possibility of lowering anesthetic dosages for patients with impaired complex I-dependent oxidative phosphorylation capacity. At the same time, the study of genetically based, non-complex I MRC disorders in a model organism suggests that individuals with these disorders may be at lower risk for anesthetic hypersensitivity and adverse anesthesia-related effects. Our data demonstrate the utility of *C. elegans* as a translational model organism in which to study the genetic basis of mitochondrial dysfunction and anesthetic sensitivity. Such an approach permits detailed characterization of a genotype-phenotype correlation confounded in humans by vastly greater complexity.

Supplemental Data

Supplemental Data include Experimental Procedures and one table and are available with this article online at: http://www.current-biology.com/cgi/content/full/16/16/1641/DC1/.

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