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Isoflurane Selectively Inhibits Distal Mitochondrial Complex I in Caenorhabditis Elegans

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Abstract

BACKGROUND—Complex I of the electron transport chain (ETC) is a possible target of volatile anesthetics (VAs). Complex I enzymatic activities are inhibited by VAs, and dysfunction of complex I can lead to hypersensitivity to VAs in worms and in people. Mutant analysis in *Caenorhabditis* (*C.) elegans* suggests that VAs may specifically interfere with complex I function at the binding site for its substrate ubiquinone. We hypothesized that isoflurane inhibits electron transport by competing with ubiquinone for binding to complex I.

METHODS—Wildtype and mutant *C. elegans* were used to study the effects of isoflurane on isolated mitochondria. Enzymatic activities of the ETC were assayed and dose-response curves determined using established techniques. Two-dimensional native gels of mitochondrial proteins were performed after exposure of mitochondria to isoflurane.

RESULTS—Complex I is the most sensitive component of the ETC to isoflurane inhibition; however the proximal portion of complex I (the flavoprotein) is relatively insensitive to isoflurane. Isoflurane and quinone do not compete for a common binding site on complex I. The absolute rate of complex I enzymatic activity in vitro does not predict immobilization of the animal by isoflurane. Isoflurane had no measurable effect on stability of mitochondrial supercomplexes.

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Reduction of ubiquinone by complex I displayed positive cooperative kinetics not disrupted by isoflurane.

CONCLUSIONS—Isoflurane directly inhibits complex I at a site distal to the flavoprotein subcomplex. However, we have excluded our original hypothesis that isoflurane and ubiquinone compete for a common hydrophobic binding site on complex I. In addition, immobilization of the nematode by isoflurane is not due to limiting absolute amounts of complex I electron transport as measured in isolated mitochondria.

The nematode *Caenorhabditis* (*C.) elegans* has been established as a tractable genetic model for studying anesthetic action. [1,2] While reversible immobility produced by volatile anesthetics (VAs) occurs at concentrations higher than minimum alveolar concentration in humans, this end point obeys the Meyer-Overton rule, exhibits stereoselectivity, is not affected by nonimmobilizers, and fits well with the LC_{50}/EC_{50} ratio seen in mammals for VAs. [3] Most importantly, the molecular mechanisms that determine this end point control the anesthetic behavior of other organisms, including humans. [4–6]

Data from both humans and *C. elegans* indicate that mitochondrial physiology plays a role in controlling anesthetic sensitivity. The electron transport chain (ETC) is a collection of interacting protein complexes and mobile electron carriers that resides within the inner mitochondrial membrane. [7–9] In *C. elegans*, animals that are defective in respiration through complex I of the ETC are hypersensitive to VAs. [4,6,10] The same is true for children who present for a muscle biopsy to diagnose mitochondrial dysfunction. [5]

Complex I is the point of entry for electrons from mitochondrial nicotinamide adenine dinucleotide (NADH) into the respiratory chain in the inner mitochondrial membrane (For excellent reviews see references [9,11,12]). Complex I transfers electrons from NADH, through a series of iron sulfur clusters, finally to the hydrogen carrier ubiquinone (UQ) (coenzyme Q, CoQ). [7,11] This molecule consists of a hydrophobic tail embedded within the inner mitochondrial membrane, and a more polar aromatic headgroup that is the redox active moiety. Driven by the free enthalpy of the electron transfer, complex I pumps protons from the matrix to the intermembrane space. This contributes to the proton motive force that energizes the inner mitochondrial membrane, and is crucial for the aerobic regeneration of adenosine triphosphate (ATP). Complex I is an enormous complex consisting of at least 45 distinct subunits (in eukaryotes). Most of these subunits are encoded by nuclear genes and have to be imported into the mitochondrion. Only the 7 most hydrophobic subunits are encoded by mitochondrial DNA and expressed inside the mitochondria. Insight into subunit composition, structure, and function has been compiled from studies of a wide variety of biological sources for both the eukaryotic and prokaryotic forms of the enzyme. [9,11,12] Both forms are roughly L-shaped with a matrix arm and a membrane arm. It is important however to appreciate that the bacterial complex I is composed of only 14 subunits while fulfilling the same basic function as the eukaryotic form. All of the prokaryotic subunits have clear orthologs in the eukaryotic enzyme. These are referred to as the core subunits. Subunits that do not have counterparts in bacteria are often called supernumerary, reflecting our present ignorance about their possible functions. The recent progress in crystallizing bacterial complex I allowed development of a detailed spatial model for the prokaryotic complex and by analogy for the arrangement of the core subunits of the eukaryotic form. [9]

In worms, one of the mutants most sensitive to VAs, *gas-1*(*fc21*), cannot readily transport electrons from complex I to complex III of the ETC. [7] The mutation alters an evolutionarily strictly conserved arginine (R280K) in a core subunit of complex I. [13] This subunit of complex 1 termed GAS-1 (orthologous to 49kD, Nuo4, NDUFS2), [14] together with another core subunit PSST, holds the terminal iron sulfur cluster N2 and forms part of the binding site for the headgroup of UQ (Fig. 1A). [7,9,11] Crystallographic data from the bacterial

complex I showed that the cavity believed to bind the UQ headgroup is in part formed by a 4-α helical bundle of the GAS-1 ortholog. [9] Amphiphilic peptides with four helical bundles have been extensively used to model the binding of halothane to membrane proteins. [15,16] Halothane binds to these synthetic peptides with an affinity approximately within physiologic ranges and had dose-dependent effects on the structure of these synthetic molecules.

Several findings suggested that VAs like isoflurane inhibit complex I near the site of UQ reduction: The *gas-1* mutation strongly affects anesthetic sensitivity; the GAS-1 protein forms part of a specific binding site of the UQ headgroup; and GAS-1 has a structural motif suspected to bind halothane. Furthermore, knockdown of the UQ binding site subunits PSST and GAS-1 also increase sensitivity to VAs. [14] Moreover, the UQ biosynthesis mutant *clk-1*(*qm30*) which can only produce demethoxyubiquinone, an UQ analog with an aberrant headgroup, is hypersensitive to anesthetics. [6] Since interference with UQ reduction by VAs is a possible site of anesthetic action, we tested the hypothesis that isoflurane competes with UQ for a common hydrophobic pocket in complex I (for a model, see Fig. 1). If so, VAs could inhibit electron flow and energy production by displacing UQ from its normal binding site at complex I.

Complex I exists primarily as a component of a very large, intricately entwined, functional structure, the mitochondrial supercomplex. [17,18] In worms, virtually all of complex I is associated with complexes III and IV, in I:III_2:IV_n supercomplexes. [19] Different members of this supercomplex have allosteric effects on the activity of complex I such that complex I activity is maximized when it is part of the I:III:IV supercomplex. [17–20] In addition, Xi et al. have shown that complex IV of the ETC binds labeled halothane. [21] It is possible that anesthetic binding to complex III or IV could indirectly change the activity of complex I in an allosteric manner, by disturbing the normal tight associations between members of the supercomplex. (e.g., Fig. 1, B and C.) To interpret the anesthetic inhibition as a direct interaction between complex I and anesthetic, we had to exclude indirect inhibition via the respiratory supercomplex. Therefore we measured activities of each of these enzymatic protein complexes. We also asked if isoflurane can indirectly inhibit complex I by dissociating mitochondrial supercomplexes or by weakening the associations between members of supercomplexes I:III_2:IV_n.

METHODS

Nematodes

C. elegans wildtype N2 (Bristol) was obtained from the *Caenorhabditis* Genetics Center (Minneapolis, MN). The mutant *gas-1*(*fc21*) was isolated in our laboratory. [1,13] Worms were maintained according to standard techniques. [22] Mitochondrial preparation was as previously described, [10] with the minor change that worms were separated from bacteria by settling in ice-cold S-basal. In addition, the protease inhibitor cocktail was omitted.

Mitochondrial Assays

Spectrophotometric ETC assays were performed as previously described. [10] Fresh mitochondria preparations were solubilized with cholate and zero order rates were determined spectrophotometrically for the following enzyme activities: rotenone-sensitive NADH cytochrome c reductase (complex I-III), succinate cytochrome c reductase (complex II-III), antimycin A-sensitive decylubiquinol: cytochrome c reductase (complex III), NADH ferricyanide reductase (NFR), rotenone-sensitive NADH decylubiquinone reductase (complex I). Assays and calculations were performed as described by Hoppel et al. [23]

Azide-sensitive TMPD\ascorbate:oxidase (complex IV) was measured with a Hansatech Oxytherm Clark electrode at 30°C. Assay conditions were: fresh intact mitochondria (0.3 mg/mL) in 0.5 mL gemisch (0.1M KCl, 0.1% bovine serum albumine, 2 mM EGTA, 50 mM MOPS, 2 mM potassium phosphate pHed to 7.4), either with or without 4 mM KCN, were energized with 25 mM ascorbate, 2.5 mM N,N,N[prime],N[prime]-tetramethyl-p-phenylene-diamine (TMPD), and 2 mM ADP.

For all assays, the isoflurane concentration was adjusted by mixing appropriate volumes of reaction buffer and buffer saturated with anesthetic before mitochondria were added. Isoflurane concentrations are given as % equivalent in air. We determined by gas chromatography that fully isoflurane-saturated air contains 38% anesthetic at 30°C. Electron transport activities were measured in methacrylate disposable cuvettes after confirming that results were indistinguishable from those achieved in optical glass cuvettes. Loss of isoflurane during the course of kinetic measurements was negligible, judged by the observation that complex I activity did not increase during runs.

Blue Native/High Resolution Clear Native Gel Electrophoresis (BN/hrCNE)

For the first dimension of blue native gels (BNG), digitonin was used to solubilize frozen (−80°C) mitochondrial samples with a detergent/protein mass ratio of 6/1 ([19,20,24] with minor modifications). For the second dimension, excised lanes from the first dimension gel were incubated for 1 h at 20°C in a gastight container in either anesthetic-free BNG or the same buffer half saturated with isoflurane (equiv. 19% isoflurane in air; ~7.4 mM). Immediately afterward each gel strip was placed horizontally on top of a 3.5%–11% clear native gradient polyacrylamide gel and run at 125V, 4C for 15 h. The cathode buffer contained 0.05% deoxycholate, and 0.02% dodecyl maltoside.

Reagents

Equine heart cytochrome c was procured from Calbiochem/E.M.D., and isoflurane from Abbot Laboratories, North Chicago, IL. All other reagents were from Sigma-Aldrich, St. Louis, MO.

Statistical Analysis

Experimental data points in all figures are given as arithmetic means of independent mitochondria isolations with error bars representing the SD. SigmaPlot 11.2 (Systat software, inc.) was used for plotting data and curve fitting. This software reports uncertainties for the fit parameters as asymptotic standard errors. Errors for IC_{50}s were calculated using the macro *Propagation* [25] for Microsoft Excel. *P* values for the comparison of fit parameters were reported as * if $P \leq 0.01$.

RESULTS

Direct Inhibition of complex I

Isoflurane inhibited complex I activity (rotenone-sensitive electron transfer from NADH to decylubiquinone) in a dose-dependent manner (Fig. 2A) with an IC_{50} of 6.1% (2.4 mM) (Table 1). The rotenone-resistant activity of complex I was an order of magnitude lower (15 to 30nmol/min/mg) and not affected by isoflurane (data not shown).

Isoflurane inhibited flow of electrons from complex I to complex III (I-III activity, NADH:cytochrome c reductase) with similar characteristics (Fig. 2B, Table 1) as in the complex I assay: IC_{50} = 8.4% (3.3 mM). By contrast, complex II-III activity (succinate:cytochrome c reductase) was very resistant to isoflurane (Fig. 2C, Table 1) such that half-maximal inhibition could not be achieved by 19% (7.4 mM) isoflurane. We next

measured the activities of complexes III (Fig. 2D) and IV (Fig. 2E) as antimycin A-sensitive decylubiquinone: cytochrome c reductase and azide-sensitive TMPD\ascorbate:oxidase respectively. Neither activity was inhibited by up to 19% isoflurane.

Site of Action within complex I

NFR (NADH:ferredoxin reductase) measures the very first step of electron transfer through complex I. [13] The artificial electron acceptor ferricyanide diverts electrons from the most proximal subcomplex of complex I, the flavoprotein (the NADH dehydrogenase). [13] The flavoprotein sits at the tip of the matrix arm of complex I (Fig. 1E). Isoflurane has no significant effect on this activity (Fig. 2F).

Supercomplex stability

Mitochondrial supercomplexes can be identified on native gels, where they retain enzymatic activity and respiratory capability. [9] Mutations in complexes III or IV can weaken the interactions within supercomplexes, altering their appearance on BNGs, and indirectly decreasing the measurable activity of complex I itself. [19,20] Analogously, isoflurane may inhibit complex I activity by weakening the integrity of the supercomplex. We investigated this possibility by single dimension BNG electrophoresis of mitochondrial proteins after exposure of the mitochondria to isoflurane. No changes were seen compared to controls (data not shown). In addition, after running a BNG in a single dimension, we exposed the gel to 19% (7.4 mM) isoflurane. Immediately after exposure to isoflurane, a lane of the BNG was run in a second dimension in a stronger detergent, maltoside. A control BNG was treated similarly without the isoflurane exposure. If the anesthetic had weakened or strengthened interactions between components of the supercomplex, then exposure to isoflurane would alter separation, compared to the untreated control, of complexes from the supercomplex in the second dimension. Increased dissociation of supercomplexes would be seen as more bands migrating below a diagonal line that represents protein complexes that are unchanged by electrophoresis in a second dimension. We observed that even after treatment of the gel with 19% isoflurane, the supercomplexes did not differ from controls in patterns of dissociation (Fig. 3). The same technique (but without isoflurane treatment) has been successfully used to demonstrate that mutations in complex III weakened interaction between complex IV and the supercomplex (data not shown).

Mechanism of Inhibition

Both UQ and isoflurane are lipophilic and interact with the distal subcomplexes of complex I. The protein encoded by *gas-1* is part of this segment of complex I. We therefore hypothesized that the substrate UQ and inhibitor isoflurane compete for binding of the free enzyme.

Complex I from bovine heart follows hyperbolic (“Michaelis-Menten”) kinetics for UQ substrates. [26,27] This type of kinetics allows differentiation among three possible mechanisms of inhibition (competitive, *non*competitive, and *un*competitive) by comparing V_{lim} and apparent K for UQ measured in the absence and presence of the inhibitor. [28] We observed 510 ± 13 and 332 ± 8 nmol/min/mg for V_{lim} in the absence and presence of 5.7% isoflurane respectively (Fig. 4, A and Table 2). These clearly distinct values excluded competition of UQ and isoflurane for free complex I as the underlying mechanism of inhibition. We also found nematode complex I systematically deviated from hyperbolic kinetics (Fig. 4A,B). Instead the data fit sigmoidal kinetics with Hill coefficients above 2 (Table 2), suggesting positive cooperation between UQ binding sites. The cooperative nature of UQ kinetics was not disrupted by the presence of anesthetic (Fig. 4A).

Complex I mutant gas-1(fc21)

In the absence of isoflurane, complexes I and I-III were severely depressed by the *gas-1* mutation alone: 62nmol/min/mg and 85nmol/min/mg respectively which is 25% and 8% of the wildtype level (Fig. 2A,B, Table 1). Isoflurane minimally inhibited these two assays in *gas-1*.

NFR activity in the mutant was about 60% of wildtype (Fig. 2F). As in wildtype, NFR activity in *gas-1* was not inhibited by isoflurane. Hence, the presence of a mutant subunit in the neighboring iron-sulfur-protein subcomplex does not induce a new target for isoflurane on the flavoprotein. Complex II-III activity of *gas-1* was similar to wildtype (Fig. 2C), as can be expected for an activity that does not involve the mutated peptide. Complexes III and IV were not studied in *gas-1* since the mutation does not change these activities from wildtype, and the wildtype activities are not inhibited by isoflurane.

DISCUSSION

We have shown for the first time that isoflurane inhibition of complex I, the most susceptible member of the ETC to inhibition by VAs, is not due to competition of the agent for the hydrophobic binding site of UQ within complex I. In addition, it appears that isoflurane has no effect on the flow of electrons through the flavoprotein, the proximal tip of the matrix arm of complex I that protrudes into the aqueous matrix of the mitochondrion. Enzymatic rates of both complex I and complex I-III were very sensitive to inhibition by isoflurane. Also, complex I activity is measured using a water-soluble form of UQ while I-III activity uses the native quinone. Since both I and I-III activity are inhibited by isoflurane, anesthetic inhibition of complex I does not involve the binding sites for the UQ isoprenyl tail.

Our results are fully consistent with selective inhibition of complex I by isoflurane at a site distal to the flavoprotein subcomplex (Fig. 1E). Considering that both cytochrome c reductase assays (I-III Fig. 2B, II-III Fig. 2C) use a shared electron path from UQ to cytochrome c, the marked difference in sensitivity to isoflurane confirms that complex I is more sensitive to the drug than complexes II or III. Our results for isoflurane inhibition of complex I in *C. elegans* are in agreement with previous findings for halothane in rat liver mitochondria [29] and for three VAs, including isoflurane, in pig heart mitochondria. [30] However, these earlier reports did not establish IC_{50}s for isoflurane nor did they definitively establish that the complex I inhibition was selective to complex I or a portion of complex I. Our study, since it interrogates each individual relevant step of electron flow, represents a comprehensive evaluation of the possible indirect effects of isoflurane on complex I that have not been previously reported.

Likewise, previous investigations of the affects of VAs on ETCs preceded our current knowledge of the importance of supercomplex I:III:IV_n for proper complex I function. in vivo complex I, III, and IV form a supercomplex that is thought to be the functional unit of respiration. [17–19] Normal associations with complexes IV and complex III are necessary for maximal electron transport activity of fully assembled complex I in vitro. [17–20] The data indicate that supercomplexes remain intact in ETC assays and that complexes III and IV can indirectly affect complex I activity. Specific interactions of VAs with complexes III and IV have been convincingly demonstrated by Xi et al. using radiolabeled halothane. [21] It is therefore also conceivable that anesthetic bound to complex III or IV could elicit activity changes in complex I (Fig. 1B). However, such an interaction would be expected to also affect the activity of complex III or IV. Our results indicate that neither complex III nor IV is the relevant target for isoflurane. Alternatively, isoflurane might bind to complexes III or IV without affecting their individual activities but indirectly inhibit complex I by

destabilizing activating interactions between complex I and its partners (Fig. 1C). Weakened interactions between members of the supercomplexes that are not detected by one-dimensional BNGs can be unmasked by two-dimensional (2D) BNGs. We have identified mutations in subunits of complex III that cause an ~40% decrease in complex I activity and a striking vulnerability of the I:III:IV supercomplex to dissociation by maltoside (unpublished data), conditions identical to those we used for our 2D native gels presented here. If isoflurane inhibited complex I by weakening the association of complexes within the supercomplex, we would expect an observable effect on stability after exposure to 19% isoflurane. However, 2D electrophoresis did not reveal any stabilizing or destabilizing effects of 19% isoflurane on the supercomplexes. Thus, isoflurane did not disrupt the binding of complexes I, III and IV within the supercomplex. These results further validate the conclusion drawn from the ETC assays, that isoflurane interacts directly with complex I.

VAs are a heterogeneous group of chemicals, not identified by a common active chemical group. Their anesthetic potency is largely determined by their gas/oil partitioning coefficient. [31] The binding sites for UQ and three classes of complex I inhibitors are believed to be located in a hydrophobic fold of the complex. [12,32,33] UQ ordinarily has a long hydrophobic poly-isoprenyl tail thought to be embedded in the mitochondrial membrane, as well as a relatively hydrophilic head portion that swings between hydrophilic domains of complex I and complex III, passing electrons through the ETC. [12,32,33] In the prokaryote *Thermus thermophilus*, crystallographic data demonstrate that the binding site for quinone in complex I is sufficiently large to chamber VA molecules. [9,34] In addition, that part of the protein Nqo4 (also termed 49 kDa), an orthologue of GAS-1, which binds to UQ is a 4 α-helix bundle, located precisely at the junction of the transmembrane and the hydrophilic domains that comprise the quinone binding site. This type of structure, a 4 α-helix bundle, has been used extensively to model the binding of VAs to membrane proteins. [15,16] Thus, the hydrophobic binding pocket, containing the UQ binding site, could be a prime candidate for the binding of VAs. Just as some of the inhibitor binding sites of complex I overlap with each other and with UQ binding sites, it is easily conceivable that anesthetics interfere with the binding of UQ and vice versa. However, such competitive inhibition is unequivocally excluded (Fig. 4, Table 2) because even very high concentrations of decylubiquinone could not out-compete a fixed concentration (5.7%) of isoflurane. We conclude that isoflurane and UQ neither share a common binding site nor do they bind to overlapping sites (Fig. 1D,E). Our results furthermore excluded another variant of competitive inhibition: heterotropic allosteric inhibition. Binding of either isoflurane or UQ does not change the conformation of the complex as to negatively affect binding of the other. However, it is likely that isoflurane binds to other sites within complex I, the I:III:IV supercomplex, or even to UQ itself.

Once kinetics indicated that cooperative binding of UQ had to be considered, the simple model to distinguish the remaining two mechanisms of inhibition was no longer applicable. Thus, we were unable to determine whether isoflurane binds and blocks exclusively a preformed enzyme-substrate complex (*un*competitive inhibition) or binds to complex I independent of UQ (*non*competitive inhibition). Having established wildtype complex I as a selective target for isoflurane, but not in direct competition with Q for a binding site, we tested whether the hypersensitivity of *gas-1* may be caused directly by hypersensitivity of its mutant complex I to isoflurane.

GAS-1, a core subunit of complex I, is 83.4% similar, at the amino acid level, to its human counterpart. [13,14] The *fc21* missense mutation (R290K) affects a residue in GAS-1 that is conserved even in simple bacterial NADH:UQ reductases. It renders *C. elegans* exquisitely sensitive to all VAs but especially so to isoflurane (wildtype EC_{50} = 7.0 ± 0.1%, *fc21* EC_{50} = 1.3 ± 0.2%). [1,10] At 7.0% isoflurane, wildtype complex I is severely inhibited to about

40% of its potential activity, suggesting the inhibition may cause immobility of the animal. However, this argument loses traction when results from the mutant are considered. The baseline activity of mutant complex I was very low yet *gas-1* is quite mobile in the absence of isoflurane. Its complex I activity measured in the absence of anesthetic is already lower than that of the wildtype mitochondria in the presence of anesthetizing concentrations of isoflurane. A similar pattern as described here for isoflurane has previously been found for halothane. [35] Thus, there is no common threshold of complex I activity (measured in vitro) for wildtype and mutant, below which the worms become immobile/anesthetized.

It would appear that although complex I baseline activity (before inhibition) is a fair predictor of the *anesthetic sensitivity* of an organism, [4] the absolute level of complex I activity (resulting from the combination of genetic background and inhibition by anesthetic) is a poor predictor of the *anesthetic state* of the animal. However, several points indicate that complex I is important in determining anesthetic sensitivity. First, when complex I activity is inhibited by knockdown of multiple subunits of complex I, anesthetic sensitivity is almost uniformly increased. [14] Second, children with complex I defects have been noted to have an increased sensitivity to VAs. [5] Third, as shown here and by others, of all complexes in the ETC, complex I is by far the most sensitive to VAs. [29,30,36] Finally, as shown in this study, complex I activity in *gas-1* was resistant to further depression by the anesthetic (the isoflurane IC_{50} for *gas-1* [mt] than for N2). Thus, while isoflurane does not compete directly with quinone for binding to complex I, a mutation in a protein that is part of the UQ binding site does cause resistance to inhibition. The simplest interpretation of these data is that the mutation alters an isoflurane target.

How can one reconcile that complex I dysfunction leads to anesthetic hypersensitivity, yet we cannot correlate levels of complex I activity with the anesthetic state? First, defects in complex I, which changes its sensitivity to isoflurane, may lead to more important secondary changes in pertinent downstream anesthetic targets, resulting in an increase in anesthetic sensitivity. Second, ETC assays measure maximal enzymatic activity, a condition probably not attained in vivo. N2 and *gas-1* animals could have similar basal rates of complex I function in vivo. However, this rate may approach *gas-1*'s maximal complex I function and render the mutant complex I more vulnerable than wildtype to even small decreases in capacity. This difference could result, in the presence of isoflurane, in a more rapid decrease by *gas-1* than N2 to a common in vivo complex I threshold for immobility. Third, chronic loss of complex I activity (as in *gas-1*) may lead to compensatory changes that allow the animal to function at low complex I rates not tolerated by the wild type animal during acute anesthetic exposure. For example, the capacity for complex II-dependent respiration is up in *gas-1* [10], and we have shown here that complex II activity is not inhibited by isoflurane. Fourth, the anesthetic state may be the result of inhibition of multiple types of targets of which mitochondria are only one. Studies to differentiate between these possibilities are underway.

Finally, we have found that UQ itself has a cooperative interaction with complex I not previously appreciated. To our knowledge we are the first to report non-Michaelis-Menten behavior of complex I. For complex I the existence of multiple UQ binding sites is supported by experimental evidence and demanded by several mechanistic models [37,38]; however, their exact number is still a matter of debate. [39] Tocilescu et al. identified residues on the 49 kDa subunit (the GAS-1 orthologue) and the adjacent subunit (PSST) that altered the interaction of complex I with UQ. 12 However, this work did not establish whether more than one UQ molecule may occupy this redox active site nor has it been excluded that additional UQ binding sites may exist elsewhere on complex I which may be merely allosteric. Our measured Hill coefficients of 2.1 ± 0.2 and 2.5 ± 0.4 are consistent with either two strongly cooperative binding sites for UQ or more than two, but weaker, interacting

sites. It is noteworthy that isoflurane does not interrupt the cooperative interaction between UQ molecules since the Hill coefficient remains unchanged by the presence of isoflurane. It is crucial to acknowledge, however, that UQ kinetics are not trivial and are affected by multiple complicated interactions. [40] The apparent cooperation we observe may be specific to *C. elegans* or even an artifact of our system. The water-soluble substrate, decylubiquinone, that is used for this assay, may have unique properties of binding that are different from the native quinone. To clarify these possibilities, such studies should be repeated in a well-defined system such as bovine mitochondrial preparations.

In summary, we have shown that, in nematodes, isoflurane directly inhibits complex I function, at a site distal to the flavoprotein within the complex. We have excluded our initial hypothesis that the VAs compete with quinones for a hydrophobic binding site on complex I. The demonstrated stability of supercomplexes in the presence of isoflurane supported that complex I inhibition is not caused by isoflurane disrupting necessary interaction with neighboring complexes III and IV. Although complex I activity does predict VA sensitivity, absolute rates of complex I activity do not predict a threshold that correlates with immobility of the animal. Furthermore, we describe non-Michaelis-Menten kinetics, suggesting interacting binding sites, for UQ within complex I.

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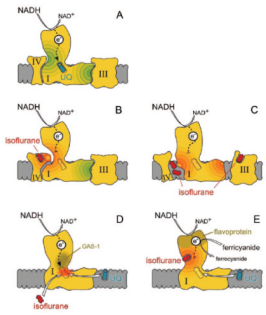

Figure 1.

Schematic model of potential mechanisms for the inhibition of complex I activity by isoflurane.

(**A**) The L-shaped complex I normally transports electrons from nicotinamide adenine dinucleotide (NADH) to ubiquinone (UQ, shown in its binding site. Complex I activity depends on proper interactions (green ripples) with associated complexes(complexes III and IV) within a supercomplex. (**B**) If complex III or IV binds isoflurane, changes in conformation (flash) could indirectly inhibit complex I activity (red ripples). (**C**) If isoflurane disrupts the integrity of the supercomplex, loss of the activating contacts (flash) may lead to indirect inhibition of complex I. For B and C, UQ is omitted for clarity. (**D**) Direct inhibition of complex I, in which isoflurane and UQ compete for a common hydrophobic binding site. The other members of the supercomplex were omitted for clarity. (**E**) Direct inhibition by isoflurane at a site distal to the flavoprotein, without preventing UQ binding. The arrows depict the flow of electrons that is measured by the NADH ferricyanide reductase (NFR) assay.

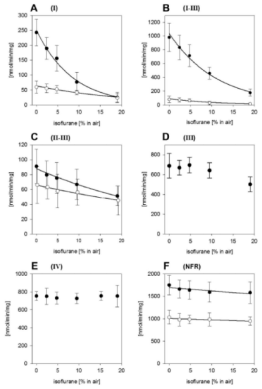

Figure 2.

Effect of isoflurane on mitochondrial electron transport.

Each point is the mean of ≥4 independent mitochondria preparations from wildtype N2 (●) and mutant *gas-1*) ([circo]) unless otherwise noted. Error bars represent standard deviations. Lines where presented, were plotted using the parameters from Table 1 for the best exponential fit. **(A)** Complex I activity of cholate-solubilized mitochondria measured as rotenone-sensitive nicotinamide adenine dinucleotide (NADH):decylubiquinone reductase. **(B)** Complex I-III activity measured as rotenone-sensitive NADH:cytochrome c reductase. **(C)** Complex II-III activity measured as antimycin A-sensitive succinate:cytochrome c reductase (Note: Data points for mutant are plotted offset by + 0.2% isoflurane from their actual positions to visually separate the error bars for the datasets.) **(D)** Wildtype N2 (●) complex III electron transport measured as antimycin A-sensitive decylubiqinol:cytochrome c reductase. (Here, $n = 3$) **(E)** Complex IV activity determined as the azide sensitive respiration of intact isolated wildtype N2 mitochondria supplied with external electron donor TMPD\ascorbate under phosphorylating conditions (“state-3”). (**F**) NFR, activity of the flavoprotein subcomplex of complex I NFR(measured as NADH:ferricyanide reductase for wildtype N2 (●) and *gas-1*.

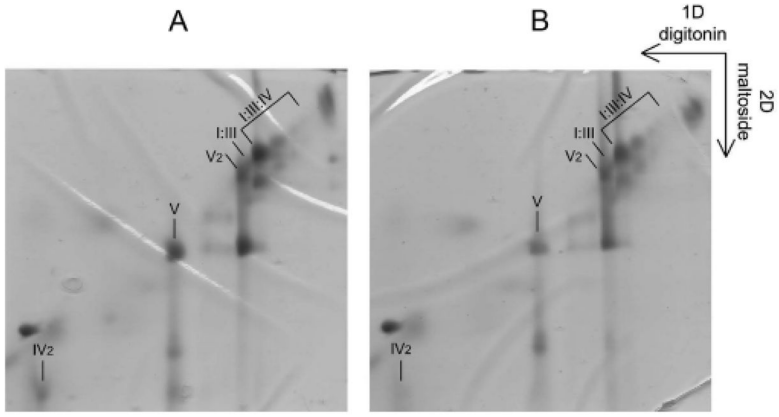

Figure 3.

Effect of isoflurane on the stability of I:III:IV supercomplexes.

BN/hrCN gel stained with Coomassie Blue. Complexes and supercomplexes of the respiratory chain were first separated by blue native gels (BNG) PAGE, then subjected to 0% (panel **A**) or 19% (panel **B**) isoflurane respectively, before being re-electrophoresed by hrCNE in the second dimension. A diagonal line is formed if electron transport chain (ETC) complexes do not separate from their original conformation when run in the second dimension in the harsher detergent, maltoside. When interactions between complexes I, III and IV are weakened, increased banding is seen below the diagonal line noted by I:III:IV. There is no difference in banding patterns after exposure to isoflurane, indicating no increase in dissociation of the $I:III_2:IV_n$ supercomplexes.

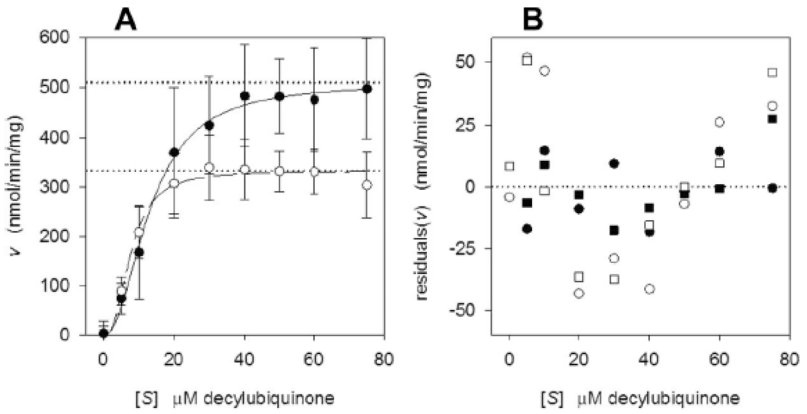

Figure 4.

Inhibition kinetics

(**A**) Initial rate *v* of complex I activity (rotenone-sensitive nicotinamide adenine dinucleotide (NADH):decylubiquinone reductase) as a function of the concentration of substrate decylubiquinone S measured in the absence (●) and presence ([circo]) of 5.7% isoflurane. Data points are arithmetic means of ≥6 independent mitochondria preparations with error bars for the SD. Solid and broken curves represent nonlinear least squares fit of data to the modified Hill Equation (Equation 1, Table 2); the calculated V_{lim} for both curves are indicated by horizontal dotted lines.

(**B**) Residuals Plot for complex I activity as a function of decylubiquinone concentration: Closed symbols (● isoflurane absent, [squlf] 5.7% isoflurane) depict the differences between measured data and the fit result for the sigmoidal model where all 3 parameters of the modified Hill equation, V_{lim}, *K* and *h*, were optimized (Table 2 and Fig. 4A). Open symbols ([circo] isoflurane absent, [squlo] 5.7% isoflurane) show the discrepancy between real data and the best fit to hyperbolic ("Michaelis-Menten") kinetics where *h* was held fixed to 1 and only V_{lim} and *K* were optimized (data not shown).

Table 1

Inhibition of electron transport activities by isoflurane in mitochondria from wildtype (N2) and complex I mutant *gas-1*.

source	activity	A_0 (nmol/min/mg)	b ($\%^{-1}$)	IC_{50} (%)
N2	NFR	1698±26	0.004±.002	
	I	250±8	0.118±.007	5.9±.3
	I-III	1037±40	0.089±.005	7.7±.4
	II-III	88±2	0.028±.002	
gas-1	NFR	1007±12	0.003±.001	
	I	64±2	0.046±.005	14.8±1.5
	I-III	91±7	0.090±.010	7.7±1.3
	II-III	66±1	0.019±.002	

Results for fitting electron transport rate v (nmol/min/mg) in response to the isoflurane concentration [lsqb]I[rsqb] (% in air) to equation **[GRAPHIC]** where A_O=rate in the absence of isoflurane and b=1st order constant. NFR (NADH:ferricyanide reductase), I (NADH:decylubiquinone reductase), I-III (NADH:cytochrome c reductase), II-III (succinate:cytochrome c reductase). Parameters A_0 and b were used to plot curves in Figs. 2A,B,C,F and to calculate the concentrations of isoflurane causing half-maximal inhibition (IC_{50}) where appropriate. Errors are the standard deviations of the fit parameters.

* different than value for N2, p≤0.01.

Table 2

Complex I activity as a function of the concentration of its substrate decylubiquinone.

	Isoflurane	
	0.0%	**5.7%**
V_{lim} (nmol/min/mg)	509.8 ± 13.0	332.0 ± 8.0
K (%)	13.2 ± 0.7	7.7 ± 0.5
h ()	2.1 ± 0.2	2.5 ± 0.4

Best fits for the parameters V_{lim}, *K* and *h* to the "modified Hill equation"[1] were determined by "non-linear least squares"; errors designate standard deviations. The parameter values shown were used to plot the curves in Fig. 4A.

[1] Equation 1. "Modified Hill Equation" to describe sigmoidal kinetics: Where velocity v denotes the initial rate of product formation (here: NAD+) when the substrate (here: decylubiquinone) is present at a concentration of [lsqb]S[rsqb]. Parameters V_{lim}, *K*, *h* are constants: V_{lim} is the limiting velocity if enzyme could be fully saturated with substrate; *K* is the substrate concentration at which half-maximal velocity is achieved and *h* is the so called "Hill coefficient". Note: for the special case of *h*=1 the equation simplifies to describe hyperbolic (Michaelis-Menten) kinetics.