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Volatile Anesthetic Preconditioning Present in the Invertebrate Caenorhabditis elegans

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Background: **Volatile anesthetics (VAs) have been found to induce a delayed protective response called preconditioning to subsequent hypoxic/ischemic injury. VA preconditioning has been primarily studied in canine and rodent heart. A more genetically tractable model of VA preconditioning would be extremely useful. Here, the authors report the development of the nematode *Caenorhabditis elegans* as a model of VA preconditioning.**

Methods: **Wild-type and mutant *C. elegans* were exposed to isoflurane, halothane, or air under otherwise identical conditions. After varying recovery periods, the animals were challenged with hypoxic, azide, or hyperthermic incubations. After recovery from these incubations, mortality was scored.**

Results: **Isoflurane- and halothane-preconditioned animals had significantly reduced mortality to all three types of injuries compared with air controls. Concentrations as low as 1 vol% isoflurane (0.64 mM) and halothane (0.71 mM) induced significant protection. The onset and duration of protection after anesthetic were 6 and 9 h, respectively. A mutation that blocks inhibition of neurotransmitter release by isoflurane did not attenuate the preconditioning effect. A loss-of-function mutation of the Apaf-1 homolog CED-4 blocked the preconditioning effect of isoflurane, but mutation of the downstream caspase CED-3 did not.**

Conclusions: **Volatile anesthetic preconditioning extends beyond the vertebrate subphylum. This markedly broadens the scope of VA preconditioning and suggests that its mechanisms are widespread across species and is a fundamental and evolutionarily conserved cellular response. *C. elegans* offers a means to dissect genetically the mechanism for VA preconditioning as illustrated by the novel finding of the requirement for the Apaf-1 homolog CED-4.**

PRECONDITIONING is a cellular adaptive response where exposure to a potentially injurious agent induces a protective response to subsequent noxious exposure. Hypoxia and ischemia are the best characterized preconditioning agents and have been well-studied in cardiac myocytes and cerebral neurons.[1,2] Both of these cell types exhibit a robust hypoxic and ischemic preconditioning response. Indeed, circumstantial evidence suggests that both angina and transient ischemic attacks may induce a protection from subsequent ischemia in humans by a preconditioning mechanism.[3–5]

Hypoxia and/or ischemia is inherently dangerous and difficult to titrate and therefore unsuitable as a means to protect against hypoxia and ischemia in the clinical setting. However, other agents besides hypoxia/ischemia have been subsequently found to induce a preconditioning-like protective response. Volatile anesthetics (VAs) are one such preconditioning drug.[6,7] Isoflurane in particular has been well documented to induce preconditioning in a variety of cell types. As for hypoxic/ischemic preconditioning, cardiac myocytes and cerebral neurons have been widely shown to be protected by anesthetic preconditioning. Limited human trials administering VAs before ischemia in patients undergoing coronary artery bypass surgery suggest that VAs can also act to protect these human cell types from hypoxia/ischemia.[8]

A number of molecules have been implicated in preconditioning mechanisms.[1,6,7,9] Reactive oxygen species have been consistently found to be important, if not essential, for both hypoxic and anesthetic preconditioning.[8,10] Adenosine triphosphate–inhibited potassium (K_{ATP}) channels have also been implicated in several different models of preconditioning, including hypoxic and anesthetic preconditioning.[7,11] Apoptosis has also been suggested by some experiments to be inhibited by preconditioning or to induce preconditioning.[9,12,13] For neuronal protection, in addition to the aforementioned mechanisms, inhibition of glutamate release has been suggested as a mechanism for VA protection and preconditioning.[6] These are only a subset of molecules with some evidence for a role in preconditioning.

Given the complexity and likelihood of thus far unidentified components of the preconditioning mechanism, a genetically tractable model for the study of preconditioning would have great potential. Not only could such a genetic model test hypotheses in ways not always feasible by other methods, it might discover components of the preconditioning mechanism not previously identified. We have developed the nematode *Caenorhabditis elegans* as a model to define molecular mediators of hypoxic cell death.[14,15] The strengths of the model are many. One can screen through large numbers of genes for those that control hypoxic injury without a requirement for a preexisting bias as to what one will find. This ability to go from phenotype (hypoxia resistance for example) to genotype (the gene variation responsible for the phenotype) enables the discovery of completely novel mechanisms. On the other hand, a large collection

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of readily available mutants and a nearly whole-genome library of RNAi plasmids that can knock down the expression of a particular gene[16] allows for rapid and affordable hypothesis testing. Finally, *C. elegans* has a well-characterized and relatively simple apoptotic cell death pathway and has evolutionarily conserved genes that encode proteins that regulate necrotic and autophagic death pathways.[17–27] Of course, a limitation of *C. elegans* for understanding hypoxic injury in humans is its evolutionary distance. It is likely that some mechanisms controlling hypoxic injury in *C. elegans* will translate to humans and some will not.

We have recently found that *C. elegans* has a mechanism for hypoxic preconditioning.[28] We tested the role of the apoptosis pathway in hypoxic preconditioning in *C. elegans* and found surprisingly that only one component of the pathway, the Apaf-1 homolog CED-4, was required for hypoxic preconditioning. Here, we test whether *C. elegans* has a mechanism for anesthetic preconditioning and test whether anesthetic and hypoxic preconditioning share a requirement for CED-4.

Materials and Methods

Culture of C. elegans *and Strains*

All strains were grown at 20° on nematode growth medium (NGM) agar plates seeded with OP50 *Escherichia coli* bacteria.[29] The wild-type *C. elegans* strain used was N2 *var* Bristol.[29] *unc-64(md130)* has been described previously.[30,31] *ced-3(n1286)* was described and kindly provided by the Horvitz laboratory (Massachusetts Institute of Technology, Cambridge, MA).[32] *ced-4(n1162)* and N2 were obtained from the *Caenorhabditis* Genetics Center funded by the National Institutes of Health National Center of Research Resources (Bethesda, MD).

Anesthetic Preconditioning

For anesthetic preconditioning, uncrowded synchronized populations of young adult animals on uncovered NGM plates with bacteria were placed in small glass chambers.[33,34] A measured volume of anesthetic (isoflurane: Isothesia, Butler Animal Health Supply, Dublin, OH; halothane: Halocarbon Laboratories, River Edge, NJ) in its liquid phase was deposited through the side port onto the roof of each chamber using a Hamilton syringe, and the side port was quickly sealed. The anesthetic completely volatilizes within 30 s. The volume of anesthetic placed in the chamber was calculated to produce a desired gas phase concentration according to the ideal gas equation, given the known volume of the chamber and the density and molecular weight of the liquid anesthetic. At the end of each assay, the actual VA concentrations in the chambers were measured by gas chromatography (HP 5890A gas chromatograph; Agilent Technologies, Inc., Life Sciences and Chemical Analysis Group, Santa Clara, CA). Chambers containing anesthetic or air control were immediately placed in a 20° temperature-controlled incubator for 4 h. After the 4-h preconditioning incubation, the agar plates with worms were then removed from the glass chambers, and the worms were allowed to recover for a given period before hypoxic, azide, or thermal challenge.

Hypoxic and Azide Killing Incubation

After anesthetic or air control preconditioning, animals were transferred from their NGM plates to a 1.5-ml Eppendorf tube with 1 ml M9 buffer,[29] the buffer was exchanged three times to remove bacteria, and the final wash was removed down to 100 μl. The tubes were placed into the hypoxia chamber as described previously,[14] incubated for 20 h at 26°, and then transferred to NGM agar plates seeded with OP50 bacterial to recover in normoxia at 20°. Oxygen tension in the hypoxia chamber was measured by an oxygen meter and electrode (OM4 meter, MI730 electrode; Microelectrodes, Bedford, NH) and was always less than 0.3%. Azide incubations were performed as described previously[14] except that azide incubations were 70 min in 0.5 M NaN_3. Animals were scored 20–24 h after completion of the hypoxic or azide incubations. Animals without pharyngeal pumping and without spontaneous or touched-evoked movement were scored as dead.

Thermal Injury

After anesthetic or air control preconditioning, young adult animals were transferred from their NGM plates to a 1.5-ml Eppendorf tube with 1 ml M9 buffer. The buffer was exchanged three times to remove bacteria, and the final wash was removed down to 100 μl. The tubes were placed into a 37° circulating water bath and incubated for 1 h. Animals were then transferred to NGM agar plates seeded with OP50 bacterial to recover in normoxia at 20°. Animals were scored 20–24 h later, and those without pharyngeal pumping and spontaneous or touched-evoked movement were scored as dead.

Statistical Analyses

Simultaneously determined experimental and control values were compared for significant differences by paired *t* test with the significance threshold set at $P < 0.01$. For determinations of divergence from control or baseline in time course and concentration–response experiments, respectively, all values for a particular time point or concentration were pooled and compared by a Fisher exact chi-square test. The significance threshold was set at $P < 0.01$ after Bonferroni correction for multiple comparisons. All statistical manipulations and testing were performed using GraphPad Prism Software (GraphPad Software, Inc., San Diego, CA).

Results

We tested the ability of two VAs, isoflurane and halothane, to produce protection of the whole *C. elegans* animal from death after normally lethal injuries. Preliminary experiments indicated that 2% isoflurane (1.28 mM) and 1% halothane (0.71 mM) followed by a 1-h recovery before exposure to the lethal injury might be efficacious. Indeed, under these conditions, both isoflurane and halothane reproducibly produced an approximately twofold reduction in lethality after a hypoxic injury (fig. 1A). Sodium azide is an inhibitor of cytochrome oxidase, the terminal protein complex in the mitochondrial electron chain, and thereby blocks utilization of oxygen for energy production.[35] Sodium azide has been used a chemical surrogate for hypoxia.[36–40] Agents or conditions that alter hypoxic sensitivity in general similarly affect azide sensitivity if the agents are in fact altering the consequences of inhibition of oxidative metabolism. Therefore, we tested the effect of isoflurane and halothane pretreatment on azide-mediated death. As for hypoxia, both anesthetics reduced azide lethality (fig. 1B). Finally, to test whether the protective response was specific for hypoxic injury or was more generalized, we measured the effect of isoflurane preconditioning against hyperthermic injury. Isoflurane did produce a significant reduction in hyperthermic death (fig. 1C). This result argues that anesthetic pretreatment in *C. elegans* induces a protective response to generalized cellular injury. Notably, hypoxic preconditioning does not protect from hyperthermic death.[28] Therefore, anesthetic preconditioning induces a protective response that is broader than that by hypoxic preconditioning and argues against metabolic suppression as a mechanism for anesthetic preconditioning.

Preconditioning is by definition a delayed protection after the preconditioning stimulus is no longer being applied. To establish that the protection by the anesthetic pretreatment was indeed preconditioning and not due to a direct protective effect of residual drug, we measured the onset and duration of the protection after isoflurane and halothane. Isoflurane significantly protected from hypoxic injury even with no recovery period after the isoflurane preconditioning before hypoxic killing (fig. 2A). The hypoxia protection significantly increased after a 1-h recovery from isoflurane and then gradually waned, returning to baseline by 5 h. For halothane, the protective effect from hypoxia appeared more transient, only significant at 1 h of recovery (fig. 2B). This determination of the time course for protection is limited by the long hypoxic incubation, 20 h, required to kill the majority of *C. elegans*.[14] Therefore, the onset of the protective preconditioning response could be at any time during or even after the 20-h hypoxic killing incubation. To get a more precise time course, we used azide killing, which can be accomplished in 70 min. With azide incubation, the protection by isoflurane and halothane was clearly delayed (figs. 2C and D). The earliest

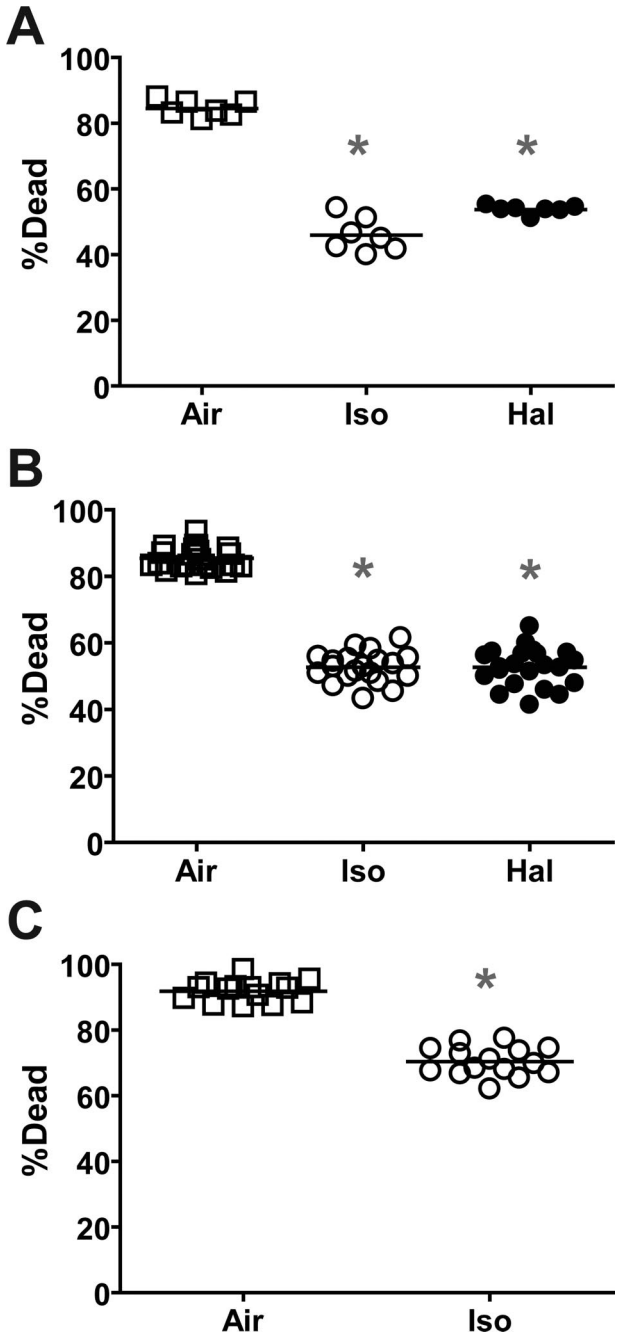

Fig. 1. Volatile anesthetic preconditioning against hypoxia, azide, thermal injury. (*A*) Isoflurane (Iso) and halothane (Hal) preconditioning against hypoxic death. Age-matched adult wild-type *Caenorhabditis elegans* were exposed to 2 vol% isoflurane, 1 vol% halothane, or air control for 4 h at 20°, were allowed to recover for 1 h in air at 20°, and were subsequently challenged for 20 h in a hypoxia chamber (<0.3% O_2) and were then allowed to recover for 24 h in air at 20° before scoring as alive or dead. Each *data point* represents an independent trial with more than 40 animals/trial. The *line* indicates the mean value. * $P < 0.01$ by paired *t* test compared with the corresponding parallel air control. (*B*) Isoflurane and halothane preconditioning against azide death. Otherwise identical to *A* except that the animals were challenged with 0.5 M sodium azide for 70 min. Twenty trials, more than 50 animals/trial. (*C*) Isoflurane preconditioning against thermal death. Otherwise identical to *A* except that animals were treated with isoflurane before being thermally challenged at 37° for 60 min. Fifteen trials, more than 50 animals/trial.

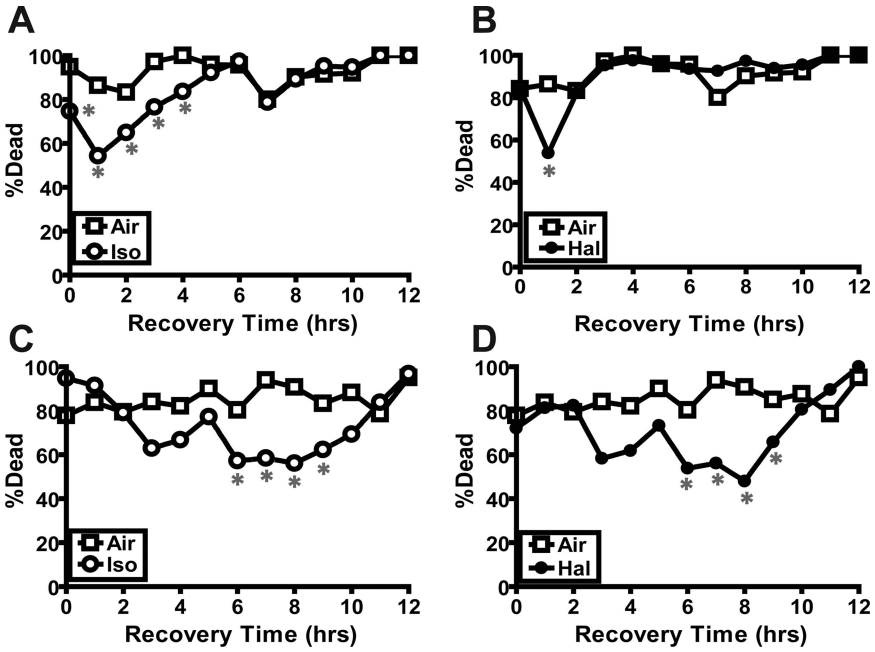

Fig. 2. Time course of preconditioning by volatile anesthetics against hypoxia and azide. (*A*) Isoflurane (Iso) preconditioning time course against hypoxic death. Age-matched adult wild-type *Caenorhabditis elegans* were exposed to 2 vol% isoflurane or air control for 4 h at 20°. Animals were allowed to recover for varying periods in air at 20° before challenge for 20 h in a hypoxia chamber (<0.3% O_2) and were then allowed to recover for 24 h in air at 20° before scoring as alive or dead. * $P < 0.01$ after Bonferroni correction *versus* corresponding air control by Fisher exact chi-square test. Two trials, more than 40 animals/trial for *A–D*. (*B*) Halothane (Hal) preconditioning time course against hypoxic death. Otherwise identical to *A* except the preconditioning exposure was to 1 vol% halothane. (*C*) Isoflurane preconditioning time course against azide death. Otherwise identical to *A* except that animals were challenged with 0.5 M sodium azide for 70 min. (*D*) Halothane preconditioning time course against azide death. Otherwise identical to *B* except that animals were challenged with 0.5 M sodium azide for 70 min.

time for statistically significant protection after anesthetic exposure was 6 h for both halothane and isoflurane, and the protection lasted up to 9 h. This delayed time course is indicative of a preconditioning action of the anesthetics and not a direct protective effect.

We next measured the concentration dependence of isoflurane and halothane preconditioning. The concentration–effect curve for both anesthetics was biphasic (figs. 3A and B). For isoflurane, significant preconditioning was observed after 1 vol% isoflurane (0.64 mM) and was maximal at 2 vol%. However, the protection was lost with higher concentrations. Similarly, significant preconditioning by halothane was observed after exposure to 1 vol% halothane (0.71 mM) with disappearance of the effect at 3 vol%. The biphasic nature of the curve suggests that at higher concentrations, both halothane and isoflurane have a second action that directly or indirectly opposes the preconditioning mechanism. For general anesthesia in mammals and for behavioral defects in *C. elegans*, halothane has an aqueous potency higher than that of isoflurane.[33,41,42] For preconditioning, the relative potencies of the two drugs are unclear because the minimum potencies and EC_{50}s for isoflurane and halothane preconditioning are not well defined, although isoflurane has been shown to precondition at a lower aqueous concentration than the lowest tested for halothane.[43] Notably, for both isoflurane and halothane, the minimally effective aqueous concentrations for preconditioning in *C. elegans* are higher than those required in mammalian cells; mammalian halothane and isoflurane EC_{50}s for preconditioning are similar to those that produce anesthesia (0.1–0.3 mM).[6,43] These concentrations are also higher than required to produce behavioral effects in *C. elegans* but are lower than required to immobilize wild-type animals.[33,34]

An important and unresolved question for anesthetic preconditioning is the relation between the mechanism for preconditioning and the mechanism for anesthesia. In particular, inhibition of neurotransmitter release, a likely contributor to anesthesia produced by

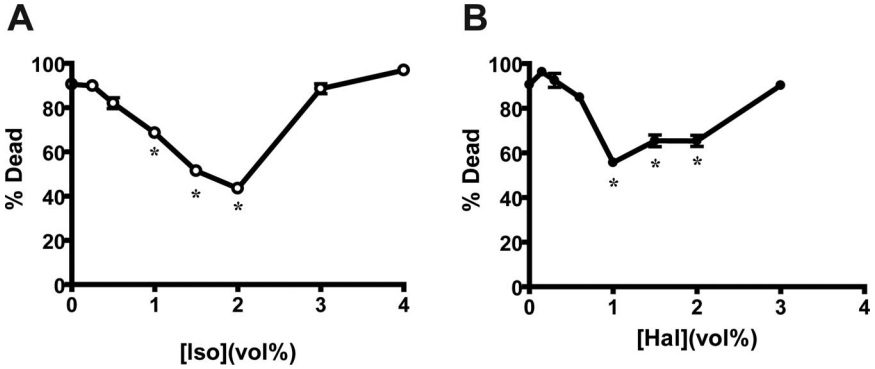

Fig. 3. Concentration dependence of volatile anesthetic preconditioning. (*A*) Isoflurane (Iso) concentration-dependence curve. Age-matched adult wild-type *Caenorhabditis elegans* were exposed to various concentrations of isoflurane for 4 h at 20°, were allowed to recover for 1 h in air at 20°, and were subsequently challenged for 20 h in a hypoxia chamber (<0.3% O2) and were then allowed to recover for 24 h in air at 20° before scoring as alive or dead. The *data points* represent the mean ± SEM of five trials with more than 50 animals/trial. * $P < 0.01$ after Bonferroni correction *versus* 0% by Fisher exact chi-square test for *A–B*. (*B*) Halothane (Hal) concentration-dependence curve. Identical to *A* except with various concentrations of halothane. The *data points* represent the mean ± SEM of five trials with more than 50 animals/trial.

VAs,[44] has also been proposed as a mechanism for VA preconditioning in the nervous system.[6] In *C. elegans*, the behavioral effects of human clinical concentrations of isoflurane can be blocked by a specific mutation (*md130*) in the gene (*unc-64*) encoding the presynaptic protein syntaxin.[31] This syntaxin mutant, *unc-64(md130)*, blocks both the behavioral effects and the inhibition of neurotransmitter release produced by isoflurane acting at clinical concentrations. Therefore, *unc-64(md130)* offers a means to ask whether inhibition of transmitter release is a necessary component of the preconditioning mechanism of isoflurane in *C. elegans*. Testing of the hypoxic sensitivity of *unc-64(md130)* without preconditioning showed that the strain was significantly hypoxia resistant at baseline compared with parallel wild-type controls (figs. 4A and B). We have observed this with some other strains like *unc-64(md130)* that have reduced locomotion and transmitter release (data not shown); therefore, this was not a surprising result. After isoflurane preconditioning, *unc-64(md130)* became significantly more hypoxia resistant compared with baseline, and the relative change in hypoxic sensitivity after preconditioning was similar to that of the wild-type strain N2 (figs. 4B and C). Therefore, we can conclude that in *C. elegans*, the mechanism of isoflurane preconditioning does not require inhibition of neurotransmitter release.

We have recently found that *C. elegans* has a mechanism for hypoxic preconditioning.[28] In testing the role in hypoxic preconditioning of the well-characterized *C. elegans* apoptosis pathway, we found an interesting and surprising result. A functional apoptosis pathway was not required for hypoxic preconditioning, but one component of the pathway was. The Apaf-1 homolog CED-4, which normally serves to activate the caspase CED-3,[17] was found to be essential for hypoxic preconditioning. However, proteins both upstream and downstream of CED-4 in the apoptosis pathway, including the caspase CED-3, were not essential for hypoxic preconditioning. Given these results, we asked here whether anesthetic preconditioning and hypoxic preconditioning in *C. elegans* share a requirement for CED-4 (fig. 5). A loss-of-function *ced-4* mutant was significantly resistant at baseline compared with the wild-type strain (figs. 5A and B). This result agrees with our previous report and indicates that apoptosis *via* CED-4 mediates a portion of the cell death after hypoxia in *C. elegans*.[28] After isoflurane preconditioning, *ced-4* mutant animals actually exhibited a higher proportion of hypoxic death compared with the air controls (figs. 5B and D). *ced-3* mutant animals lacking the CED-3 caspase were hypoxia resistant at baseline like the *ced-4* mutant, but unlike the *ced-4* mutant they exhibited a normal preconditioning response to isoflurane (figs. 5C and D). Therefore, as for hypoxic preconditioning, isoflurane preconditioning requires CED-4 but not CED-3.

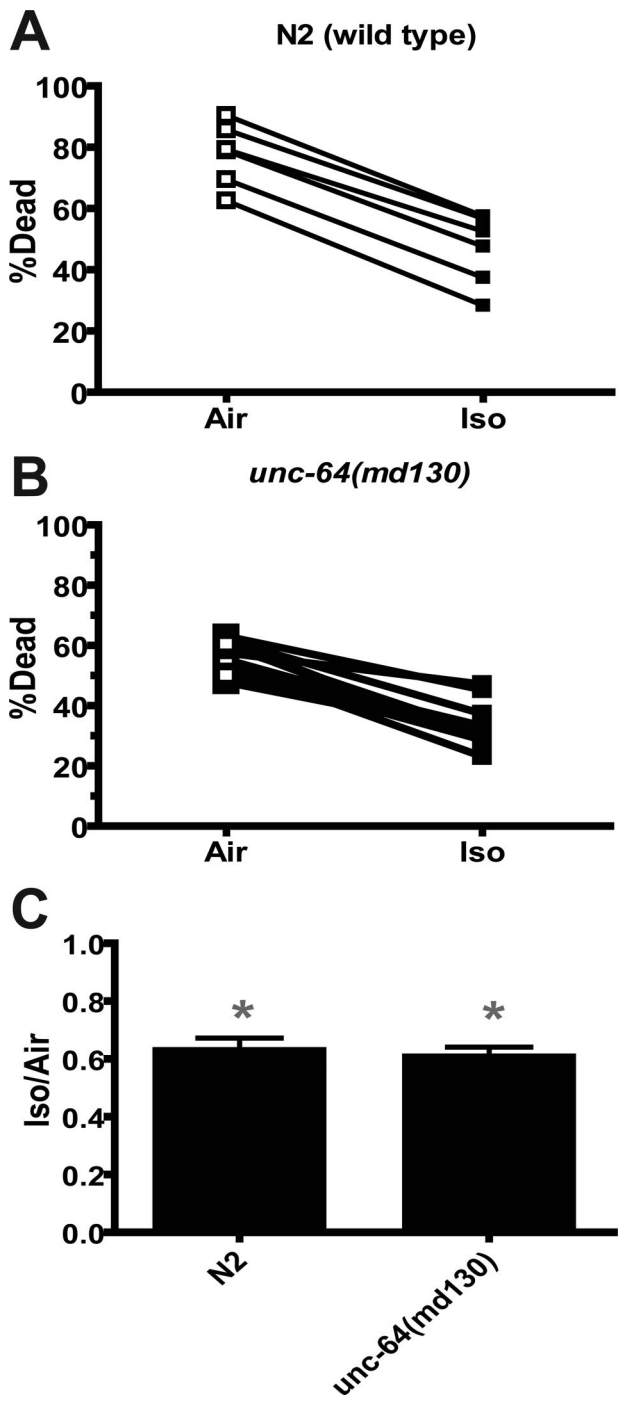

Fig. 4. Inhibition of neurotransmitter release not required for isoflurane preconditioning. (*A*) Wild-type *Caenorhabditis elegans* (N2) were preconditioned by 2% isoflurane or air for 4 h at 20°, recovered for 1 h, and were then challenged with hypoxia for 20 h. The percentages of dead animals were scored 24 h after hypoxia. The results from the six trials (>40 animals/trial) done as wild-type controls for part B are shown. (*B*) *unc-64(md130)* animals tested under identical conditions as in *A*. Seven trials, more than 40 animals/trial. (*C*) Ratio of percentage dead for isoflurane *versus* air (Iso/Air) for each paired trial. * Iso/Air <1, $P < 0.01$, paired *t* test.

Discussion

The data reported here have shown that the nematode *C. elegans* has a mechanism for VA preconditioning. The

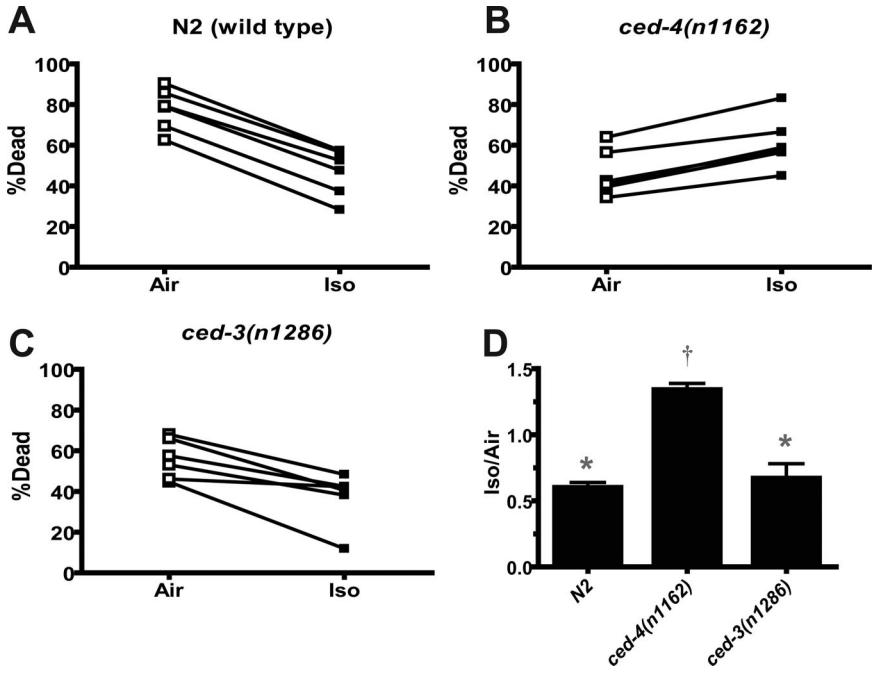

Fig. 5. Requirement for CED-4 but not CED-3 in isoflurane preconditioning. Isoflurane preconditioning protocol identical to that for figure 4 for all strains. (*A*) Wild-type N2 parallel positive control isoflurane preconditioning. (*B*) *ced-4(n1162)* isoflurane preconditioning; *n1162* is a null allele of *ced-4*. (*C*) *ced-3(n1286)* isoflurane preconditioning; *n1286* is severe, likely null allele of *ced-3*. (*D*) Summary bar graph. Ratio of percentage dead for isoflurane *versus* air (Iso/Air) for each paired trial. Six trials, more than 30 animals/trial for each strain. * Iso/Air <1, $P < 0.01$, paired *t* test. † Iso/Air >1, $P < 0.01$, paired *t* test.

time course of the protection after anesthetic exposure is not immediate and meets the definition of preconditioning. The preconditioning effect is concentration dependent, with the minimally effective concentration for isoflurane in the high human clinical range. The preconditioning mechanism and the anesthetic mechanism acting at these concentrations in *C. elegans* are genetically separable; unlike the anesthetic mechanism against *C. elegans* behavior, the anesthetic preconditioning mechanism does not require inhibition of neurotransmitter release. Finally, apoptosis pathway mutants show that anesthetic preconditioning shares with hypoxic preconditioning a requirement for the Apaf-1 homolog CED-4 but that this mechanism is novel, not acting through the downstream caspase.

The discovery that an invertebrate has a mechanism for isoflurane and halothane preconditioning tremendously increases the phylogenetic breadth of anesthetic preconditioning. Previously, only mammals have been reported to have anesthetic preconditioning. This discovery of anesthetic preconditioning in invertebrates suggests that at least a portion of the mechanisms of anesthetic preconditioning are likely to be ancient and conserved throughout most metazoan phyla. Therefore, vertebrate-specific molecules are not essential or fundamental to preconditioning mechanisms. One such protein is the sarcolemmal K_{ATP} channel. A variety of inhibitors of this channel have suggested its role in both hypoxic and anesthetic preconditioning in cardiac muscle.[8] However, *C. elegans* does not have a clear homolog for the sarcolemmal K_{ATP} channel.[45] Although it is possible that homology searches have not identified an authentic *C. elegans* homolog, it is more likely that *C. elegans* does not have such a protein. Of course, one cannot rule out that another potassium channel is regulated by adenosine triphosphate in a manner similar to the mammalian K_{ATP} channel and might serve the same function. One such possibility is the hypothetical mitochondrial K_{ATP}, which has been identified only pharmacologically. The amino acid sequence and gene identity are unknown for the mammalian mitochondrial K_{ATP}; therefore, *C. elegans* might well have one. Of course, these data do not rule out a role of the sarcolemmal K_{ATP} channel in mammalian preconditioning; rather, these findings indicate that this channel is not essential for anesthetic preconditioning in all organisms and cell types.

The window of protection produced by anesthetic preconditioning in *C. elegans* is not typical of either early or late preconditioning. Cytoprotection after early preconditioning has been seen in as little as 15 min after the preconditioning incubation and typically is lost in 4 h.[1,2,46] Protection after late preconditioning is typically seen at approximately 12 h and lasts at least 24 h after the preconditioning incubation. Hypoxic preconditioning in *C. elegans* has a time course corresponding well with late preconditioning as defined in other species.[28] Onset of hypoxic preconditioning in *C. elegans* is approximately 16 h after preconditioning incubation, and the duration is at least 36 h. In contrast, anesthetic preconditioning in *C. elegans* begins approximately 6 h after removal of anesthetic and lasts approximately an additional 3 h. Even if one adds the 4-h anesthetic exposure time to the onset and duration (*i.e.*, 10–13 *vs.* 6–9 h), the duration of the protective effect is less than typical for hypoxic preconditioning. However, this time course is consistent with induction of new transcription and translation, such as that which has been observed

during the delayed window of protection after hypoxic preconditioning.[47–49] If the protection does require new transcription and translation, the newly synthesized protective molecules must be relatively short-lived compared with hypoxic preconditioning. Alternatively, anesthetics might induce a toxic response that offsets the preconditioning protective response at later time points, and indeed there is some precedent for this.[50] The hypothesis of a toxic effect of the anesthetics is supported by the fact that in the *ced-4* mutant, preexposure to isoflurane actually increases the lethality of hypoxia (figs. 5B and D).

An intriguing finding is the requirement for CED-4, the Apaf-1 homolog, in isoflurane preconditioning. These results agree with those from a more extensive set of experiments examining the role of the apoptosis pathway in hypoxic preconditioning in *C. elegans*.[28] Given that CED-4 is required for both isoflurane and hypoxic preconditioning, CED-4 is unlikely to mediate sensing of hypoxia or isoflurane in any direct way. Rather, CED-4 is more likely to be a component of a downstream signal transduction or induced protection mechanism that is common to both anesthetic and hypoxic preconditioning. How might CED-4 transduce or mediate hypoxic and anesthetic preconditioning? Our original hypothesis for hypoxic preconditioning was that hypoxic preconditioning might induce a protective mechanism that acts exclusively or primarily to inhibit apoptotic cell death. If so, mutants that lack essential components of the apoptotic machinery should be both resistant to hypoxia at baseline and incapable of becoming more resistant after hypoxic preconditioning. The data for both hypoxic and anesthetic preconditioning show that although apoptosis is mediating a portion of hypoxic injury in *C. elegans* as evidenced by the baseline hypoxia resistance, the apoptosis pathway in general is not required for preconditioning. Therefore, the original hypothesis is incorrect. Rather within the apoptosis pathway, CED-4 is uniquely required for hypoxic and anesthetic preconditioning. CED-4 has no known function in the absence of the other components of the apoptosis pathway. Therefore, we have difficulty speculating as to its role in preconditioning. One reasonable hypothesis is that CED-4 activity is induced or in some way increased after hypoxic or anesthetic stress and that this increase in activity of an apoptosis protein serves as a panic signal that induces a counterprotective response. Motivated by this hypothesis, future experiments will attempt to identify genes regulated in a CED-4–dependent manner after hypoxic and anesthetic preconditioning. Such genes, if evolutionarily conserved, could lead to a better understanding of the preconditioning mechanism in higher organisms and potentially to therapies for hypoxia-mediated disease states.

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