


Sevoflurane Preconditioning Increases Stress Resistance via IMB-2/DAF-16 in *Caenorhabditis Elegans*

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Yue Cao, MMed¹, Yongchen Cui, MMed¹, Junling Liao, MMed¹, Chente Gao, MMed¹, Zhe Zhao, MD², and Junfeng Zhang, MD¹ 

Abstract

Sevoflurane preconditioning has been proved to possess therapeutic effects on stress. However, the mechanism by which sevoflurane preconditioning protects against stress remains unclear. In this study, an acute model of heat stress in *C.elegans* was established. We investigated the dose-response of sevoflurane exposure on coordinated movement in *C.elegans* and time course for protection against heat stress of sevoflurane preconditioning to determine the optimal concentration and time point in the following experiments. EC99 of sevoflurane is 1.7% (1.3EC50) and sevoflurane preconditioning exerts the maximal protection at 6 hours after incubation, and these 2 parameters were used in our following experiments. We found that sevoflurane preconditioning increased DAF-16 nuclear translocation and enhanced the expression of DAF-16 during heat stress in N2 strain of *C.elegans*. DAF-16 mutation abolished the sevoflurane preconditioning-induced protection for heat stress. Furthermore, suppression of IMB-2 by RNAi prevented the upregulation of DAF-16 and enhancement of stress resistance caused by sevoflurane preconditioning. Overall, this work reveals that sevoflurane preconditioning increases the expression of DAF-16 via IMB-2 to enhance the stress resistance of *C.elegans*.

Keywords

sevoflurane preconditioning, stress resistance, DAF-16, IMB-2, *caenorhabditis elegans*

Introduction

Heat stress occurs when animals or cells are exposed to a temperature higher than normal body temperature. Components of heat stress pathway are also important for human diseases.¹ *Caenorhabditis (C.) elegans* serves as an important model to study stress response for its anatomic and genetic simplicity compared with other animals.² Besides, there are many evolutionarily conserved pathways between *C.elegans* and human.³ Recently, anesthetic preconditioning in clinically relevant doses has been proved to exert protective effects on thermal stress in *C.elegans* and *Saccharomyces cerevisiae* yeast.^{4,5} Volatile anesthetic-induced preconditioning, compared with traditional hormetic stress treatment, is of feasible general practice.⁶ However, the mechanism of anesthetic preconditioning remains unclear.

There are many effective pathways to fight against heat stress, and the most well-studied of which is the IGF/insulin-like signaling (ILS) pathway.¹ In *C.elegans*, the lifespan-

extending effects of ILS are completely dependent on its downstream transcription factor DAF-16, a homolog of human FOXO, which has been shown to be strongly associated with extreme human longevity.⁷ In fact, changes in gene expression

¹Department of Anesthesiology, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, China

²Department of Geriatrics, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, China

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Corresponding Authors:

Junfeng Zhang, Department of Anesthesiology, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, No. 600, Yishan Road, Xuhui District, Shanghai 116044, China.

Email: zhangjunfeng@sjtu.edu.cn

Zhe Zhao, Department of Geriatrics, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai, China.

Email: sevofurane@163.com



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that increase *C.elegans* longevity also increase stress resistance. Recent studies have identified the conserved forkhead transcription factor DAF-16/FOXO as a key player in many stress response pathways.⁸ It has been shown that DAF-16 exerts its function through nuclear translocation and IMB-2, the *C.elegans* homolog of the nuclear import receptor transportin-1 (TNPO1), is required for FOXO4 nuclear translocation upon increased ROS levels. This redox-dependent nuclear transport mechanism is conserved from humans to *C.elegans*.⁹ This is consistent with findings that many foods and drugs promote stress resistance via stress factor DAF-16/FOXO.¹⁰

In this study, we test the hypothesis that sevoflurane preconditioning can protect *C.elegans* from heat stress via IMB-2/DAF-16. The analysis of wild-type *C.elegans* and TJ356 mutant reveals that sevoflurane preconditioning significantly upregulated the expression of DAF-16 of *C.elegans* during heat stress. Moreover, survival analysis reveals that DAF-16 is required in the protection offered by sevoflurane preconditioning against heat stress. Importantly, we find that IMB-2 is required for the enhanced stress resistance caused by sevoflurane preconditioning. Our study indicates that SEVO preconditioning upregulates DAF-16 gene expression via IMB-2 during heat stress, resulting in enhanced heat stress resistance.

Methods

C.elegans Strains

The wild-type *C.elegans* strain N2 (Bristol), transgenic *C.elegans* strain TJ356 DAF-16 (zls356) IV, CF1038 DAF-16 (mu86) I, and *Escherichia coli* OP50 were obtained from the *Caenorhabditis* Genetics Center (CGC) at the University of Minnesota (Minneapolis, MN, USA).

C.elegans strains were maintained and cultured in NGM nematode growth medium using *E. coli* OP50 strain (CGC, University of Minnesota, Minneapolis, MN, USA) as the major food source. The incubation temperature was 20°C. The worms were maintained according to the standard protocol as previously described.¹¹ For RNAi experiments, animals were grown on HT115 bacteria since hatch (see below).

RNA Extraction and qPCR

Total RNA was extracted from some 800 synchronized worms with a 37°C heat stress for 25 min using the EZB RNA kit (EZBioscience, USA) according to the manufacturer's directions. RNA was DNase treated according to a protocol. RNA purity and integrity were evaluated by the ratio of absorbance at 260 nm–280 nm (OD 260/280 ratio), and the ratio of absorbance at 260 nm to 230 nm (OD 260/230 ratio) using a NanoDrop (ND-2000, Thermo Science, USA). 1 µg of RNA was used to synthesize cDNA using an EZBioscience cDNA synthesis kit. Real-time qPCR was performed by using the SYBR Green PCR Master Mix kit (EZBioscience, USA) in an AP Biosystems RT-PCR machine. The thermocycling conditions were as follows:

Initial denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 15 sec, annealing at 55°C for 30 sec and extension at 70°C for 25 sec. Data from 3 biological repeats were analyzed using the comparative $2^{-\Delta\Delta C_t}$ method. The following primer sequences were used for qPCR: CDC-42 (ctgctggacaggaa-gattacg, ctggacattctcgaatgaag)

DAF-16 (tcctcattcactcccgattc, ccgggtgattcatgaacgtg)

Behavioral Assays. The radial dispersal assay was used to measure the uncoordinated movement produced by VAs as described previously.¹² In brief, synchronized 300–500 young adult worms were collected from NGM plates with 1 mL of S-basal. After washed twice with S-basal and a final wash with ddH₂O, 50–100 worms in 10 µl distilled water were placed in the center of 9.5-cm NGM plates where a .5-cm ring of OP50 *E. coli* was seeded in the edge of them 1 day prior to the assay. After the chamber was sealed, liquid sevoflurane was delivered using a Hamilton syringe. The plates were then rapidly put into an air-tight glass chamber without their lids on and the volume of liquid sevoflurane injected was calculated to acquire the desired sevoflurane concentration in the gas phase with the known volume of the glass chamber.¹³ Immediately after the 10 µL sample went dry, the glass chamber was gently shaken to get worms unclamped and dispersed. The assay was then left untouched at room temperature (21 to 24°C) for 45 min. Then, the dispersal index was scored as the number of animals in the bacterial ring was divided by the total number of animals in the plate as previously described.^{12,14} At the end of the assay, the concentration of VAs was measured by gas chromatography and determined by comparison to a known standard.¹⁵ EC50 was calculated by methods shown in statistical analysis.

Preconditioning Treatment

All experiments were performed on adult animals. For sevoflurane preconditioning, four 35 mm uncovered NGM agar plates containing 20 animals were placed on a glass chamber. The plates were then rapidly put into an air-tight glass chamber and exposed to 1.7% (1.3EC50) sevoflurane which was determined in the behavioral assays above. Chambers containing sevoflurane or air was placed in a 20°C incubator for 4 hours. After the concentration of sevoflurane was measured by gas chromatography, worms are put back into the 20°C incubator for some time for recovery before heat stress. For starvation preconditioning, N2 animals were cultured as normal until they reached the young adult stage. Then, they were transferred to new NGM plates with or without OP50 for 4 hours. After a 6-hour recovery period, the worms were exposed to heat stress.

Heat Stress

After sevoflurane or air control preconditioning, NGM plates containing worms were placed in a 37°C incubator for 2 hours which could kill most of the worms. The plates were then put

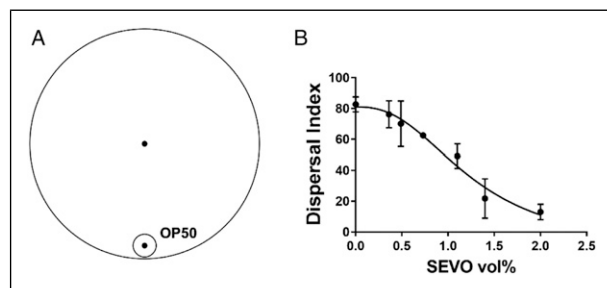


Figure 1. Potency assay of sevoflurane for disrupting coordinated movement in N2 strains. (A) A plate for radial dispersal assay: 50-100 worms in 10 μ l distilled water were aliquoted onto the center of a dispersal assay plate, which is a 9.5-cm NGM plate which was seeded with a thin ring of OP50 *Escherichia coli* at its edge 1 day prior to the assay. (B) Sevoflurane concentration-response curves for the wild-type N2 strains. 50-100 animals per data point were scored for reaching the bacterial ring in the edge of the 9.5-cm agar pad in 45 min. Median effective concentration and standard errors are estimated by logistic regression. The EC₅₀ for N2 animals in sevoflurane is 1.3 vol%.

in a 20°C incubator for recovery. Survival rates were counted 24 hours later.

RNAi Assay

RNAi assay was performed as previously described.¹⁵ In short, a single colony of *E. coli* HT115 (Addgene, USA) from LB-Tet-Amp plates was grown on LB media containing 50 μ g/mL carbenicillin (Shenggong, China) overnight. Bacteria were concentrated 5X before seeding plates. Bacterial culture was seeded on NGM plates containing 1 μ g/mL IPTG (Sigma, USA) and 50 μ g/mL carbenicillin and was allowed to dry overnight at room temperature. Twenty 4-day-old worms were transferred to an NGM plates with no *E. coli* on it. The worms were allowed to crawl freely for 2 minutes to get rid of *E. coli* on their body. Then, the worms were transferred to an RNAi plate where they laid eggs for 2 hours. Remove the worms 2 hours later for whole-life RNAi.

Imaging of Fluorescent Reporter Strains

The bright green fluorescent dots were measured on day 1 of adult TJ356 strain which was incubated on NGM/HT115 plates after treated with or without RNAi bacteria from birth. After 25 minutes of heat stress at 37°C, worms were placed on a 2% agarose pad and paralyzed with levamisole (Yuanye, China). The expression of GFP was detected by fluorescence microscopy. The intensity of fluorescence was analyzed using ImageJ.

Statistical Analysis

GraphPad Prism 7 was used for all other statistical analyses. Concentration/response data were fitted by nonlinear regression to estimate EC₅₀ \pm SE values. Differences between 2

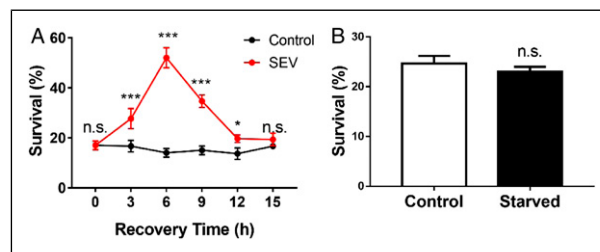


Figure 2. Time course for sevoflurane preconditioning-induced protective response. (A) Time course of sevoflurane preconditioning against heat stress. At least 100 synchronized adult wild-type N2 animals were exposed to 1.3EC₅₀ (1.7%) sevoflurane or air control for 4 hours at 20°C. Worms were allowed to recover for 0, 3, 6, 9, 12, and 15 hours in air at 20°C. The worms were then exposed to a 37°C heat stress for 2 hours. After recovery for 24 hours in air at 20°C, survival was determined. (B) Starvation prior to 48 hours of heat stress has no impact on survival. **P*-value < .05; ****P*-value < .001; n.s. No significance.

groups were assessed using Student's *t*-test. For comparisons involving more than two genotypes or treatments, the ANOVA was used to test the significance of differences. *P*-values < .05 were considered to be significant.

Results

The Potency of Sevoflurane-Induced Coordinated Movement Defects in *C. elegans*

C. elegans has multiple behaviors which can be used as anesthetic endpoints. In this article, the coordinated movement under different levels of sevoflurane was scored by the radial dispersal assay which measures worms' ability to move from starting point at center of the plate to the OP50 ring at the edge (Figure 1A). The EC₅₀ of sevoflurane was 1.3% and the 1.3EC₅₀ was 1.7%, which was used in the following experiments (Figure 1B).

Time Course of Preconditioning by Sevoflurane Against Heat Stress

Sevoflurane preconditioning augments resistance to subsequent heat stress.⁴ The time course for protection of preconditioning was determined by exposing *C. elegans* to heat stress at 0th, 3th, 6th, 9th, 12th, and 15th hour after a 4-hour sevoflurane incubation. Our results showed that the effect of protection after anesthetic exposure began at 3th hour, peaked at 6th hour, and lasted up to 12th hour (Figure 2A). This delayed time course indicated that the protection is the result of preconditioning effect rather than direct anesthetic effect. As a result, we chose 6th hour as our experimental time point for sevoflurane preconditioning recovery for its maximal protective effects. Because sevoflurane could impair the ability of *C. elegans* to intake OP50, we wondered if protection offered by sevoflurane preconditioning was achieved by impeding food intake of *C. elegans*. This was also supported

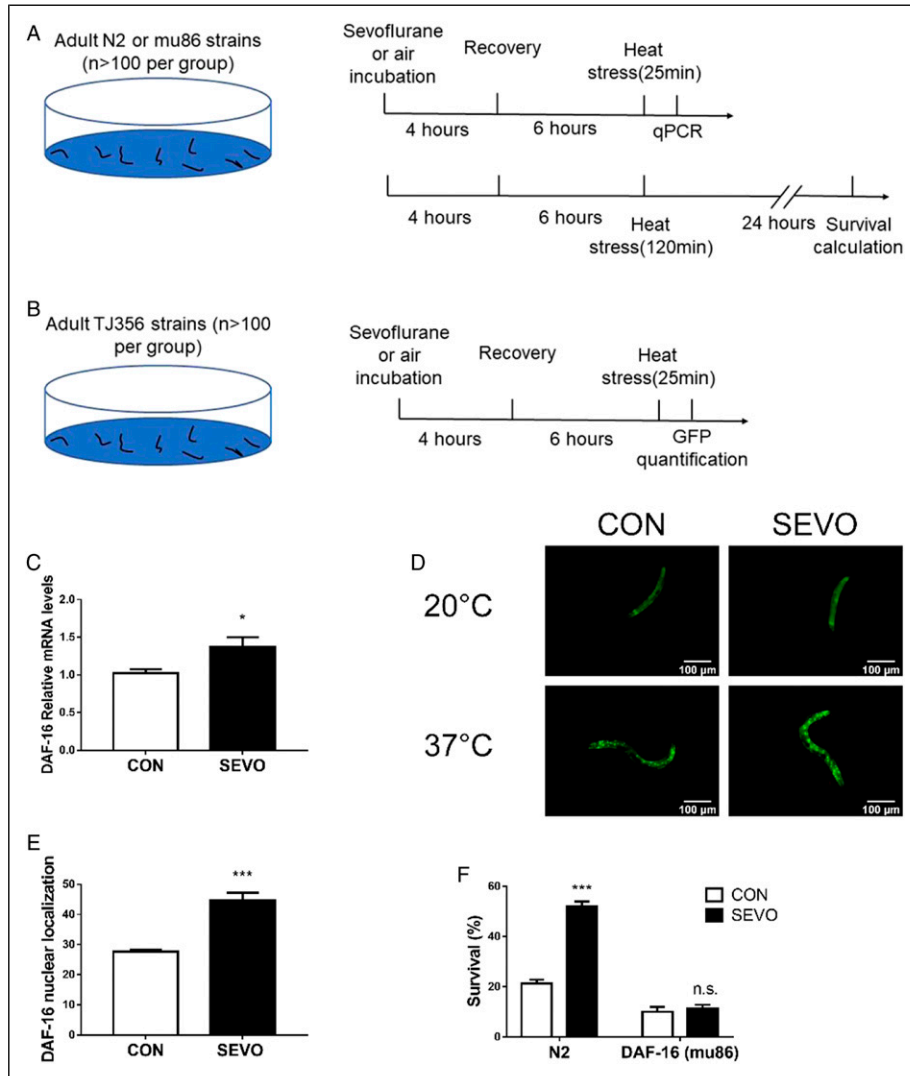


Figure 3. DAF-16/FOXO mediates sevoflurane preconditioning-induced stress resistance. (A) Protocol for qPCR, GFP quantification and survival test. (B) Effects of 4-hour 1.3EC50 (1.7%) sevoflurane preconditioning on the relative mRNA levels of DAF-16 in wild-type animals. (C) Sevoflurane preconditioning increased DAF-16:GFP nuclear localization during heat stress. The bright green fluorescent dots signal the nuclear translocation of DAF-16. (D) The number of spots on worms exposed to heat stress was counted and was represented in the diagram. (E) Sevoflurane preconditioning significantly extended survival of N2 animals after a 37°C heat shock but failed to extend survival of DAF-16 mutant worms regarding heat shock resistance. * P -value<.05; *** P -value <.001; n.s, no significance.

by previous research that dietary restriction can enhance longevity and stress resistance.^{16,17} To explore this issue, we transferred well-synchronized young adult worms into unseeded NGM plates for 4 hours. Then we transferred them into NGM plates seeded with OP50 and kept them for 6 hours before heat stress assay. We found that food restriction for 4 hours did not improve survival from heat stress (Figure 2B).

DAF-16 is Required for Sevoflurane Preconditioning-Induced Stress Resistance

To unravel the correlation between sevoflurane preconditioning and the IGF signaling pathway, we measured the

relative expression levels of DAF-16 in response to heat stress through quantitative Real-time PCR. *C.elegans* was exposed to heat stress following a 6-hour recovery period after a 4-hour sevoflurane or air incubation. (Figure 3A). The expression level of DAF-16 was significantly increasing in N2 animals (Figure 3B). In order to test the impact of sevoflurane preconditioning on the intracellular localization of DAF-16 under stress, we employed the TJ356 strain which stably expresses a DAF-16:GFP fusion protein. Our results showed sevoflurane preconditioning further increased DAF-16:GFP nuclear localization following a 37°C heat shock in Wild-type worms (Figure 3C-D). These results suggest that sevoflurane preconditioning enhances the expression of DAF-16 during heat

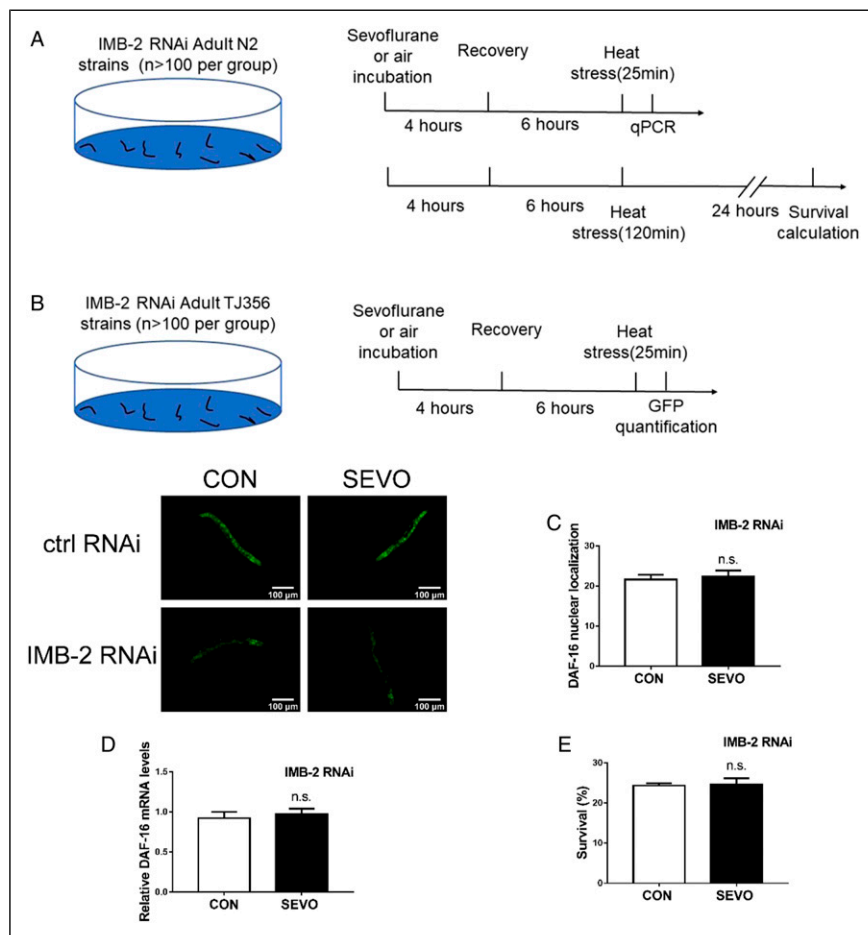


Figure 4. IMB-2 RNAi abolishes increased DAF-16 nuclear translocation and enhanced survival caused by sevoflurane preconditioning. (A) Protocol for qPCR, GFP quantification and survival test of RNAi animals. (B) IMB-2 RNAi prevented sevoflurane preconditioning-induced DAF-16 translocating into the nucleus during heat stress. (C) The number of spots on worms exposed to heat stress was counted and was represented in the diagram. (D) IMB-2 RNAi prevented DAF-16 upregulation of N2 strain against heat stress. (E) IMB-2 RNAi abolished sevoflurane preconditioning-induced protection for N2 strain against heat stress. n.s., no significance.

stress. To establish genetically the role of DAF-16 in sevoflurane preconditioning-induced stress resistance, we examined the effect of sevoflurane preconditioning on the null *C.elegans* DAF-16 (*mu86*) mutant strain. Consistent with a previous study,⁴ we found that sevoflurane preconditioning protects N2 animals from heat stress (Figure 3E). However, DAF-16 mutation compromised sevoflurane preconditioning-induced enhanced stress resistance, indicating that sevoflurane preconditioning is dependent on DAF-16 to enhance stress resistance (Figure 3F). These data suggest that sevoflurane preconditioning induces the nuclear translocation of DAF-16 which mediates enhanced stress resistance against heat stress.

Sevoflurane Preconditioning Upregulates DAF-16 Through IMB-2

Since we have demonstrated that DAF-16 was required for the stress resistance of worms exposed to sevoflurane

preconditioning, we next sought to determine its possible upstream mechanism. IMB-2 is required for DAF-16 nuclear localization,^{9,15} so we wondered whether IMB-2 mediates the sevoflurane preconditioning-induced DAF-16 nuclear localization in *C.elegans* during heat stress. We tested the effect of IMB-2 knockdown on the DAF-16 nuclear localization in the TJ356 strain of *C.elegans* (Figure 4A). We found that the knockdown of IMB-2 substantially decreased the DAF-16 nuclear localization of TJ356 worms under heat stress (Figure 4B-C). Consistent with this, DAF-16 upregulation caused by sevoflurane preconditioning was abolished (Figure 4D). To investigate the role of IMB-2 in sevoflurane preconditioning-induced stress resistance. The heat stress survival assay was performed after sevoflurane preconditioning in the presence or absence of the IMB-2 gene. Knockdown of IMB-2 with RNAi significantly impaired the protective effect of sevoflurane preconditioning compared with control worms (Figure 4E). These results indicate that sevoflurane

preconditioning-induced DAF-16 upregulation during heat stress is mediated by IMB-2.

Discussion

C.elegans serves as an important model to study stress response for its anatomic and genetic simplicity. Like other animals, *C.elegans* has developed sophisticated stress response pathways to stress-inducing stimuli, which signal potential danger in the environment, to maintain cellular homeostasis.¹⁸ The heat shock responses occur when animals or cells are exposed to a temperature higher than normal body temperature. In this study, we found that sevoflurane preconditioning increased heat stress resistance in *C.elegans*. Sevoflurane preconditioning upregulated the expression of DAF-16 in *C.elegans* while DAF-16 mutation abrogated the protection against heat stress offered by sevoflurane preconditioning. Furthermore, IMB-2 RNAi prevented sevoflurane preconditioning-induced DAF-16 upregulation and abolished sevoflurane preconditioning-induced protection for N2 strain against heat stress.

Preconditioning refers to a series of phenomena in which a brief, sub-lethal stimulus confers protection from a subsequent prolonged, lethal stimulus. Volatile anesthetic (VA)-induced preconditioning has been proved to contribute to both cardiac and cerebral protection in I/R injury with equivalent potency of ischemic preconditioning. Anesthetic preconditioning is an important anesthetic property, but whose exact mechanism is now poorly understood. A more potent therapeutic method calls for a better understanding of the mechanism of anesthetic preconditioning.

In this study, we test if anesthetic preconditioning can make a difference to the heat stress resistance of *C.elegans*. We found that anesthetic preconditioning can be applied to stress caused by temperature higher than optimal conditions. This is consistent with the results of previous studies.^{4,5} Of note, this phenomenon is independent of food restriction. Of all the pathways underlying stress resistance, the most notable one is the insulin/IGF-1 signaling pathway whose function is dependent on the *C.elegans* FOXO homolog DAF-16. To figure out the exact mechanism of stress resistance effect conferred by sevoflurane preconditioning, we employed the TJ356 strain which stably expresses a DAF-16:GFP fusion protein. We found that sevoflurane preconditioning increased the translocation of DAF-16 into the nucleus under heat stress, which was consistent with our qPCR results.

Previous research has proved that disulfide-dependent binding of FOXO4 to TNPO1 could potentially lead to FOXO nuclear localization under elevated ROS levels.⁹ And this phenomenon is conserved in *C.elegans*, as DAF-16 and IMB-2 can form a complex in *C.elegans* under heat stress and the complex leads to DAF-16 nuclear translocation.⁹ There is evidence that volatile anesthetics cause the release of small amounts of ROS which is the main effector of anesthetic preconditioning.¹⁹⁻²¹ We suspect that

sevoflurane preconditioning-induced ROS may be the underlying cause of the binding of DAF-16 and IMB-2.

Of note, knockdown of IMB-2 can abolish the nuclear translocation of DAF-16.⁹ In this study, we found that IMB-2 RNAi abolished increased DAF-16 nuclear translocation and enhanced survival caused by sevoflurane preconditioning. Taken together, our data demonstrated that the IMB-2/DAF-16 pathway is involved in the process of sevoflurane preconditioning-induced stress resistance in *C.elegans*.

Volatile anesthetics have been recommended as the main anesthetic procedure during cardiac surgery due to their protective effects.²² Our results show that SEVO preconditioning enhances the expression of DAF-16 and improve worms' ability to cope with heat stress. As DAF-16 plays an important role in complex human diseases, such as cardiovascular disease, cancer, and Type 2 diabetes,²³ our finding suggests SEVO preconditioning can have a therapeutic end beyond its perioperative use.

In conclusion, we prove that sevoflurane preconditioning upregulates the expression of DAF-16 through IMB-2, and therefore enhances the heat stress resistance of *C.elegans*.

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Declaration of Conflicting Interests

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ORCID iD

Junfeng Zhang  <https://orcid.org/0000-0003-0150-6989>

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