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Anesthetic Isoflurane Induces DNA Damage Through Oxidative Stress and p53 Pathway

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Abstract

DNA damage is associated with aging and neurological disorders, including Alzheimer's disease. Isoflurane is a commonly used anesthetic. It remains largely unknown whether isoflurane induces DNA damage. Phosphorylation of the histone protein H2A variant X at Ser139 (γ H2A.X) is a marker of DNA damage. We therefore set out to assess the effects of isoflurane on γ H2A.X level in H4 human neuroglioma cells and in brain tissues of mice. Oxidative stress, caspase-activated DNase (CAD), and the p53 signaling pathway are involved in DNA damage. Thus, we determined the interaction of isoflurane with reactive oxygen species (ROS), CAD, and p53 to illustrate the underlying mechanisms. The cells were treated with 2 % isoflurane for 3 or 6 h. The mice were anesthetized with 1.4 % isoflurane for 2 h. Western blot, immunostaining and live cell fluorescence staining were used in the experiments. We showed that isoflurane increased levels of γ H2A.X, cleaved caspase-3, and nucleus translocation of CAD and decreased levels of inhibitor of CAD (ICAD) and p53. Isoflurane enhanced the nucleus level of γ H2A.X. Moreover, caspase inhibitor Z-VAD and ROS generation inhibitor N-acetyl-L-cysteine (NAC) attenuated the isoflurane-induced increase in γ H2A.X level. However, NAC did not significantly alter the isoflurane-induced reduction in p53 level. Finally, p53 activator (actinomycin D) and inhibitor (pifithrin- α) attenuated and potentiated the isoflurane-induced increase in γ H2A.X level, respectively. These findings suggest that isoflurane might induce DNA damage, as represented by

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Compliance with Ethical Standard The animal protocol was approved by the Massachusetts General Hospital (Boston, Massachusetts) Standing Committee on the Use of Animals in Research and Teaching. All experiments followed the National Institutes of Health guidelines, and efforts were made to minimize the number of animals used.

Conflict of Interest The authors declare that they have no competing interests.

Author Contributions C.N., C.L., and Y.D. performed experiments, generated and analyzed the data, contributed to project design and manuscript writing. Y.Z. and X.G. contributed to the design of experiments and data analysis. Z.X. designed and directed the project, participated in experiments, and wrote the manuscript. All authors read and approved the manuscript.

increased γ H2A.X level, via induction of oxidative stress and inhibition of the repair of DNA damage through the p53 signaling pathway.

Keywords

Anesthesia; DNA damage; ROS; Caspase-3; p53

Introduction

Previous studies have shown that isoflurane, a commonly used inhalation anesthetic, may induce the neurotoxicity associated with Alzheimer's disease (AD) neuropathogenesis, including accumulation of β -amyloid protein and phosphorylation of Tau protein [1–11], and cause neurobehavior deficits [9, 12–19]. However, it remains largely unknown whether isoflurane can also cause DNA damage.

DNA damage is associated with aging [20], as genotoxic drugs and ionizing radiation [21, 22] have been shown to cause DNA damage during aging. DNA damage decreases cellular replication, leading to cellular senescence or cell death, further contributing to the onset of the aging process [23]. Moreover, DNA damage is associated with memory formation [24], neurodegeneration and brain tumors [25], AD [26], and ischemic stroke [27].

DNA damage induces DNA double-strand breaks (DSBs) [28], which can trigger protein kinase ataxia telangiectasia mutated (ATM) activation. ATM marks chromatin break sites through phosphorylation of the histone protein H2A variant X at Ser139 (γ H2A.X) at the site of DNA damage within minutes following the DNA damage [29]. Thus, elevation of the γ H2A.X level could be used as a marker for DNA damage [30–32]. We therefore assessed the effects of anesthetic isoflurane on γ H2A.X level in H4 human neuroglioma cells and brain tissues of mice to determine whether isoflurane was able to induce DNA damage.

Reactive oxygen species (ROS) generation [33] has been shown to contribute to DNA damage. In addition, p53 promotes proper repair of DNA damage [34]. We therefore also determined the potential underlying mechanisms of the isoflurane-induced DNA damage by investigating the interaction of isoflurane, ROS, and p53 on γ H2A.X level. The hypothesis was that isoflurane is able to induce DNA damage through cellular mechanisms associated with ROS and p53 signaling pathways. Finally, caspase-activated DNase (CAD) binds to its inhibitor (ICAD) to form an inactive ICAD-CAD complex in the cytoplasm [35]. Cleaved caspase-3, resulted from caspase-3 activation, can cleave ICAD into 24- and 12-kDa fragments and therefore release CAD from ICAD inhibition. The released CAD can translocate to the nucleus and cleave genomic DNA [36], leading to DNA damage [37]. We therefore assessed the effects of isoflurane on the levels of ICAD and CAD and on nucleus level of CAD in the present studies.

Materials and Methods

Cell Line

H4 human neuroglioma cells (H4 cells) and H4 cells stably transfected to express full-length human amyloid precursor protein (H4-APP cells) were used in the studies. We chose this particular cell line (H4-APP cells) because we had already shown the isoflurane-induced neurotoxicity in the H4-APP cells [4, 6, 18, 38]. The cells were cultured in DMEM (high glucose), containing 9 % heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 ug/ml streptomycin, and 2 mM L-glutamine, and were supplemented with 220 µg/ml G418.

Cell Treatment

Isoflurane was delivered from an anesthesia machine to a sealed plastic box in a 37 °C incubator containing 6-well plates or 96-well plates; the 6-well plates were seeded with one million cells in 1.5 ml cell culture media per well, and the 96-well plates were seeded with fifty thousand cells in 200 µl cell culture media per well, as described in our previous studies [18]. A Datex infrared gas analyzer (Puritan-Bennett, Tewksbury, MA) was used to continuously monitor the delivered concentrations of carbon dioxide, oxygen, and isoflurane. The cells were treated with 2 % isoflurane, plus 21 % O₂ and 5 % CO₂, for a duration of 6 h as described by Xie et al. [4] and Zhang et al. [39] in the studies. Fifty µM Z-VAD-FMK (Z-VAD, Abcam Plc, Cambridge, UK), 1 mM N-acetyl-L-cysteine (NAC, Sigma-Aldrich Corporation, St. Louis, MO), 100 ng/ml actinomycin D [p53 activator [40], Santa Cruz Biotechnology, Santa Cruz, CA], or 10 ng/ml pifithrin-α [p53 inhibitor [41], Sigma-Aldrich Corporation, St. Louis, MO] was given to the cells 60 min before the isoflurane treatment.

Mouse Anesthesia

The animal protocol was approved by the Massachusetts General Hospital (Boston, Massachusetts) Standing Committee on the Use of Animals in Research and Teaching. All experiments followed the National Institutes of Health guidelines, and efforts were made to minimize the number of animals used. Eighteen-month-old mice (National Institute of Aging, Bethesda, MD) were used in the studies. The mice were randomly assigned to an anesthesia or control group. Mice randomized to the anesthesia group received 1.4 % isoflurane in 100 % oxygen for 2 h in an anesthetizing chamber, whereas the control group received 100 % oxygen at an identical flow rate for 2 h in an identical chamber. The mice breathed spontaneously, and anesthetic and oxygen concentrations were measured continuously (Datex, Tewksbury, MA). Temperature of the anesthetizing chamber was controlled to maintain rectal temperature of the animals at 37 ± 0.5 °C. This isoflurane anesthesia has been shown not to significantly alter values of blood pressure and blood gas [8]. Anesthesia was terminated by discontinuing isoflurane and placing animals in a chamber containing 100 % oxygen until 20 min after return of righting reflex. The animals were then returned to individual home cages until sacrifice. Mice were sacrificed by decapitation at the end of the experiments. The brain tissues were removed rapidly, and the prefrontal cortex was dissected out and frozen in liquid nitrogen for subsequent processing for determinations of γH2A.X level.

Lysis and Protein Quantification for Brain Tissue and Cells

The harvested brain tissues and cells were detergent-extracted on ice using an immunoprecipitation buffer plus protease inhibitors, as described in our previous studies [42]. The lysates were collected, centrifuged at 13,000 rpm for 15 min, and quantified for total protein amount by a bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

Western Blot Analyses

Western blot analyses were performed as described in our previous studies [18, 38, 42]. Specifically, γ H2A.X antibody (1:1000 dilution; Cell Signaling Technology, Danvers, MA) was used to detect γ H2A.X (15 kDa), caspase-3 antibody (1:1000 dilution; Cell Signaling Technology, Danvers, MA) was used to recognize full-length caspase-3 (35 kDa) and cleaved caspase-3 fragments (17 kDa), ICAD and CAD antibodies (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) were used to detect ICAD and CAD (45 and 40 kDa), p53 antibody (1:1000 dilution; Santa Cruz Biotechnology) was used to detect p53 (53 kDa), and β -actin antibody (1:10,000, Sigma, St. Louis, MO) was used to detect β -actin (42 kDa). Each band in the Western blot represented an independent experiment. The results were averaged from six independent experiments for the in vitro studies and were averaged from three independent experiments for the in vivo mice studies. The intensity of signals was analyzed using the National Institute of Health image program. We quantified the Western blots in two steps. First, we used β -actin levels to normalize protein levels (e.g., determining the ratio of caspase-3 fragment to β -actin amount) and to control for loading differences in the total protein amount. Second, we presented the changes in protein level in the anesthesia group as a percentage of those in the control group. For the purpose of comparison to the experimental conditions, 100 % of the protein level changes refer to the control levels.

Immunofluorescence

At the end of the treatment, cells were washed with PBS buffer and subsequently fixed in -20°C methanol for 20 min. Then, cells were incubated in 0.2 % Triton X-100 for 10 min and in 1 % BSA for 1 h to block nonspecific binding sites. After washing with PBS buffer, cells were stained using γ H2A.X antibody (1:500 dilution; Cell Signaling Technology) or CAD antibody (1:100 dilution; Santa Cruz Biotechnology) at 4°C overnight with gentle rocking and then probed with FITC conjugated secondary antibody (1:500 dilution; Thermo Fisher Scientific, Waltham, MA) at room temperature for 1 h with gentle rocking. The cells were stained with Hoechst 33342 (1:1000 dilution; Sigma-Aldrich Corporation, St. Louis, MO) at room temperature for 10 min. Finally, the fluorescent images were taken in mounting medium using a Nikon Eclipse Ti confocal microscope with $\times 40$ objective lens.

ROS Measurement

An OxiSelect™ Intracellular ROS Assay Kit (Cell Biolabs, San Diego, CA) was used to measure the amount of ROS in cells, according to the protocol provided by the company and our previous studies [18, 38, 42]. Briefly, the cultured H4-APP cells were placed in a clear 96-well cell culture plate in the incubator overnight. We then added the 2',7'-dichlorofluorescein-diacetate (DCFH-DA) media solution to the cells. The DCFH-DA loaded H4-APP cells were subsequently exposed to 2 % isoflurane for 6 h. These treated

cells were first lysed by adding 100 μ l of cell lysis buffer and then mixed thoroughly and incubated for 5 min at room temperature. One hundred fifty microliters of the mixture was transferred to each well of a 96-well plate to be used for fluorescence measurement. Finally, the fluorescence was read with a fluorometric plate reader at 480/530 nm.

Statistical Analyses

Data were expressed as means $\pm$ standard deviation (SD). The number of samples was six per group for the in vitro studies, and the power calculation was performed using information collected from a preliminary study that was conducted under the same conditions. Based on the preliminary data, assuming a two-sided Student's *t* test, samples of six for each control and treatment group would lead to 90 % power and 95 % significance. There were three mice in the anesthesia group and three mice in the control group. The generated data were normally distributed. A two-way ANOVA was used to assess the interaction of Z-VAD, NAC, actinomycin D, or pifithrin- α with isoflurane and to test the hypothesis that isoflurane was able to induce DNA damage through cellular mechanisms associated with ROS, caspase-3, and p53 signaling pathways. Post hoc analyses were conducted if the main effects were found to be statistically significant. The cut-off *P* value was Bonferroni adjusted to correct for sub-set analysis, e.g., comparing the level of γ H2A.X between Z-VAD, NAC, actinomycin D, or pifithrin- α and saline treatments. The nature of the hypothesis testing was two-tailed. *P* values less than 0.05 were considered statistically significant. Prism 6 software (GraphPad software, La Jolla, CA) was used to analyze the data.

Results

Anesthetic Isoflurane Increased γ H2A.X Level in H4-APP Cells and in Brain Tissues of Mice

The objective of the current study was to determine the effects of anesthetic isoflurane on DNA damage and to illustrate the underlying mechanisms. Given the fact that the increase in γ H2A.X level represents DNA damage, we first assessed the effect of isoflurane on γ H2A.X level in H4-APP cells. Immunoblotting of γ H2A.X showed that treatment with 2 % isoflurane for 6 h (lanes 4 to 6) induced visible increases in the density of bands representing γ H2A.X in the Western blot images as compared to the control condition (lanes 1 to 3) (Fig. 1a). There was no significant difference in β -actin level between the isoflurane treatment and control condition (Fig. 1a). Quantification of the Western blot (Fig. 1b), based on the ratio of γ H2A.X to β -actin, showed that the isoflurane treatment (black bar) increased the γ H2A.X level as compared to the control condition (white bar): 291 versus 100 %, *P* < 0.0001. Immunostaining of γ H2A.X and Hoechst showed that the isoflurane treatment (bottom row) increased γ H2A.X level in the nucleus (column 1: γ H2A.X; column 2: Hoechst; column 3: merge of γ H2A.X and Hoechst; column 4: enlarged merge of γ H2A.X and Hoechst) as compared to the control condition (upper row) (Fig. 1c). Next, we found that treatment with 2 % isoflurane for 3 h (lanes 4 to 6 in Fig. 1d; black bar in Fig. 1e) did not increase the γ H2A.X level as compared to the control condition (lanes 1 to 3 in Fig. 1d; white bar in Fig. 1e) in H4-APP cells (*P* = 0.7525, Fig. 1e). Taken together, these data suggested that anesthetic isoflurane increased γ H2A.X level in a time-dependent manner.

Finally, we determined the in vivo relevance of the in vitro findings in mice. Anesthesia with 1.4 % isoflurane for 2 h (lanes 4 to 6) increased the γ H2A.X level as compared to the control condition (lanes 1 to 3) in the brain tissues of mice (Fig. 1f). There was no significant difference in β -actin level between isoflurane anesthesia and the control condition (Fig. 1f). Quantification of the Western blot, based on the ratio of γ H2A.X to β -actin, showed that isoflurane (black bar) was able to increase the γ H2A.X level as compared to the control condition (white bar): 239 versus 100 %, $P = 0.0005$ (Fig. 1g).

Given the finding that isoflurane increased γ H2A.X level in a time-dependent manner, we next asked whether isoflurane was able to induce caspase-3 activation in a time-dependent manner. Immunoblotting of caspase-3 showed that treatment with 2 % isoflurane for 6 h (lanes 5 to 8) increased the levels of bands representing cleaved caspase-3 as compared to the control condition (lanes 1 to 4) (Fig. 2a). There was no significant difference in β -actin level between the isoflurane treatment and control condition (Fig. 2a). Quantification of the Western blot, based on the ratio of caspase-3 fragment to full-length caspase-3, showed that the isoflurane treatment (black bar) induced caspase-3 activation as compared to the control condition (white bar): 240 versus 100 %, $P < 0.0001$ (Fig. 2b). Treatment with 2 % isoflurane for 3 h, however, did not induce caspase-3 activation (Fig. 2c, d). These data suggested that anesthetic isoflurane was able to induce caspase-3 activation in a time-dependent manner as well.

Z-VAD Attenuated the Isoflurane-Induced Increase in γ H2A.X Level in H4-APP Cells

Given the findings that isoflurane was able to induce caspase-3 activation and to increase γ H2A.X level, next, we assessed whether caspase activation inhibitor Z-VAD could attenuate the isoflurane-induced increase in the γ H2A.X level. First, we showed that Z-VAD was able to attenuate the isoflurane-induced caspase-3 activation in the cells (Fig. 3a, b). Then, we found that Z-VAD alone (lanes 3 and 4) did not significantly affect the γ H2A.X level as compared to the control condition (lanes 1 and 2), but Z-VAD (lanes 7 and 8) attenuated the isoflurane-induced increase in H2A.X level (lanes 5 and 6) (Fig. 3c). Quantification of the Western blot showed that there was a significant interaction between Z-VAD and isoflurane ($F = 9.972$, $P = 0.005$) (Fig. 3d), and Z-VAD attenuated the isoflurane-induced increase in H2A.X level: 92 versus 168 %, $P < 0.0001$ (Fig. 3d).

Isoflurane-Induced CAD Activation in H4-APP Cells

ICAD prevents translocation of CAD from the cytoplasm into the nucleus. We thus assessed the effects of isoflurane on the levels of both ICAD and CAD, as well as the nucleus level of CAD. Immunoblotting of ICAD showed that treatment with 2 % isoflurane for 6 h (lanes 4 to 6) decreased ICAD levels as compared to the control condition (lanes 1 to 3) (Fig. 4a). There was no significant difference in the β -actin levels between the isoflurane treatment and the control condition. Quantification of the Western blot showed that the isoflurane treatment (black bar) reduced the ICAD levels as compared to the control condition (white bar): 54 versus 100 %, $P = 0.0001$ (Fig. 4b). Treatment with 2 % isoflurane for 6 h did not significantly change the CAD levels as compared to the control condition (Fig. 4c, d). However, immunostaining of CAD and Hoechst showed that the isoflurane treatment (bottom row) increased level of CAD in the nucleus (column 1: CAD; column 2: Hoechst;

column 3: merge of CAD and Hoechst; column 4: enlarged merge of CAD and Hoechst) as compared to the control condition (upper row) (Fig. 4e). These data suggested that isoflurane might reduce ICAD levels to release CAD, which then translocated to the nucleus to cleave genomic DNA [36], leading to DNA damage [37].

NAC Attenuated the Isoflurane-Induced Increase in γ H2A.X Level in H4-APP Cells

NAC is an inhibitor of ROS accumulation [43, 44]. We thus determined whether NAC could inhibit the isoflurane-induced caspase-3 activation and increase in the γ H2A.X level. Treatment with 2 % isoflurane for 6 h was able to increase ROS levels as compared to the control condition in the present studies: 139 versus 100 %, $P = 0.0006$ (Fig. 5a). Then, we found that NAC inhibited the isoflurane-induced ROS accumulation ($F = 9.728$, $P = 0.0021$) (Fig. 5b) and caspase-3 activation (Fig. 5c, d, $F = 20.100$, $P = 0.0002$). γ H2A.X immunoblotting showed that NAC alone (lanes 3 and 4) did not significantly alter γ H2A.X level as compared to the control condition (lanes 1 and 2), but the NAC treatment (lanes 7 and 8) attenuated the isoflurane-induced increase in the γ H2A.X level (lanes 5 and 6) (Fig. 5e). Quantification of the Western blot showed that there was a significant interaction of NAC and isoflurane on γ H2A.X level ($F = 8.838$, $P = 0.0075$), and NAC reduced the isoflurane-induced increase in the γ H2A.X level: 110 versus 198 %, $P < 0.0001$ (Fig. 5e). These data suggested that the isoflurane-induced increase in γ H2A.X level could be dependent on ROS accumulation.

NAC Did Not Affect the Isoflurane-Induced Increase in p53 Level in H4-APP Cells

A major cellular stress sensor known as p53 contributes to the repair of DNA damage [45]. We therefore determined the effects of isoflurane on p53 level. Immunoblotting of p53 showed that treatment with 2 % isoflurane for 6 h (lanes 4 to 6) decreased p53 level as compared to the control condition (lanes 1 to 3) (Fig. 6a). Quantification of the Western blot showed that the isoflurane treatment (black bar) decreased p53 level as compared to the control condition (white bar): 45 versus 100 %, (Fig. 6b, $P = 0.0005$). Immunoblotting of p53 showed that NAC treatment alone (lanes 3 and 4) decreased p53 level as compared to the control condition (lanes 1 and 2), but NAC treatment (lanes 7 and 8) did not significantly affect the isoflurane-induced reduction in p53 level (lanes 5 and 6) (Fig. 6c). Quantification of the Western blot showed significant interaction of isoflurane and NAC with p53 level ($F = 11.020$, $P = 0.0034$) (Fig. 6d). However, NAC did not affect the isoflurane-induced reduction in p53 level: 58 versus 46 %, $P = 0.0645$ (Fig. 6d). These data suggested that isoflurane decreased p53 level, which would be independent of ROS accumulation.

p53 Activator Attenuated, but p53 Inhibitor Potentiated, the Isoflurane-Induced Increase in γ H2A.X Level in H4-APP Cells

Given the finding that isoflurane was able to reduce p53 level, next, we asked whether there were interactions between isoflurane and p53 activator or inhibitor on γ H2A.X level. Immunoblotting of γ H2A.X showed that whereas treatment with p53 activator actinomycin D alone (lanes 3 and 4) did not significantly alter γ H2A.X level as compared to the control condition (lanes 1 and 2), the actinomycin D treatment (lanes 7 and 8) attenuated the isoflurane-induced increase in γ H2A.X level (lanes 5 and 6) (Fig. 7a). Quantification of the Western blot showed that there was interaction of isoflurane and actinomycin D on γ H2A.X

level ($F=7.128$, $P=0.0147$), and actinomycin D attenuated the isoflurane-induced increase in γ H2A.X level: 147 versus 201 %, $P=0.0207$ (Fig. 7b). However, treatment with p53 inhibitor pifithrin- α potentiated the isoflurane-induced increase in γ H2A.X level: lanes 7 and 8 versus lanes 5 and 6 (Fig. 7c). Quantification of the Western blot showed that there was interaction of isoflurane and pifithrin- α on γ H2A.X level ($F=25.250$, $P<0.0001$), and pifithrin- α potentiated the isoflurane-induced increase in γ H2A.X level: 933 versus 460 %, $P<0.0001$, (Fig. 7d). These results suggested that p53 signaling could be involved in the isoflurane-induced increase in γ H2A.X level, and activation or inhibition of p53 might lead to attenuation or potentiation of the isoflurane-induced increase in γ H2A.X level, respectively.

Discussion

DNA double-strand break (DSB), representing DNA damage, is a serious lesion that can cause genomic instability [46, 47], which triggers DNA repair [48]. Histone protein H2AX is a key component of DNA repair; H2AX can be phosphorylated on a serine (Ser139) four residues from the carboxyl-terminus to form γ H2A.X at the sites of nascent DNA double-strand break [49]. Thus, the elevation of γ H2A.X suggests DNA damage. Note that presence of γ H2A.X could also be involved in mitosis and meiosis, neural development, and neurogenesis in the adult brain [50–52]. The formation of γ H2A.X is associated with cell cycle suspension in proliferating cells [29], as well as NMDA/AMPA receptor stimulation in differentiated neuronal cells [53]. Finally, the presence of γ H2A.X predicts neuronal endangerment and death following a pathological state in the adult brain [54]. In the present studies, we detected γ H2A.X level to study whether anesthetic isoflurane could induce DNA damage as demonstrated by increase in γ H2A.X level. We showed that treatment with a clinically relevant concentration of isoflurane (2 %) increased γ H2A.X level following six, but not three, hours of treatment (Fig. 1). Moreover, the finding that isoflurane was able to increase γ H2A.X level in the nucleus further suggested that isoflurane could induce DNA damage (Fig. 1c). Taken together, these data suggested that anesthetic isoflurane might induce DNA damage in a time-dependent manner. Note that isoflurane also increased γ H2A.X level in H4 naïve cells (data not shown), which suggests that the isoflurane-induced increase in γ H2A.X level was not dependent on the overexpression of APP. In addition, we demonstrated in vivo relevance, as isoflurane increased γ H2A.X level in brain tissues of mice (Fig. 1f, g). Isoflurane induced a time-dependent caspase-3 activation in the same system (Fig. 2), and caspase inhibitor Z-VAD attenuated both the isoflurane-induced caspase-3 activation and the isoflurane-induced increase in γ H2A.X level (Fig. 3). Collectively, these results suggested that the isoflurane-induced increase in γ H2A.X level could be dependent on the isoflurane-induced caspase-3 activation.

Caspase-activated deoxyribonuclease (CAD) and its inhibitor (ICAD) bind to each other and exist in the cytosol [35, 36]. It has been reported that cleaved caspase-3 can cleave ICAD and therefore release CAD from inhibition by ICAD [36]. Finally, CAD translocates to the nucleus and cleaves genomic DNA [36], causing DNA damage [37]. Isoflurane induced caspase-3 activation (Fig. 2), increased γ H2A.X level, and caused DNA damage (Figs. 1 and 3). Isoflurane also reduced the level of ICAD (but not CAD) and increased the level of CAD in the nucleus (Fig. 4). Collectively, these findings suggested that isoflurane was able to

induce caspase-3 activation, which cleaved ICAD and released CAD. CAD then entered the nucleus to induce DNA damage, as represented by the increase in γ H2A.X level (Fig. 8).

Furthermore, we found that isoflurane increased ROS level (Fig. 5), and ROS generation inhibitor NAC was able to inhibit the isoflurane-induced caspase-3 activation and isoflurane-induced increase in γ H2A.X level (Fig. 5). These findings suggested that ROS could contribute, at least partially, to the isoflurane-induced DNA damage, and oxidative stress could be one of the up-stream mechanisms of the isoflurane-induced DNA damage (Fig. 8). This conclusion was also supported by the findings obtained from other studies that ROS accumulation could lead to DNA damage [55, 56].

DNA damage can trigger activation of p53 [[57, 58], reviewed in [59, 60]]. Activated p53, in response to DNA damage, then binds to DNA and mediates transcriptional activation, leading to the activation of a number of genes which promote DNA repair [[45, 61, 62], reviewed in [63]]. We found that isoflurane was able to reduce p53 level (Fig. 6a, b). Moreover, the isoflurane-induced reduction in p53 level was not affected by NAC. These data suggested that the isoflurane-induced reduction in p53 levels was independent of ROS accumulation. Finally, the isoflurane-induced increase in the γ H2A.X level was attenuated by p53 activator actinomycin D and was potentiated by the p53 inhibitor pifithrin- α (Fig. 7). These data suggested that p53 signaling would, independent of oxidative stress, contribute to the underlying mechanisms by which isoflurane induced DNA damage, and isoflurane might reduce p53 level, leading to inhibition of the DNA repair.

Collectively, the hypothesized pathway of the isoflurane-induced DNA damage is that isoflurane induces oxidative stress to activate caspase-3, which then triggers CAD activation, leading to DNA damage. On the other hand, isoflurane reduces p53 level, which then mitigates the repair of DNA damage, leading to further DNA damage (Fig. 8).

Brozovic et al. reported that isoflurane induced DNA damage in peripheral blood leucocytes and kidney cells of Swiss albino mice by using alkaline comet assay [64]. However, other studies did not investigate the underlying mechanisms. Clinical studies showed that isoflurane did not induce DNA damage in lymphocytes of people working in the operating room [65]. The negative findings could be due to the very low concentration of isoflurane to which the people were exposed. Employing comet assay and micronucleus test, Leffa et al. demonstrated that anesthetic ketamine with and without xylazine could induce DNA damage in blood and brain cells of mice [66]. However, the dose of ketamine used in the experiment was not clinically relevant (140 mg/kg) [66]. In the current studies, we used a clinically relevant concentration of isoflurane (2 %) and found that the treatment with a clinically relevant concentration of isoflurane was able to induce DNA damage. Moreover, we elucidated the underlying mechanisms associated with oxidative stress and the p53 signaling pathway.

DNA damage has been reported to be associated with Alzheimer's disease neuropathogenesis. Early studies have reported that β -amyloid protein (A β) 42 can cause DNA damage, which would be an early event to lead to neuronal death, contributing to AD neuropathogenesis [67]. Recently, many studies have linked the DNA damage and

neurodegenerative disease, including AD [reviewed in [68]]. Collectively, our findings that isoflurane induced DNA damage would further suggest the potential association between anesthesia and AD neuropathogenesis. Further research to investigate the anesthesia neurotoxicity associated with AD neuropathogenesis would lead to safer anesthesia and better postoperative outcomes for AD patients who need surgery.

DNA damage has also been reported to be associated with aging, especially stem cell and tissue aging [59]. Many studies have reported accumulation of DNA damage in tissues, stem cells, and compartments in an age-dependent manner [69–75]. There is an association between DNA damage and the development of aging-associated diseases, which include neurodegenerative diseases, bone marrow failure, cardiac disease, hypertension, and type 2 diabetes [74]. DNA damage is also associated with disease progression and organ failure, including cirrhosis and ulcerative colitis [76, 77]. The current finding that isoflurane induced DNA damage would promote more studies to determine whether anesthesia can stimulate the aging process. Given the fact that there are more than 234 million major surgeries under anesthesia every year in the world [78], such studies would be important and urgent.

The current studies have several limitations. First, we only used one method (increase in γ H2A.X level) to assess DNA damage. However, the purpose of the current studies was to demonstrate that isoflurane could increase γ H2A.X level and moreover to elucidate the underlying mechanism (oxidative stress and p53 signaling) by which isoflurane increased γ H2A.X level. The findings obtained from the current studies would lead to future research to assess the effects of anesthesia on other markers of DNA damage (e.g., using comet assay). Second, we only performed both in vitro and in vivo experiments in the current studies to show that isoflurane was able to increase γ H2A.X level in cultured cells and in brain tissues of mice, and we will determine the in vivo relevance of other in vitro findings (e.g., p53, ICAD, and CAD levels) in animals and even in humans in the further studies. Third, we did not identify the cell type(s) in mouse brain tissues that demonstrated DNA damage following the isoflurane anesthesia. However, the main objectives of the current studies were to show that anesthetic isoflurane was able to induce DNA damage in vitro and in vivo and to elucidate the underlying mechanisms. We will use the established system to investigate whether anesthesia could induce the DNA damage in specific cell types (e.g., neuron versus microglia) in the future.

In conclusion, we found that treatment with a clinically relevant concentration of anesthetic isoflurane was able to induce DNA damage as evidenced by the increase in γ H2A.X level in H4-APP cells. Moreover, we demonstrated the following potential underlying mechanism: (1) isoflurane-induced oxidative stress, which caused caspase-3 activation and consequently CAD activation, leading to induction of DNA damage; (2) isoflurane inhibited the repair of DNA damage by reducing p53 levels. Given the facts that DNA damage is associated with aging and neurological disorders, such as AD, and that more than 234 million people receive anesthesia each year in the world, these findings are important and would promote further studies to determine the potential adverse effects of anesthesia, especially the neurotoxicity associated with AD neuropathogenesis.

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Abbreviation

AD	Alzheimer's disease
Aβ	β -amyloid protein
DSBs	DNA double-strand breaks
ATM	Ataxia telangiectasia mutated
γH2A.X	Phosphorylation of histone protein H2A variant X at Ser139
ROS	Reactive oxygen species
CAD	Caspase-activated DNase
ICAD	Inhibitor of caspase-activated DNase
NAC	N-acetyl-L-cysteine
DCFH-DA	2',7'-Dichlorofluorescein-diacetate

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Fig. 1.

Anesthetic isoflurane increases γ H2A.X level in H4-APP cells in a time-dependent manner.

a Treatment with 2 % isoflurane for 6 h (*lanes 4 to 6*) increases γ H2A.X level as compared to the control condition (*lanes 1 to 3*) in the H4-APP cells. There is no significant difference in the β -actin level between the isoflurane treatment and control condition. **b** Quantification of the Western blot, normalized to β -actin level, shows that the isoflurane treatment (*black bar*) increases γ H2A.X level as compared to the control condition (*white bar*) in the cells. **c** Immunostaining shows that the isoflurane treatment (*bottom row*) increases γ H2A.X level

as compared to the control condition (*upper row*) in the H4-APP cells. *Column 1* represents the image of γ H2A.X immunostaining; *column 2* represents the image of Hoechst immunostaining; *column 3* represents the merged image of γ H2A.X and Hoechst immunostaining; and *column 4* represents an enlarged image of a single cell, magnification $\times 400$. **d** Treatment with 2 % isoflurane for 3 h (*lanes 4 to 6*) does not significantly increase γ H2A.X level as compared to the control condition (*lanes 1 to 3*) in the H4-APP cells. There is no significant difference in the β -actin level between the isoflurane treatment and control condition. **e** Quantification of the Western blot, normalized to β -actin level, shows that the isoflurane treatment (*black bar*) does not significantly increase γ H2A.X level as compared to the control condition (*white bar*) in the cells. **f** Anesthesia with 1.4 % isoflurane for 2 h (*lanes 4 to 6*) increases γ H2A.X level as compared to the control condition (*lanes 1 to 3*) in the brain tissues of mice. There is no significant difference in the β -actin level between the isoflurane anesthesia and control condition. **g** Quantification of the Western blot, normalized to β -actin level, shows that the isoflurane treatment (*black bar*) increases γ H2A.X level as compared to the control condition (*white bar*) in the brain tissues of mice. APP amyloid protein precursor. $N = 6$ in each group in the in vitro studies, $N = 3$ in each group in the in vivo studies

Fig. 2.

Anesthetic isoflurane induces caspase-3 activation in H4-APP cells in a time-dependent manner. **a** Treatment with 2 % isoflurane for 6 h (*lanes 5 to 8*) increases cleaved caspase-3 level as compared to the control condition (*lanes 1 to 4*) in the H4-APP cells. There is no significant difference in the β -actin level between the isoflurane treatment and control condition. **b** Quantification of the Western blot, normalized to β -actin level, shows that the isoflurane treatment (*black bar*) increases cleaved caspase-3 level as compared to the control condition (*white bar*) in the cells. **c** Treatment with 2 % isoflurane for 3 h (*lanes 4 to 6*) does not increase cleaved caspase-3 level as compared to the control condition (*lanes 1 to 3*) in the H4-APP cells. There is no significant difference in the β -actin level between the isoflurane treatment and control condition. **d** Quantification of the Western blot, normalized to β -actin level, shows that the isoflurane treatment (*black bar*) does not significantly increase cleaved caspase-3 level as compared to the control condition (*white bar*) in the cells. *APP* amyloid protein precursor. *N* = 6 in each group

Fig. 3.

Z-VAD attenuates the isoflurane-induced caspase-3 activation and increase in γ H2A.X level in H4-APP cells. **a** Treatment with 2 % isoflurane for 6 h (*lanes 5 and 6*) increases cleaved caspase-3 level as compared to the control condition (*lanes 1 and 2*) in the H4-APP cells. Z-VAD treatment alone (*lanes 3 and 4*) does not significantly alter cleaved caspase-3 level as compared to the control condition (*lanes 1 and 2*), but Z-VAD attenuates the isoflurane-induced increase in cleaved caspase-3 level (*lanes 7 and 8*). There is no significant difference in the β -actin levels among these treatments and control condition. **b** Quantification of the Western blot, normalized to β -actin level, shows that the isoflurane treatment (*black bar*) increases cleaved caspase-3 level as compared to the control condition (*white bar*) in the cells. Z-VAD treatment alone (*net bar*) does not significantly alter the cleaved caspase-3 level as compared to the control condition (*white bar*), but Z-VAD treatment significantly attenuates the isoflurane-induced increase in cleaved caspase-3 level (*dot bar*). **c** Treatment with 2 % isoflurane for 6 h (*lanes 5 and 6*) increases γ H2A.X level as compared to the control condition (*lanes 1 and 2*) in the H4-APP cells. Z-VAD treatment alone (*lanes 3 and 4*) does not significantly alter γ H2A.X level as compared to the control condition (*lanes 1 and 2*), but Z-VAD treatment attenuates the isoflurane-induced increase in γ H2A.X level (*lanes 7 and 8*). There is no significant difference in the β -actin level among these treatments and control condition. **d** Quantification of the Western blot, normalized to β -actin level, shows that the isoflurane treatment (*black bar*) increases γ H2A.X level as compared to the control condition (*white bar*) in the cells. Z-VAD treatment alone (*net bar*) does not significantly alter the γ H2A.X level as compared to the control condition (*white bar*) but significantly attenuates the isoflurane-induced increase in γ H2A.X level (*dot bar*). APP amyloid protein precursor. $N = 6$ in each group

Fig. 4.

Anesthetic isoflurane decreases cytosol ICAD level and increases CAD level in the nucleus in H4-APP cells. **a** Treatment with 2 % isoflurane for 6 h (*lanes 4 to 6*) decreases ICAD level as compared to the control condition (*lanes 1 to 3*) in the H4-APP cells. There is no significant difference in the β -actin level between the isoflurane treatment and control condition. **b** Quantification of the Western blot, normalized to β -actin levels, shows that the isoflurane treatment (*black bar*) decreases ICAD level as compared to the control condition (*white bar*) in the cells. **c** Treatment with 2 % isoflurane for 6 h (*lanes 4 to 6*) does not significantly alter CAD level as compared to the control condition (*lanes 1 to 3*) in the H4-APP cells. There is no significant difference in the β -actin level between the isoflurane treatment and control condition. **d** Quantification of the Western blot, normalized to β -actin level, shows that the isoflurane treatment (*black bar*) does not significantly alter CAD level as compared to the control condition (*white bar*) in the cells. **e** Immunostaining shows that the isoflurane treatment (*bottom row*) increases level of CAD in nucleus as compared to the control condition (*upper row*) in the H4-APP cells. *Column 1* represents the image of CAD immunostaining; *column 2* represents the image of Hoechst immunostaining; *column 3* represents the merged image of CAD and Hoechst immunostaining; and *column 4* represents

an enlarged image of a single cell, magnification $\times 400$. *APP* amyloid protein precursor, *CAD* caspase-activated DNase, *ICAD* inhibitor of caspase-activated DNase. *N* = 6 in each group

Fig. 5.

NAC attenuates the isoflurane-induced caspase-3 activation and increase in γ H2A.X level in H4-APP cells. **a** Treatment with 2 % isoflurane for 6 h (*black bar*) increases ROS level as compared to the control condition (*white bar*) in the H4-APP cells. **b** The isoflurane treatment (*black bar*) increases ROS level as compared to the control condition (*white bar*) in the cells. NAC treatment alone (*net bar*) does not significantly alter the ROS level as compared to the control condition (*white bar*), but NAC treatment significantly attenuates the isoflurane-induced increase in ROS level (*dot bar*). **c** Treatment with 2 % isoflurane for 6 h (*lanes 5 and 6*) increases cleaved caspase-3 level as compared to the control condition (*lanes 1 and 2*) in the H4-APP cells. NAC treatment alone (*lanes 3 and 4*) does not significantly alter cleaved caspase-3 level as compared to the control condition (*lanes 1 and 2*), but NAC treatment attenuates the isoflurane-induced increase in cleaved caspase-3 level (*lanes 7 and 8*). There is no significant difference in the β -actin level among these treatments and control condition. **d** Quantification of the Western blot, normalized to β -actin level, shows that the isoflurane treatment (*black bar*) increases cleaved caspase-3 level as compared to the control condition (*white bar*) in the cells. NAC treatment alone (*net bar*)

does not significantly alter the cleaved caspase-3 level as compared to the control condition (*white bar*), but the NAC treatment significantly attenuates the isoflurane-induced increase in cleaved caspase-3 level (*dot bar*). **e** Treatment with 2 % isoflurane for 6 h (*lanes 5 and 6*) increases γ H2A.X level as compared to the control condition (*lanes 1 and 2*) in the H4-APP cells. NAC treatment alone (*lanes 3 and 4*) does not significantly alter γ H2A.X levels as compared to the control condition (*lanes 1 and 2*), but the NAC treatment attenuates the isoflurane-induced increase in γ H2A.X level (*lanes 7 and 8*). There is no significant difference in the β -actin level among these treatments and control condition. **f** Quantification of the Western blot, normalized to β -actin level, shows that the isoflurane treatment (*black bar*) increases γ H2A.X level as compared to the control condition (*white bar*) in the cells. NAC treatment alone (*net bar*) does not significantly alter the γ H2A.X level as compared to the control condition (*white bar*) but significantly reduces the isoflurane-induced increase in γ H2A.X level (*dot bar*). *APP* amyloid protein precursor, *NAC* N-acetyl-L-cysteine, *ROS* reactive oxygen species. *N* = 6 in each group

Fig. 6.

NAC does not affect isoflurane-induced reduction in p53 level in H4-APP cells. **a** Treatment with 2 % isoflurane for 6 h (*lanes 4 to 6*) decreases p53 level as compared to the control condition (*lanes 1 to 3*) in the H4-APP cells. **b** Quantification of the Western blot, normalized to β -actin level, shows that the isoflurane treatment (*black bar*) decreases p53 level as compared to the control condition (*white bar*) in the cells. **c** Treatment with 2 % isoflurane for 6 h (*lanes 5 and 6*) decreases p53 level as compared to the control condition (*lanes 1 and 2*) in the H4-APP cells. NAC treatment alone (*lanes 3 and 4*) decreases p53 level as compared to the control condition (*lanes 1 and 2*), but NAC does not affect the isoflurane-induced reduction in p53 level (*lanes 7 and 8*). There is no significant difference in the β -actin levels among these treatments and control condition. **d** Quantification of the Western blot, normalized to β -actin level, shows that the isoflurane treatment (*black bar*) decreases p53 level as compared to the control condition (*white bar*) in the cells. NAC treatment alone (*net bar*) decreases the p53 level as compared to the control condition (*white bar*), but NAC treatment does not significantly affect the isoflurane-induced reduction in p53 level (*dot bar*). APP amyloid protein precursor, NAC N-acetyl-L-cysteine. $N = 6$ in each group

Fig. 7.

Actinomycin D attenuates, but pifithrin- α potentiates, the isoflurane-induced increase in γ H2A.X levels in H4-APP cells. **a** Treatment with 2 % isoflurane for 6 h (*lanes 5 and 6*) increases γ H2A.X level as compared to the control condition (*lanes 1 and 2*) in the H4-APP cells. Actinomycin D treatment alone (*lanes 3 and 4*) does not significantly alter γ H2A.X level as compared to the control condition (*lanes 1 and 2*) but attenuates the isoflurane-induced increase in γ H2A.X level (*lanes 7 and 8*). There is no significant difference in the β -actin level among these treatments and control condition. **b** Quantification of the Western blot, normalized to β -actin level, shows that the isoflurane treatment (*black bar*) increases γ H2A.X level as compared to the control condition (*white bar*) in the cells. Actinomycin D treatment alone (*net bar*) does not significantly alter the γ H2A.X level as compared to the control condition (*white bar*) but significantly attenuates the isoflurane-induced increase in γ H2A.X level (*dot bar*). **c** Treatment with 2 % isoflurane for 6 h (*lanes 5 and 6*) increases γ H2A.X level as compared to the control condition (*lanes 1 and 2*) in the H4-APP cells. Pifithrin- α treatment alone (*lanes 3 and 4*) does not significantly alter γ H2A.X level as compared to the control condition (*lanes 1 and 2*) but potentiates the isoflurane-induced increase in γ H2A.X level (*lanes 7 and 8*). There is no significant difference in the β -actin levels among these treatments and control condition. **d** Quantification of the Western blot, normalized to β -actin level, shows that the isoflurane treatment (*black bar*) increases γ H2A.X level as compared to the control condition (*white bar*) in the cells. Pifithrin- α treatment alone (*net bar*) does not significantly alter the γ H2A.X level as compared to the control condition (*white bar*) but significantly potentiates the isoflurane-induced increase in γ H2A.X level (*dot bar*). APP amyloid protein precursor. $N = 6$ in each group

Fig. 8.

Hypothesized pathway of isoflurane-induced DNA damage. Anesthetic isoflurane induces oxidative stress, which causes caspase-3 activation, leading to CAD activation and, consequently, induction of DNA damage. Isoflurane also decreases p53 levels, which causes inhibition of repair of DNA damage. The combination effect of induction of DNA damage and inhibition of repair of DNA damage leads to DNA damage