British Journal of Anaesthesia 113 (4): 695–707 (2014)
Advance Access publication 3 April 2014 · doi:10.1093/bja/aeu053

BJA

TRANSLATIONAL RESEARCH

Isoflurane induces endoplasmic reticulum stress and caspase activation through ryanodine receptors

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Editor's key points

- Isoflurane has been suggested to cause neurotoxicity by several mechanisms including by induction of caspase-3.
- In this study, isoflurane increased endoplasmic reticulum (ER) stress and activated caspase-3 using mouse neurones.
- Effects depended on the concentration and duration of exposure and were attenuated by dantrolene.
- These data suggest that caspase 3 activation may be mediated by ryanodine receptors and ER stress.
- Further data are required.

Background. Isoflurane has been reported to induce caspase-3 activation, which may induce neurotoxicity and contribute to the pathogenesis of Alzheimer's disease. However, the underlying mechanism is largely unknown, especially whether or not isoflurane can induce ryanodine receptors (RyRs)-associated endoplasmic reticulum (ER) stress, leading to caspase-3 activation. We therefore assessed the effects of isoflurane on RyRs-associated ER stress.

Methods. We treated primary neurones from wild-type (C57BL/6J) mice with 1% and 2% isoflurane for 1, 3, or 6 h. We then measured levels of C/EBP homologous protein (CHOP) and caspase-12, two ER stress markers, using immunocytochemistry staining and western blotting analysis. Dantrolene (5 μM), the antagonist of RyRs, was used to investigate the role of RyRs in the isoflurane-induced ER stress and caspase-3 activation.

Results. Isoflurane 2% for 6 h treatment increased the levels of CHOP (876% *vs* 100%, $P=0.00009$) and caspase-12 (276% *vs* 100%, $P=0.006$), and induced caspase-3 activation in the neurones. The administration of 2% isoflurane for 3 h (shorter duration), however, only increased the levels of CHOP (309% *vs* 100%, $P=0.003$) and caspase-12 (266% *vs* 100%, $P=0.001$), without causing caspase-3 activation. The isoflurane-induced ER stress (CHOP: $F=16.64$, $P=0.0022$; caspase-12: $F=6.13$, $P=0.0383$) and caspase-3 activation ($F=32.06$, $P=0.0005$) were attenuated by the dantrolene treatment.

Conclusions. These data imply that isoflurane might induce caspase-3 activation by causing ER stress through RyRs, and dantrolene could attenuate the isoflurane-induced ER stress and caspase-3 activation. Further investigations of the potential neurotoxicity of isoflurane are needed.

Keywords: endoplasmic reticulum; inhalation anaesthetics, isoflurane; receptors, ryanodine

Accepted for publication: 22 November 2013

Around the world, ~8.5 million patients with Alzheimer's disease (AD) need surgical care under anaesthesia each year.[1] In addition, a much greater number of senior patients who are vulnerable to the development of AD also need surgical care under anaesthesia.[2] Anaesthesia, surgery, or both have been suggested to cause cognitive dysfunction, to which AD and senior patients are at risk to develop (Moller and colleagues,[3] reviewed in Querfurth and LaFerla[4] and Terrando and colleagues).[5] Thus, it is significant to study and identify anaesthetics that could potentially advance AD pathology, and to investigate the underlying mechanisms.

The common inhalation anaesthetic isoflurane has been reported to induce caspase-3 activation and other cellular damages in cultured cells and in animals,[6–14] which may then cause β-amyloid protein (Aβ) accumulation,[14] contributing to AD pathology.[15–19] Isoflurane has also been shown to induce caspase-3 activation in the brain tissues of young rodents.[20–22] However, the up-stream mechanism by which isoflurane induces caspase-3 activation remains largely unknown. Recent studies have suggested that isoflurane may cause cell death by disrupting intracellular calcium homeostasis.[13 23 24] Endoplasmic reticulum (ER) is the main source of

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cytosolic calcium in neurones and plays an important role in maintaining intracellular calcium homeostasis, protein synthesis, cell survival, and caspase activation.[25–28] There are two types of Ca^{2+}-release channels in ER: inositol 1,4,5-triphosphate receptors (IP_3R) and ryanodine receptors (RyRs).[29] Isoflurane has been shown to induce apoptosis via activation of inositol 1,4,5-trisphosphate receptors.[13] However, the effects of isoflurane on the ER still remain largely to be determined; specifically, it is unknown whether isoflurane can induce RyRs-associated ER stress, leading to caspase-3 activation. Such studies would elucidate the underlying up-stream mechanisms of the isoflurane-induced caspase-3 activation and offer the targeted intervention(s). Thus, the outcomes from these studies are novel and important.

ER stress involves the C/EBP homologous protein (CHOP).[30 31] CHOP is a proapoptotic transcription factor; its levels are very low under normal conditions but are strongly activated upon ER stress.[30] Caspase-12, another ER resident pro-caspase, is proteolysed after ER stress.[32] Taken together, we investigated a hypothesis that isoflurane could act on RyRs to increase the levels of CHOP and caspase-12, which then leads to caspase-3 activation in the primary neurones of mice.

Methods

Preparation of primary neurones

The procedure was approved by the Massachusetts General Hospital (Boston, MA, USA) Standing Committee on the Use of Animals in Research and Teaching. The relevant aspects of the ARRIVE guidelines were adhered to as appropriate. We used incremental increases in the concentration of carbon dioxide to kill the wild-type (C57BL/6J) mice at the gestation stage of day 15. The embryos were removed through Caesarean sections and they were decapitated in a 100 mm dish with 20 ml phosphate-buffered saline. We then put the harvested heads in a 100 mm dish, separated out the cortex, and removed meninges. We dissociated the neurones by using trypsinization and trituration. We then re-suspended the dissociated neurones in neurobasal medium with serum for 1 h, and finally, we placed the neurones in serum-free B27/neurobasal medium in six-well plates with a confluent rate of 25%. On the 7–10th day after the harvest, we treated the neurones with isoflurane, dantrolene, or both.

Treatments of primary neurones

We treated the primary neurones with 1% or 2% isoflurane plus 21% O_2 and 5% CO_2 for 1, 3, and 6 h, as described in our previous studies.[10 33] An anaesthesia machine was used to deliver isoflurane to a sealed plastic box in a 37°C incubator. The plastic box contained six-well plates which were seeded with 0.25 million neurones in 1.5 ml neurone culture media. We used the Datex infrared gas analyzer (Puritan-Bennett, Tewksbury, MA, USA) to continuously monitor the delivered concentrations of carbon dioxide, oxygen, and isoflurane. For the interaction studies, we administered dantrolene (5 μM) to the neurones 1 h before the treatment of isoflurane as described in a previous study.[34] Dimethyl sulfoxide (DMSO) (1:1500) was used as the solvent of dantrolene.

CHOP immunocytochemistry staining

We used the protocol provided by the company (Abcam Inc., Cambridge, MA, USA) to detect intracellular CHOP proteins. Briefly, we placed the neurones on coverslips for the treatments. At the end of the treatments, we fixed the cells in 100% methanol for 20 min on ice. We washed the neurones three times with phosphate-buffered saline, then we incubated the neurones with 0.1% TritonX-100 at 4°C for 10 min. We used 10% normal goat serum for 1 h at room temperature to block the non-specific reaction. Then, we incubated the neurones with anti-CHOP monoclonal antibody (1:200, Abcam Inc.) overnight at 4°C. The next day, we washed the neurones three times with phosphate-buffered saline and incubated the neurones with the secondary antibody (1:1000, goat anti-mouse IgG conjugated to Alexa Fluor® 488, Invitrogen, San Francisco, CA, USA) for 1 h at room temperature. Finally, we incubated the coverslips with Prolong® Gold Antifade Reagent (Invitrogen) and analysed the neurones in mounting medium using a 20× and 60× objective lens fluorescence microscope. We used the Image J (NIH, Bethesda, MD, USA) to determine the immunofluorescence intensity in the cytosol and nucleus. To determine the cytosolic fluorescence, an area surrounding the nucleus was used for counting. For the nuclear fluorescence, the value of fluorescence was acquired from the total nuclear area. Cytosolic CHOP level was expressed as the ratio of cytosolic amount of fluorescence over nuclear amount of fluorescence, which was consistent with the methods described in a previous study.[35]

Cell lysis and protein amount quantification

The pellets of primary neurones were detergent-extracted on ice with an immunoprecipitation buffer (2 mM EDTA, 150 mM NaCl, 10 mM Tris–HCl, pH 7.4, 0.5% non-idet P-40) plus protease inhibitors (1 μg ml^{-1} aprotinin, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin A). We collected the lysates, centrifuged them at 18 000 g for 15 min, and quantified them for total proteins by using a bicinchoninic acid protein assay kit (Pierce, Iselin, NJ, USA).

Western blotting analysis

The harvested primary neurones were used for western blot analyses as described in our previous study.[36] We used CHOP antibody (1:1000 dilution; Abcam Inc.) to recognize CHOP (31 kDa), caspase-12 antibody (1:1000 dilution; Cell Signaling Technology, Inc., Danvers, MA) to recognize caspase-12 (42 kDa), caspase-3 antibody (1:1000 dilution; Cell Signaling Technology, Inc.) to recognize FL-caspase-3 (35–40 kDa) and caspase-3 fragment (17–20 kDa) resulting from cleavage at asparate position 175. Finally, we used anti-β-actin antibody (1:10 000, Sigma, St Louis, MO, USA) to recognize β-actin (42 kDa). Each band in the western blot represented an independent experiment. We averaged results from six to eight independent experiments. The quantification of western blots was performed using the methods described in a previous study.[10]

Briefly, we used the National Institute of Health image program (National Institute of Health Image 1.62, Bethesda, MD, USA) to analyse the signal intensity. We then quantified the western blots in two steps. First, we used the levels of β-actin to normalize (e.g. determining ratio of FL-caspase-3 amount to β-actin amount) the levels of CHOP, caspase-12, and caspase-3, which may reduce the influence of loading differences in total protein amounts. Secondly, we presented the changes in the levels of CHOP, caspase-12, and caspase-3 in treated neurones as percentages of those in control neurones.

Statistics

There was background of CHOP levels and caspase activation in the neurones; therefore, we did not use absolute values, rather we presented their changes in treated neurones as fold or percentage of those in neurones after the control condition. We expressed the data as mean (SD). The number of samples varied from six to eight, and the samples were normally distributed (data not shown). We used two-way analysis of variance (ANOVA) or *t*-test to determine the difference between the control and treatments. We considered *P*-values of <0.05 (*) and 0.01 (**) as statistically significant. The significance testing was two-tailed, and we used Prism 6 software (La Jolla, CA, USA) to analyse the data.

Results

Treatment with 2% isoflurane for 6 h increased CHOP levels and induced caspase-12 activation in primary neurones

The neurones were harvested at the end of the treatment with 2% isoflurane for 6 h and were subjected to CHOP immunocytochemistry staining (Fig. 1A: 20 × and Fig. 1B: 60 ×). The CHOP immunostaining illustrated that the isoflurane treatment enhanced CHOP levels in cytosol. Specifically, column 1 of Figure 1A and B illustrates the image of CHOP (green), column 2 demonstrates the nuclei of the neurones (blue), and column 3 is the merged image. These images indicated that the levels of CHOP detected by the immunostaining were likely located in the cytosol and the isoflurane treatment (row b of Fig. 1A and B) increased the CHOP levels when compared with the control condition (row a of Fig. 1A and B). Quantification of the immunostaining images demonstrated that the isoflurane treatment enhanced CHOP levels when compared with the control condition: 228% *vs* 100%, $P=0.0001$ (Fig. 1C).

Next, we used western blot analysis to assess the effects of isoflurane on CHOP levels in primary neurones. The CHOP immunoblotting showed that there were observable increases in CHOP levels (31 kDa) after the isoflurane treatment when compared with the control condition in the neurones (Fig. 2A). The quantification of the western blot revealed that the isoflurane treatment increased CHOP levels: 876% *vs* 100%, $P=0.00009$ (Fig. 2B). These data suggested that isoflurane might increase CHOP levels, the marker of ER stress.[30]

The findings that isoflurane might increase CHOP levels in the neurones suggested that isoflurane might induce ER stress. Thus, we assessed whether the isoflurane treatment could also cause activation of caspase-12, another marker of ER stress.[32] Caspase-12 immunoblotting demonstrated noticeable increases in cleaved caspase-12 levels (activated) after the isoflurane treatment when compared with the control condition (Fig. 2C) in the neurones. The western blot quantification illustrated that the isoflurane treatment increased cleaved caspase-12 levels: 276% *vs* 100%, $P=0.006$ (Fig. 2D). CHOP and caspase-12 are the markers of ER stress;[28] thus, these data implied that isoflurane might induce ER stress in the primary neurones. Finally, we found that the treatment with 2% isoflurane for 6 h also induced caspase-3 activation, as evidenced by the enhancement of cleaved caspase-3 (Fig. 2E and F), which was consistent with our previous studies.[36]

Treatment with 2% isoflurane for 3 h enhanced CHOP levels and induced caspase-12 activation, but not caspase-3 activation

Given that the treatment with 2% isoflurane for 6 h induced ER stress (Figs 1 and 2) and activation of caspase-3 in primary neurones [(Fig. 2E and F) and our previous studies],[36] we then assessed whether the isoflurane-induced ER stress could occur before the isoflurane-induced activation of capsase-3. We therefore determined the effects of 2% isoflurane for 3 h (shorter duration) treatment on both ER stress and caspase-3 activation.

The neurones were harvested at the end of the isoflurane treatment and were exposed to western blot analysis. The CHOP immunoblotting illustrated noticeable enhancement in CHOP levels in the neurones after the treatment with 2% isoflurane for 3 h when compared with the control condition (Fig. 3A). The western blot quantification showed that the isoflurane treatment (2% isoflurane for 3 h) enhanced CHOP levels compared with the control condition: 309% *vs* 100%, $P=0.003$ (Fig. 3B). Caspase-12 immunoblotting demonstrated that the 2% isoflurane for 3 h treatment increased the levels of cleaved caspase-12 when compared with control condition (Fig. 3C). The western blot quantification illustrated that the isoflurane treatment (2% isoflurane for 3 h) increased the levels of cleaved caspase-12 when compared with the control condition: 266% *vs* 100%, $P=0.001$ (Fig. 3D).

However, the caspase-3 immunoblotting demonstrated that the 2% isoflurane for 3 h treatment did not cause caspase-3 activation when compared with the control condition (Fig. 3E and F). These data, that the treatment with 2% isoflurane for 3 h induced ER stress without caspase-3 activation, suggested that the isoflurane-induced ER stress might precede the isoflurane-induced caspase-3 activation.

Effects of treatment with 1% or 2% isoflurane for 1, 3, and 6 h on levels of CHOP, caspase-12, and caspase-3 activation in primary neurones of mice

Next, we asked whether the effects of isoflurane on the levels of CHOP, caspase-12, and caspase-3 activation in the primary neurones were concentration- and time-dependent. We therefore assessed the effects of treatment with 2% isoflurane for

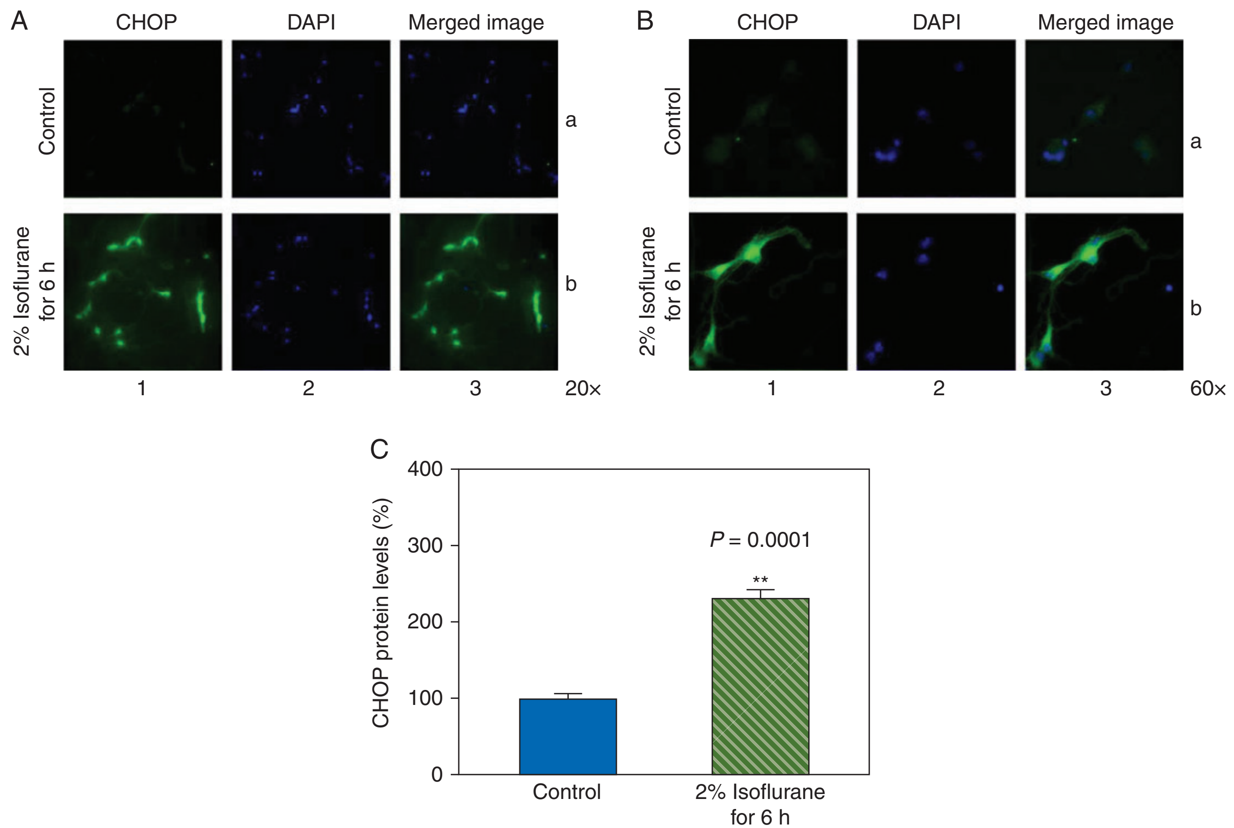

Fig 1 Isoflurane increases CHOP levels in the primary neurones. (A) Immunohistochemistry staining of CHOP (magnification 20 ×). (B) Immunohistochemistry staining of CHOP (magnification 60 ×). Column 1 is the image of CHOP (green), column 2 is the image of nuclei (blue), and column 3 is the merged image. Row a is the cells following the control condition and row b is the cells treated with 2% isoflurane for 6 h. (C) Quantification of the immunohistochemistry staining shows that the isoflurane treatment (green striped bar) increases CHOP levels compared with the control condition (blue bar).

1 h, and treatments with 1% isoflurane for 1, 3, and 6 h on the levels of CHOP, caspase-12, and caspase-3 in the primary neurones of mice. We found that these treatments did not increase the levels of CHOP (Fig. 4), and did not induce activation of caspase-12 (Fig. 5) and caspase-3 (Fig. 6) in the neurones. Instead, the treatments with 2% isoflurane for 1 h, 1% isoflurane for 3 h, and 1% isoflurane for 6 h were found to decrease the caspase-3 activation when compared with the control condition. These data suggested that the effects of isoflurane on the levels of CHOP, caspase-12, and caspase-3 activation were concentration- and time-dependent.

Dantrolene attenuated the isoflurane-induced ER stress and caspase-3 activation in primary neurones

Given the findings that isoflurane induced both ER stress and activation of caspase-3, and the fact that dantrolene is the antagonist of RyRs,[37] we then determined whether dantrolene could mitigate the isoflurane-induced ER stress and activation of caspase-3. Dantrolene (5 μM)[34] was administered to the primary neurones 1 h before the 2% isoflurane, for 6 h treatment. CHOP immunoblotting illustrated that 2% isoflurane for 6 h treatment enhanced CHOP levels when compared with the control condition in the primary neurones. Dantrolene alone did not significantly alter CHOP levels in the primary neurones when compared with the control condition, but the dantrolene treatment attenuated the isoflurane-induced increases in CHOP levels (Fig. 7A). Two-way ANOVA, based on the quantification of the western blot images, showed the significant interaction of group (control and isoflurane) and treatment (DMSO and dantrolene) (F=16.64, P=0.0022) (Fig. 7B). These data suggested that dantrolene attenuated the isoflurane-induced increases in the CHOP levels.

We then asked whether dantrolene could also attenuate the isoflurane-induced activation of caspase-12. Quantitative western blot analysis demonstrated that the dantrolene treatment attenuated the isoflurane-induced activation of caspase-12 (F=6.13, P=0.0383, two-way ANOVA) (Fig. 7C and D).

Given that dantrolene rescued the ER stress induced by isoflurane, we asked whether dantrolene could also attenuate the isoflurane-induced caspase-3 activation in the primary neurones. As shown in Figure 7E, 2% isoflurane for 6 h treatment (lanes 7–9) caused activation of caspase-3 when compared with the control condition (lanes 1–3) in the primary neurones.

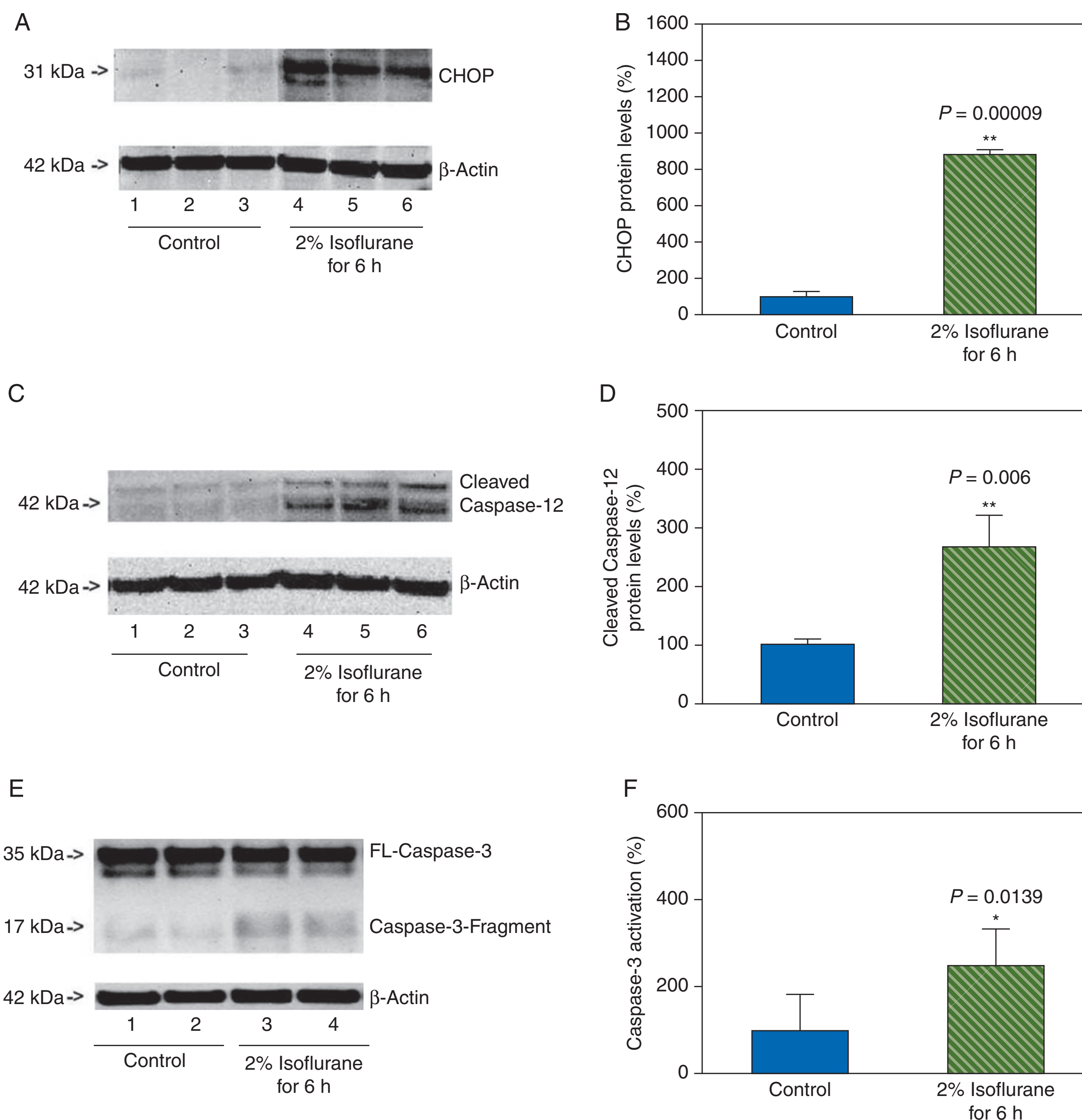

Fig 2 Isoflurane increases the levels of CHOP and caspase-12 in the primary neurones. (A) Treatment with 2% isoflurane for 6 h (lanes 4–6) increases CHOP levels when compared with the control condition (lanes 1–3) in the primary neurones. There is no significant difference in the amounts of β-actin in the control condition- or isoflurane-treated neurones. (B) Quantification of the western blot shows that isoflurane treatment (green striped bar) increases CHOP levels compared with the control condition (blue bar), normalized to β-actin levels. (C) Treatment with 2% isoflurane for 6 h (lanes 4–6) increases cleaved caspase-12 levels when compared with the control condition (lanes 1–3) in the primary neurones. There is no significant difference in the amounts of β-actin in the control condition- or isoflurane-treated neurones. (D) Quantification of the western blot shows that the isoflurane treatment (green striped bar) increases cleaved caspase-12 levels compared with the control condition (blue bar), normalized to β-actin levels. (E) Treatment with 2% isoflurane for 6 h (lanes 3 and 4) increased cleaved caspase-3 levels when compared with the control condition (lanes 1 and 2). There is no significant difference in the amounts of β-actin in the control condition- or isoflurane-treated neurons. (F) The quantification of western blot shows that the isoflurane treatment (green striped bar) induces caspase-3 activation when compared with control condition (blue bar).

Treatment with isoflurane plus dantrolene (lanes 10–12) led to a lesser degree of caspase-3 activation compared with the treatment with isoflurane plus DMSO (lanes 7–9). The western blot quantification showed that the dantrolene treatment attenuated the isoflurane-induced activation of caspase-3: F=32.06, P=0.0005 (two-way ANOVA) (Fig. 7F).

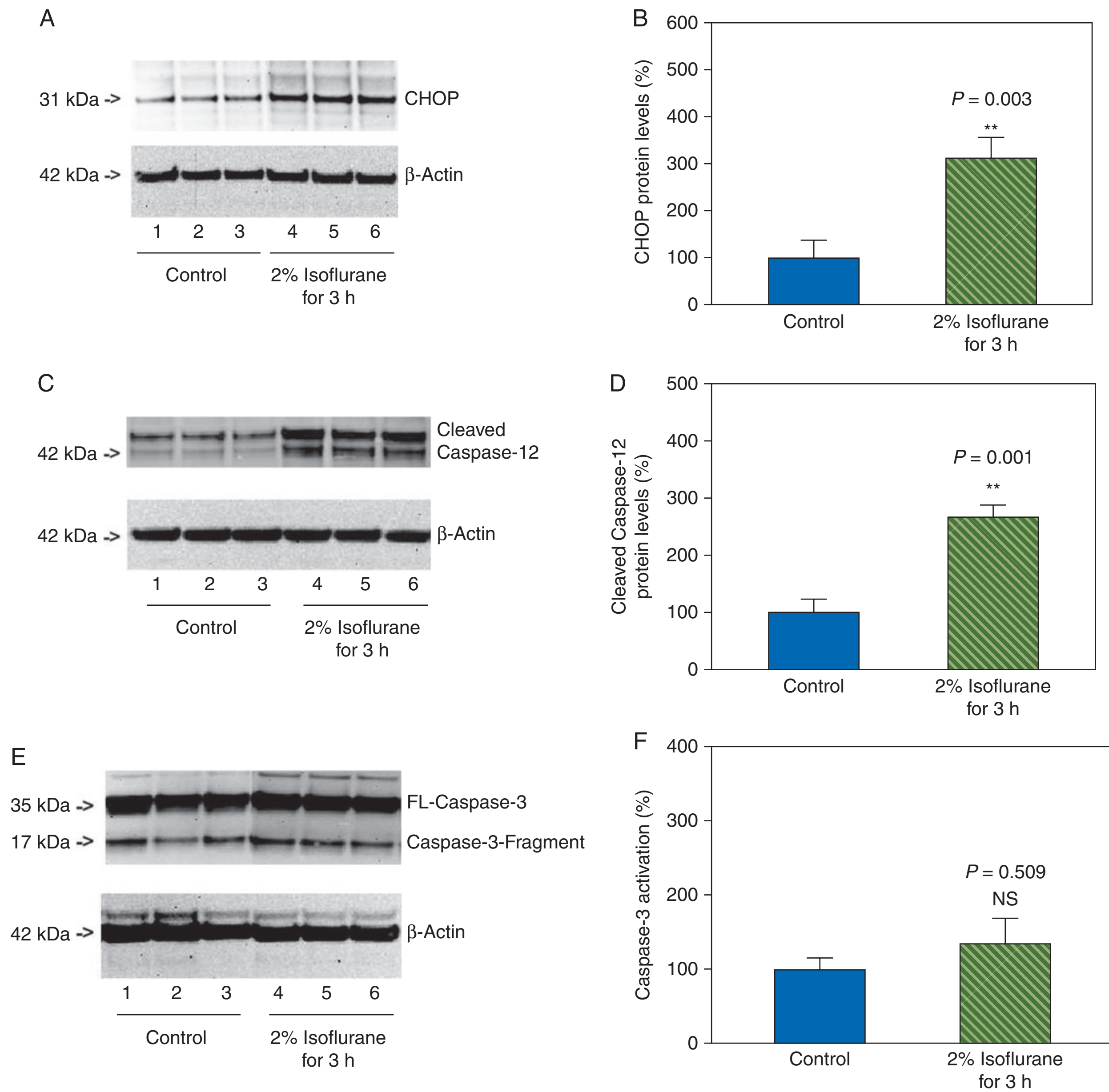

Fig 3 Treatment with 2% isoflurane for 3 h induces ER stress without caspase-3 activation in the primary neurones. (A) Treatment with 2% isoflurane for 3 h (lanes 4–6) increases CHOP levels when compared with the control condition (lanes 1–3) in the primary neurones. There is no significant difference in the amounts of β-actin in the control condition- or isoflurane-treated neurones. (B). Quantification of the western blot shows that the isoflurane treatment (green striped bar) increases CHOP levels compared with the control condition (blue bar), normalized to β-actin levels. (C) Treatment with 2% isoflurane for 3 h (lanes 4 and 6) increases cleaved caspase-12 levels when compared with the control condition (lanes 1–3) in the primary neurones. There is no significant difference in the amounts of β-actin in the control condition- or isoflurane-treated neurones. (D) Quantification of the western blot shows that the isoflurane treatment (green striped bar) increases the cleaved caspase-12 levels compared with the control condition (blue bar), normalized to β-actin levels. (E) Treatment with 2% isoflurane for 3 h (lanes 4–6) does not induce caspase-3 activation when compared with the control condition (lanes 1–3) in the primary neurones. (F) Quantification of the western blot shows that the isoflurane treatment (green striped bar) does not induce caspase-3 activation compared with the control condition (blue bar), normalized to β-actin levels.

Discussion

Given that CHOP and caspase-12 are the markers of ER stress, we assessed the effects of isoflurane on the levels of CHOP, caspase-12, and caspase-3 in the primary neurones from wild-type mice. We found that 2% isoflurane for 6 h of treatment increased the levels of CHOP (Figs 1A–C and 2A and B), and cleaved caspase-12 (Fig. 2C and D) in the primary neurones. These results suggested that isoflurane might induce ER stress.

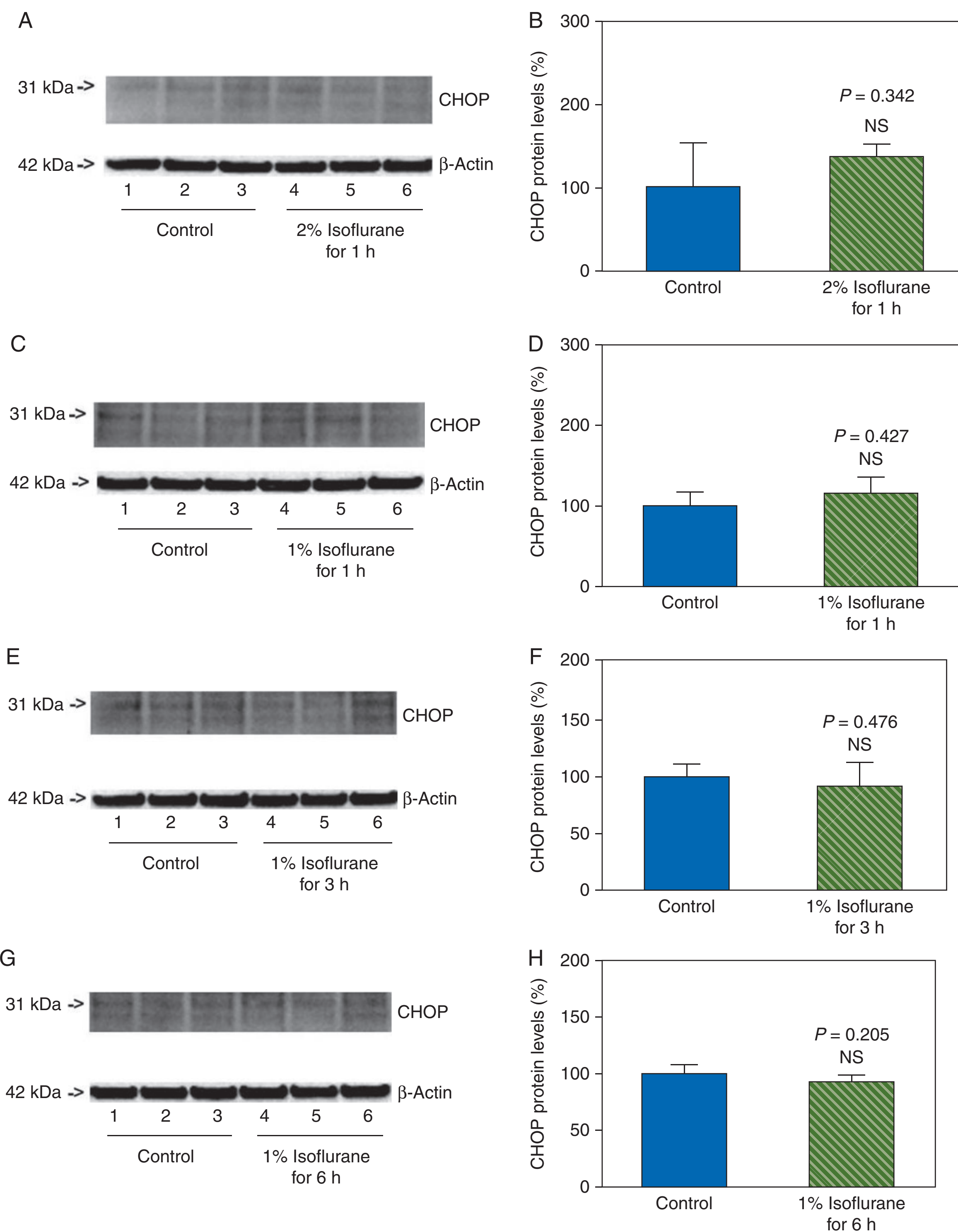

Fig 4 Treatments with 1% or 2% isoflurane for 1, 3, and 6 h on CHOP levels in primary neurones of mice. Treatment with 2% isoflurane for 1 h does not increase CHOP levels in the neurones (A and B). The treatments with 1% isoflurane for 1 (C and D), 3 (E and F), and 6 (G and H) h do not increase CHOP levels in the neurones.

We then found that isoflurane could induce activation of caspase-3 in the neurones (Fig. 2), and more importantly, treatment with isoflurane for a shorter time only induced ER stress and not activation of caspase-3 in the current experiments (Fig. 3). The data suggested that the isoflurane-induced ER stress preceded the isoflurane-caused activation

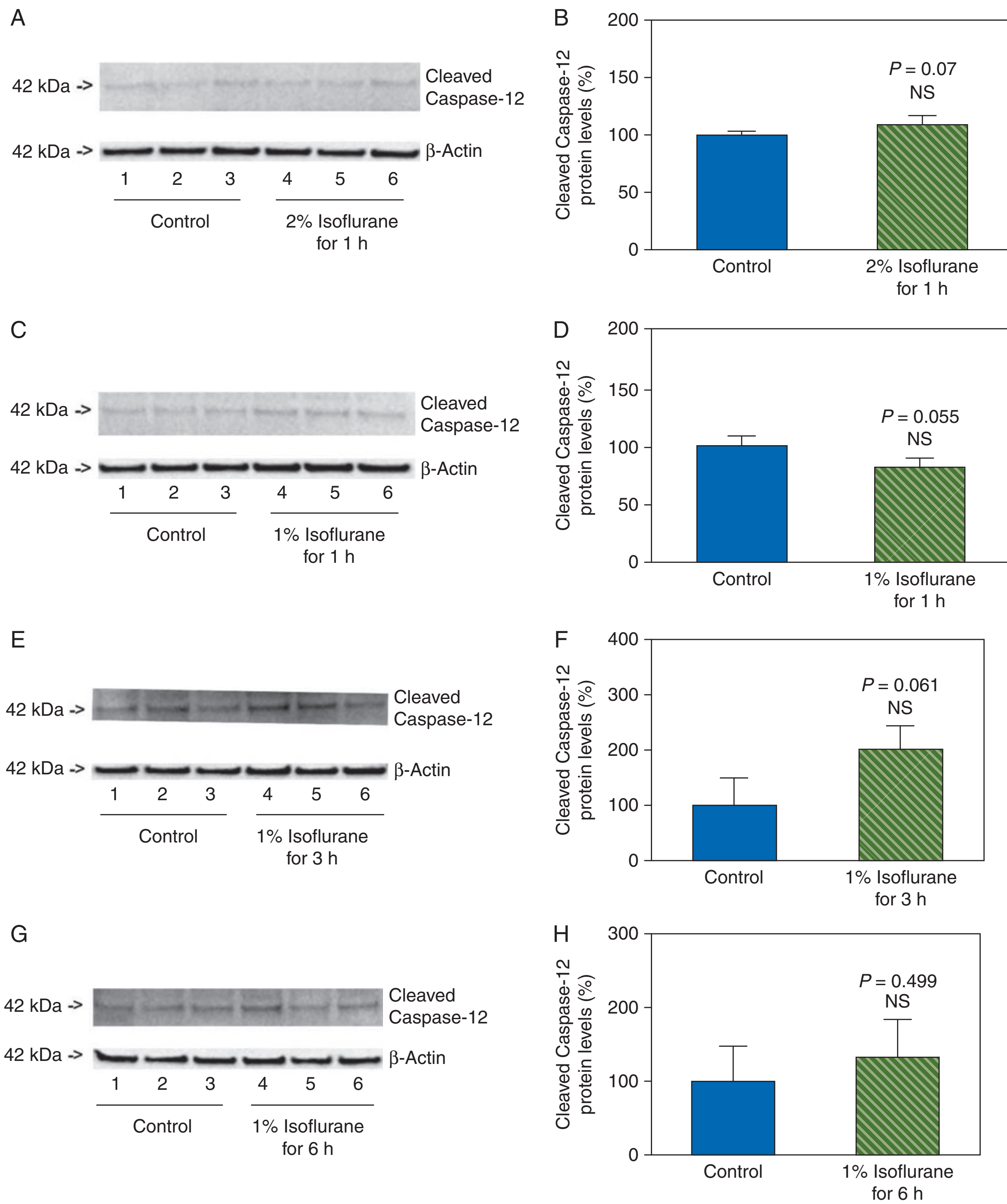

Fig 5 Treatments with 1% or 2% isoflurane for 1, 3, and 6 h on caspase-12 activation in primary neurones of mice. Treatment with 2% isoflurane for 1 h does not induce caspase-12 activation in the neurones (A and B). The treatments with 1% isoflurane for 1 (C and D), 3 (E and F), and 6 (G and H) h do not induce caspase-12 activation in the neurones.

of caspase-3, and furthermore, isoflurane might produce activation of caspase-3 via ER stress.

Moreover, we found that the effects of isoflurane on the levels of CHOP, and activation of caspase-12, and caspase-3 in the neurones were concentration- and time-dependent, and treatment with 2% isoflurane for 1 h, and treatments with 1% isoflurane for 1, 3, and 6 h did not increase the levels of CHOP (Fig. 4), and did not induce activation of caspase-12 (Fig. 5) and caspase-3 (Fig. 6) in the neurones.

Finally, we found that dantrolene, the antagonist of RyRs,[37] attenuated the isoflurane-induced ER stress and the isoflurane-caused activation of caspase-3 (Fig. 7). These

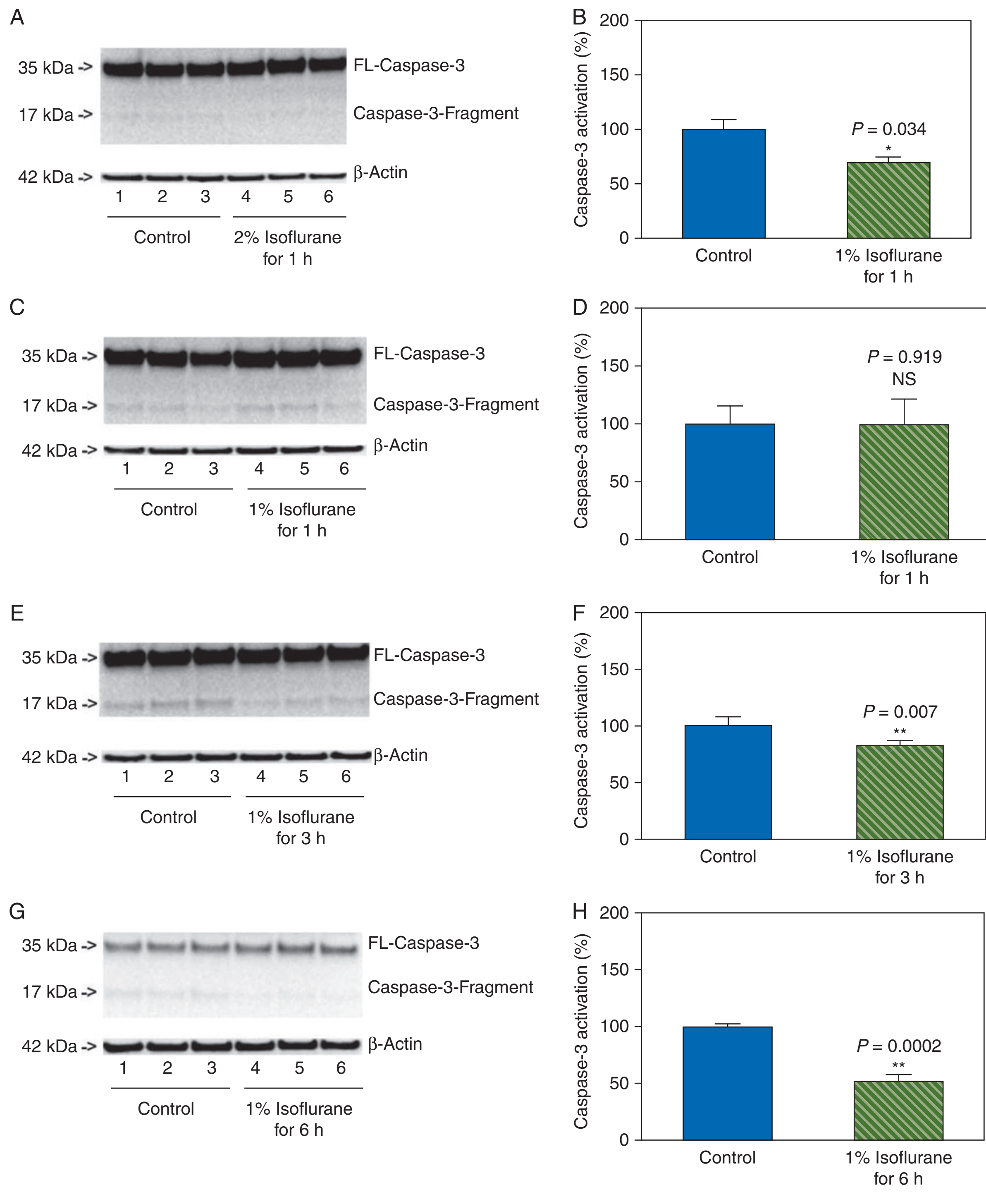

Fig 6 Treatments with 1% or 2% isoflurane for 1, 3, and 6 h on caspase-3 activation in primary neurones of mice. Treatment with 2% isoflurane for 1 h does not induce caspase-3 activation in the neurones (A and B). The treatments with 1% isoflurane for 1 (C and D), 3 (E and F), and 6 (G and H) h do not induce caspase-3 activation in the neurones. The treatments with 2% isoflurane for 1 h (A and B), 1% isoflurane for 3 h (E and F), and 1% isoflurane for 6 h (G and H) decrease the caspase-3 activation.

findings further implied that isoflurane might cause activation of capase-3 via RyRs-associated ER stress.

Our previous studies have suggested that isoflurane may cause mitochondrial dysfunction as evidenced by opening of mitochondria permeability transition pores and facilitating release of cytochrome *c* from the mitochondria to the cytosol.[1 36] The current findings suggested that isoflurane might also induce ER stress. The RyRs family has three isoforms,

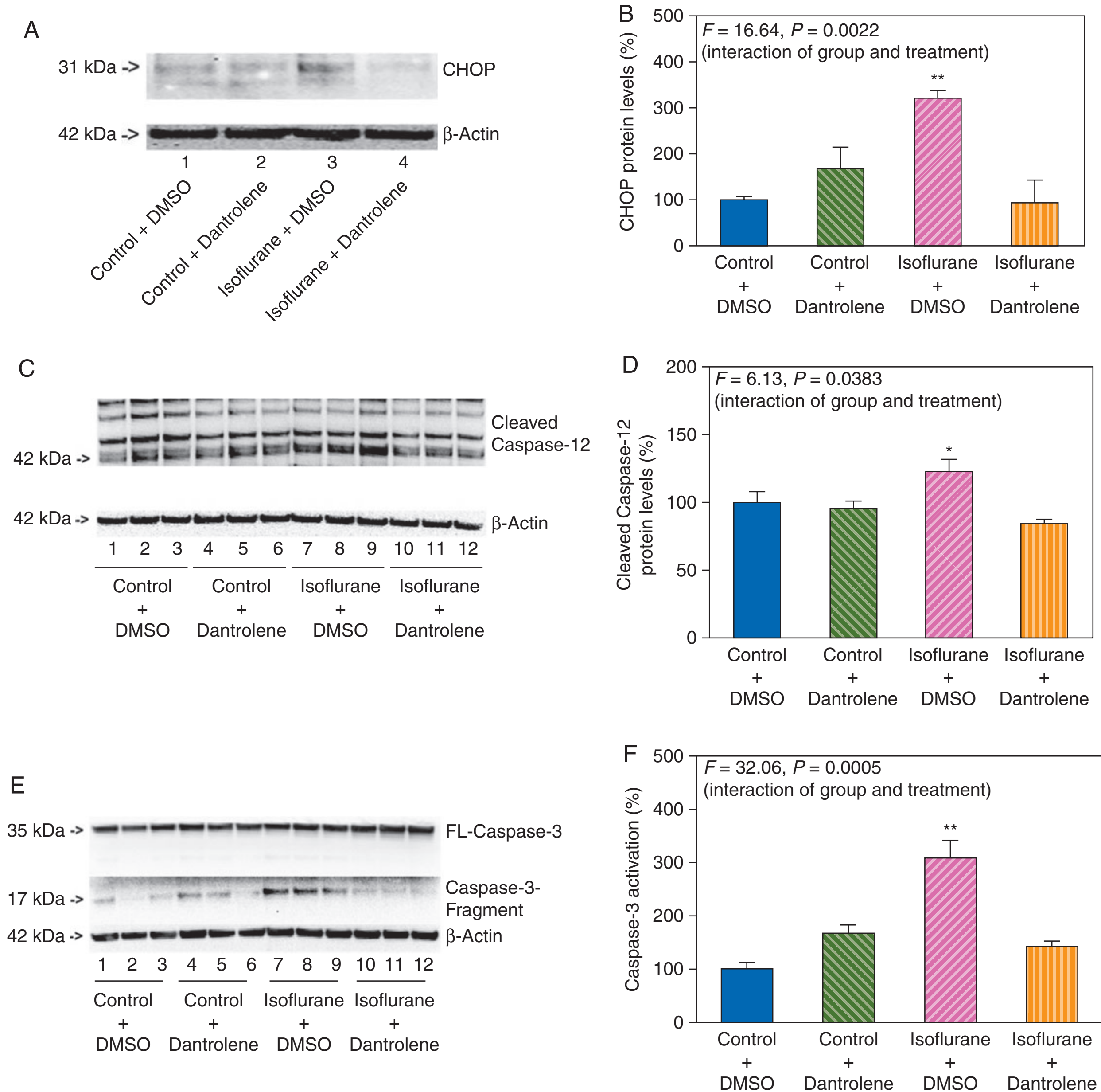

Fig 7 Dantrolene attenuates the isoflurane-induced ER stress and caspase-3 activation in the primary neurones. (A) Treatment with 2% isoflurane plus DMSO for 6 h (lane 3) increases CHOP levels when compared with the control plus DMSO condition (lane 1) in the primary neurones. Treatment with isoflurane plus dantrolene (lane 4) leads to a reduction in the CHOP levels when compared with the treatment with isoflurane plus DMSO (lane 3). There is no significant difference in amounts of β-actin among the different groups. (B) Quantification of the western blot shows that isoflurane plus DMSO treatment (pink striped bar) increases CHOP levels when compared with the control plus DMSO condition (blue bar), normalized to β-actin levels, whereas dantrolene (orange striped bar) attenuates the isoflurane-induced increases in the CHOP levels, normalized to β-actin levels. (C) Treatment with 2% isoflurane plus DMSO for 6 h (lanes 7–9) increases the cleaved caspase-12 levels when compared with the control plus DMSO condition (lanes 1–3) in the primary neurones. Treatment with isoflurane plus dantrolene (lanes 10–12) leads to a lesser degree of cleaved caspase-12 levels compared with the treatment with isoflurane plus DMSO (lanes 7–9). There is no significant difference in amounts of β-actin among different groups. (D) Quantification of the western blot shows that isoflurane plus DMSO treatment (pink striped bar) increases the cleaved caspase-12 levels compared with the control plus DMSO condition (blue bar), normalized to β-actin levels, whereas dantrolene (orange striped bar) attenuates the activation of caspase-12 induced by isoflurane plus DMSO (pink striped bar), normalized to β-actin levels. (E) Treatment with 2% isoflurane plus DMSO for 6 h (lanes 7–9) induces caspase-3 activation when compared with the control plus DMSO condition (lanes 1–3) in the primary neurones. Treatment with isoflurane plus dantrolene (lanes 10–12) induces a lesser degree of caspase-3 activation when compared with the treatment with isoflurane plus DMSO (lanes 7–9). There is no significant difference in the amounts of β-actin among different groups. (F) Quantification of the western blot shows that isoflurane plus DMSO treatment (pink striped bar) induces caspase-3 activation compared with the control plus DMSO condition (blue bar), normalized to β-actin levels, whereas dantrolene (orange striped bar) attenuates the isoflurane-induced caspase-3 activation, normalized to β-actin levels.

which are expressed in the brain. The RyRs have multiple allosteric Ca^{2+} binding sites that are responsible for prompting Ca^{2+}-induced Ca^{2+} release to the cytosol.[38] The findings that dantrolene, the antagonist of RyRs, attenuated the isoflurane-induced ER stress and activation of caspase-3 suggested that isoflurane might act on RyRs in the ER of the primary neurones, leading to ER stress and activation of caspase-3. Previous studies showed that reduction in IP3 receptor could attenuate the isoflurane-induced caspase-3 activation.[13 24] The current findings suggested that antagonism of either IP3 receptor or RyRs alone was sufficient in attenuating the isoflurane-induced ER stress-associated caspase-3 activation. However, it remains to be investigated whether the isoflurane-induced mitochondrial dysfunction and the isoflurane-induced IP3 receptor or RyRs-associated ER stress can interact with each other (potentiation or attenuation), leading to various degrees of caspase-3 activation and cellular toxicity.

ER stress and activation of RyRs contribute to malignant hyperthermia, a life-threatening disease with a dramatic increase in body temperature and skeletal muscle rigidity. Malignant hyperthermia can be triggered by inhalation anaesthetics including isoflurane. Dantrolene is the only medicine for the treatment of malignant hyperthermia and a recent study has suggested that dantrolene can ameliorate the cognitive decline and neuropathology in AD transgenic mice.[39 40] In the current study, dantrolene was shown to inhibit the isoflurane-induced ER stress and caspase-3 activation. Isoflurane-induced caspase-3 activation has been suggested to contribute to cognitive impairment in animals,[41] and isoflurane has also been suggested to be associated with postoperative cognitive dysfunction in humans.[41] Collectively, these findings imply the potential association between malignant hyperthermia and cognitive impairment or postoperative cognitive dysfunction. We therefore have postulated that the patients who have a history of malignant hyperthermia may have a higher risk in developing postoperative cognitive dysfunction, pending further studies. Future experiments to test this hypothesis are needed.

Even though isoflurane has been reported to induce caspase activation and cause apoptosis, other reports suggest that isoflurane may protect against apoptosis.[42–51] This discrepancy could be due to differences in the duration and concentration of isoflurane exposure as demonstrated in other studies.[52–54] Specifically, our previous studies showed that low concentration and short treatment time of isoflurane attenuated while high concentration and long isoflurane treatment time potentiated the hypoxia- and Aβ-induced caspase-3 activation.[52–54] Consistently, a recent study by Shu and colleagues[20] showed that prolonged exposure to isoflurane plus nitrous oxide also caused caspase-3 activation in brain tissues of 7-day-old rats. Taken together, we hypothesize that isoflurane may have concentration- and time-dependent dual effects (attenuation *vs* potentiation) on neurotoxicity, which has been supported by a recent study.[55] Future research to test this hypothesis is warranted.

One caveat of the current study is that we cannot extrapolate the *in vitro* findings to the brain. However, the majority of previous *in vitro* studies of isoflurane neurotoxicity used cultured tumour cells. Therefore, the outcomes from the current studies in primary neurones would be considered more clinically relevant. Nevertheless, future experiments are required to investigate the *in vivo* relevance of these *in vitro* findings, which may include the studies to assess whether dantrolene can mitigate the isoflurane-induced cognitive impairment in rodents. Secondly, the CHOP levels in the experiments varied even in the control condition. The variations most likely resulted from different exposure times with super strength reagents of western blot analysis. Nevertheless, the data were still able to illustrate the dose- and time-dependent effects of isoflurane on the level of CHOP in the primary neurones of mice.

In conclusion, we found that isoflurane could cause ER stress (enhancing the levels of CHOP and inducing caspase-12 activation) by acting on RyRs in primary neurones. The isoflurane-induced ER stress might precede the isoflurane-induced activation of caspase-3. RyRs antagonist dantrolene attenuated the isoflurane-induced ER stress and activation of caspase-3. These data suggested that ER stress could be one of the up-stream mechanisms by which isoflurane caused activation of caspase-3. Finally, mitigation of RyRs-associated ER stress could be a potential target for the treatment of anaesthesia neurotoxicity. More studies are needed to determine anaesthesia neurotoxicity, especially the underlying mechanisms, and targeted interventions.

Authors' contributions

H.W., Y.D., J.Z., G.W., Y.Z., and Z. Xie: conceived and designed the experiments. H.W., Y.D., J.Z., and Z. Xu: performed the experiments. J.Z. and Y.D.: analysed the data. Z. Xie, C.S., and Y.Z.: wrote the paper.

Acknowledgements

Anaesthetic isoflurane was generously provided by the Department of Anaesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA. These studies are attributed to the Department of Anaesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital and Harvard Medical School.

Declaration of interest

None declared.

Funding

This research was supported by R21AG029856, R21AG038994, R01 GM088801, and R01 AG041274 from National Institutes of Health, Bethesda, MD, Investigator-initiated Research grant from Alzheimer's Association, Chicago, IL, and Cure Alzheimer's Fund, Wellesley, MA to Z. Xie.

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Handling editor: J. P. Thompson