Interrelationship of Delirium and Dementia

Isoflurane-Induced Apoptosis: A Potential Pathogenic Link Between Delirium and Dementia

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Background. Dementia and delirium have been postulated to share common pathophysiologic mechanisms; however, identification of these unifying mechanisms has remained elusive. The inhalation anesthetic isoflurane has been shown to enhance β-amyloid protein (Aβ) oligomerization and generation, to potentiate the cytotoxicity of Aβ, and to induce apoptosis. To address the molecular mechanisms of dementia and delirium associated with anesthesia and surgery, we assessed whether the Aβ fibrillar aggregation inhibitor Congo red can attenuate isoflurane-induced caspase-3 activation in H4 human neuroglioma cells overexpressing human β-amyloid precursor protein (APP).

Methods. H4 human neuroglioma cells stably transfected to express human full-length wild-type APP were exposed to 2% isoflurane for 6 hours. The cells were harvested at the end of the treatment. Caspase-3 activation was measured with quantitative Western blotting.

Results. We found that isoflurane induces cellular apoptosis in a dose-dependent manner, and that Congo red inhibits isoflurane-induced apoptosis in H4 human neuroglioma cells overexpressing APP. Interestingly, Congo red also inhibits staurosporine-induced apoptosis.

Conclusion. The demonstration that isoflurane contributes to well-described mechanisms of Alzheimer’s neuropathogenesis provides a plausible link between the acute effects of anesthesia, a well-described risk factor for delirium, and the more long-term sequelae of dementia. These findings suggest that isoflurane-induced Aβ oligomerization and apoptosis may contribute to the risk of postoperative cognitive dysfunction and provide a potential pathogenic link between delirium and dementia.

The short-term and long-term effects of general anesthesia on brain functioning represent a burgeoning area of interest in clinical research. In the short term, general anesthesia has been identified as an important risk factor for postoperative delirium, which occurs in 15%–53% of surgical patients (1). In addition, general anesthesia may contribute to more subtle forms of postoperative cognitive dysfunction, which have been demonstrated to occur in 27% and 10% of 1218 surgical patients at 1 week and 3 months postsurgery (2). Moreover, following coronary artery bypass graft surgery, postoperative cognitive dysfunction is demonstrable in 53%, 36%, 24%, and 42% of patients at discharge, 6 weeks, 6 months, and 5 years, respectively (3). These findings suggest that anesthesia and surgery may lead to cognitive deficits that may be chronic and persistent. Three separate studies showed an odds ratio of between 1.2 and 1.6 for the association of previous surgery and Alzheimer’s disease (AD), and the age of onset of AD was inversely related to the cumulative exposure to anesthesia before age 50 (4–6). A recent study also reported that patients having coronary artery bypass graft surgery under general anesthesia were at increased risk for the emergence of AD compared to those having percutaneous transluminal coronary angioplasty under local anesthesia (7). However, the relationship of general anesthesia to dementia and AD remains unclear.

Delirium and dementia have long been considered to be entirely separate conditions, but recent evidence has increasingly highlighted their inter-relationship. First, epidemiologic studies have documented that dementia is the leading risk factor for delirium [(8), see review in (9)]. Second, delirium leads to dementia in some cases [(8), see review in (9)]. Third, previous studies have postulated shared pathophysiological mechanisms, such as decreased cerebral metabolism, cholinergic deficiency, and inflammation [(10), see review in (9)]. However, it remains unclear whether these mechanisms are truly causal or represent epiphenomena of the underlying conditions. To date, the unifying molecular mechanisms to link delirium and dementia have remained elusive.
β-amyloid protein (Aβ) generation and accumulation are major hallmarks of AD neuropathogenesis [see review in (11)]. Increasing evidence suggests that caspase activation and apoptosis also play important roles in AD neuropathogenesis [see review in (12)].

Isoflurane is an inhalation anesthetic which is widely used for general anesthesia. Isoflurane has been previously reported to have protective effects against apoptosis (13–19); other studies, however, have demonstrated that isoflurane can cause apoptosis (20–23). Recent studies illustrated that a 6-hour treatment with 1.2%–2.5% isoflurane can enhance Aβ oligomerization and cytotoxicity in pheochromocytoma cells (24). Our recent studies also revealed that isoflurane can increase Aβ levels, decrease the ratio of β-amyloid precursor protein (APP)-C-terminal fragments (CTFs) to APP-full length (FL), induce caspase-3 activation, and decrease cell viability in H4 human neuroglioma cells overexpressing human APP (25).

We hypothesized that isoflurane, with its demonstrated ability to induce apoptosis and enhance Aβ oligomerization, can initiate a vicious cycle of apoptosis and Aβ oligomerization and/or fibrillation. Congo red, an amyloid fibril–binding dye, has been reported to inhibit Aβ fibrillar aggregation and to prevent neurotoxicity (26). We therefore assessed the effect of Congo red on isoflurane-induced caspase-3 activation. Staurosporine (STS), a cell stressor that has been shown to cause apoptosis in our H4 human neuroglioma cells (27), was used as a positive control in our experiment. These studies would suggest that anesthesia with isoflurane may serve as a risk factor for the development of AD by causing apoptosis and enhancing Aβ oligomerization, which are important components of AD neuropathogenesis. The demonstration that isoflurane contributes to well-described mechanisms of Alzheimer’s neuropathogenesis would also provide a plausible link between the common acute effects of anesthesia, a well-described risk factor for delirium, and the more long-term sequelae of dementia.

METHODS

Cell Lines
We used H4 human neuroglioma cells stably transfected to express the APP-FL (H4-APP-FL cells) in the experiments. All cell lines were cultured in Dulbecco’s Modified Eagle’s Medium (high glucose) containing 9% heat-inactivated fetal calf serum, penicillin at 100 U/mL, streptomycin at 100 μg/mL, L-glutamine at 2 mM, and G418 at 200 μg/mL.

Cell Treatment
Two percent isoflurane, which produces a concentration in cell culture media typical of what is observed in the brain during clinical use, was delivered from an anesthesia machine to cells as described by Xie and colleagues (25). Specifically, 21% O₂, 5% CO₂, and 2% isoflurane were delivered from an anesthesia machine to a sealed plastic box in a 37°C incubator containing six-well plates seeded with 1 million cells in 1.5 mL of cell culture media. A Datex infrared gas analyzer (Puritan Bennett, Tewksbury, MA) was used to continuously monitor the delivered CO₂, O₂, and isoflurane concentrations. We treated the cells with 2% isoflurane for 6 hours, during which time the cells were incubated in serum-free media. Control conditions included 5% CO₂ plus 21% O₂, which did not affect caspase-3 activation, cell viability, APP processing, or Aβ generation (data not shown). Congo red (100 μM) was added to the cell culture media 1 hour before the isoflurane or STS (100 nM) treatment.

Western Blot Analysis
The cells were harvested at the end of the experiments and were subjected to quantitative Western blots as described by Xie and colleagues (25). Caspase-3 (1:1000 dilution; Cell Signaling Technology, Beverly, MA) and anti-β-actin (1:2000, Sigma, St. Louis, MO) antibodies were used to recognize the caspase-3 fragment (17–20 kD) resulting from cleavage at aspartate position 175 and caspase-3 FL (35–40 kD) and β-actin (42 kD), respectively.

Statistics
Changes in caspase-3 activation was presented as a percentage of changes in the control group. One hundred percent (100%) caspase-3 activation refers to control levels for purposes of comparison to experimental conditions. Data were expressed as mean ± standard deviation. The number of samples varied from 3 to 10, and the samples were normally distributed. We used a two-tailed t test to compare the difference between the experimental groups. Values of p less than .05 with confidence intervals of 95% were considered statistically significant.

RESULTS

Isoflurane Induced Apoptosis in H4-APP-FL Cells in a Dose-Dependent Manner
As can be seen in Figure 1A, a 6-hour treatment with 1% isoflurane did not cause visible increases in the protein levels of caspase-3 fragment (Figure 1A, Lanes 1 and 2) compared to the control conditions (Figure 1A, Lanes 3 and 4). As expected, a 6-hour treatment with 2% isoflurane (Figure 1A, Lanes 5 and 6) caused visible increases in the protein levels of caspase-3 fragment as compared to control conditions (Figure 1A, Lanes 7 and 8). There was no significant difference in the amount of β-actin in the control condition- or isoflurane-treated H4-APP-FL cells. Quantitation of the ratio of cleaved (activated) versus FL caspase-3, normalized to levels of β-actin, revealed that the 2% isoflurane treatment (Figure 1B, black bar of right panel) led to a 291% increase in caspase-3 activation as compared to control conditions (**p < .01), whereas 1% isoflurane treatment (Figure 1B, black bar of left panel) did not lead to a significant increase in caspase-3 activation as compared to control conditions (not statistically significant).

Isoflurane has been reported to enhance Aβ oligomerization (24) and to induce caspase-3 activation. We therefore
hypothesized that isoflurane can cause a vicious cycle of apoptosis and Aβ oligomerization and/or fibrillar aggregation. We then asked whether the Aβ fibrillar aggregation inhibitor Congo red can decrease isoflurane-induced caspase-3 activation.

Congo Red Inhibited Isoflurane-Induced Apoptosis

Congo red has been reported to inhibit Aβ fibrillar aggregation (26). Thus, we treated the H4-APP-FL cells with 100 µM Congo red, followed by an additional 6 hours of treatment with 2% isoflurane. As can be seen in Figure 2, caspase-3 immunoblotting revealed that 2% isoflurane plus saline treatment (Figure 2A, Lanes 5 and 6; Figure 2B, black bar) induced a visible increase in caspase-3 fragment and a visible decrease in caspase-3-FL in H4-APP-FL cells as compared to the control conditions plus saline treatment (Figure 2A, Lanes 1 and 2; Figure 2B, white bar), normalized to β-actin levels (*p < .05, **p < .01). The control conditions plus Congo red treatment (Figure 2A, Lanes 7 and 8; Figure 2B, gray bar) did not induce caspase-3 activation as compared to control plus saline treatment. Caspase-3 immunoblotting revealed less caspase activation in the cells treated with 2% isoflurane plus Congo red (Figure 2A, Lanes 5 and 4) than in the cells treated with 2% isoflurane plus saline (Figure 2A, Lanes 5 and 6). Quantitation of the Western blots, based on the ratio of caspase-3 fragment to caspase-3 FL, revealed that treatment with Congo red (Figure 2B, hatched bar) significantly attenuated isoflurane-induced caspase-3 activation (Figure 2B,
black bar) in H4-APP-FL cells, normalized to β-actin levels (# p < .05; 293% versus 180%).

Congo Red Inhibited STS-Induced Apoptosis

Next, we asked whether Congo red can also attenuate caspase-3 activation induced by other cell stressors. We found the same concentration of Congo red also inhibited the caspase-3 activation induced by STS in H4-APP-FL cells, based on the ratio of caspase-3 fragment to caspase-3 FL. As can be seen in Figure 3A, caspase-3 immunoblotting showed that 100 nM STS plus saline treatment (Figure 3A, Lanes 5 and 6) induced a visible increase in caspase-3 fragment and visible decrease in caspase-3-FL in H4-APP-FL cells as compared to control conditions plus saline treatment (Figure 2). Two percent isoflurane plus CR treatment (hatched bar) induced less caspase-3 activation as compared to that induced by the treatment of 2% isoflurane plus saline (black bar). * = p < 0.05; ** = p < 0.01, the difference between isoflurane treatment and control condition; # = p < 0.05, the difference between Congo red treatment and saline treatment.

Figure 2. Congo red (CR) inhibited caspase-3 activation induced by 2% isoflurane in H4-APP-FL cells. A, Two percent isoflurane plus saline treatment (Lanes 5 and 6), but not the control conditions plus CR treatment (Lanes 7 and 8), caused caspase-3 activation compared with control conditions plus saline treatment (Lanes 1 and 2). The 2% isoflurane plus CR treatment (Lanes 3 and 4) caused less caspase-3 activation than did 2% isoflurane plus saline treatment (Lanes 5 and 6). B, Quantitation of the Western blots showed that 2% isoflurane plus saline treatment (black bar) induced caspase-3 activation as compared to the treatment with either control conditions plus saline (white bar) or control conditions plus CR (gray bar). Two percent isoflurane plus CR treatment (hatched bar) induced less caspase-3 activation as compared to that induced by the treatment of 2% isoflurane plus saline (black bar). * = p < 0.05; ** = p < 0.01, the difference between isoflurane treatment and control condition; # = p < 0.05, the difference between Congo red treatment and saline treatment.
treatment (Figure 3A, Lanes 1 and 2). Neither control conditions plus Congo red treatment (Figure 3A, Lanes 3 and 4) nor STS plus Congo red treatment (Figure 3A, Lanes 7 and 8) induced caspase-3 activation. There was no significant difference in the amount of β-actin in the control condition- or STS-treated H4-APP-FL cells. Quantitation of the ratio of cleaved (activated) versus FL caspase-3, normalized to levels of β-actin, revealed that the STS plus saline treatment (black bar) led to a 293% increase in caspase-3 activation as compared to control conditions plus saline treatment (Figure 3B; **p < .01), whereas neither control conditions plus Congo red treatment (gray bar) nor STS plus Congo red treatment (hatched bar) induced a significant caspase-3 activation, as compared to control conditions plus saline treatment (white bar) (Figure 3B; not statistically significant). Collectively, these data show that
Congo red effectively inhibits isoflurane- and STS-induced caspase-3 activation in H4-APP-FL cells.

DISCUSSION

Increasing evidence suggests the inter-relationship of delirium and dementia, including the possibility of shared pathophysiologic mechanisms [(8,28,29), see review in (9)]. The current results are significant in providing a plausible and direct link between the acute effects of inhalational anesthetics, which are recognized risk factors for delirium, and the hallmark mechanisms of AD neuropathogenesis, namely Aβ generation and/or accumulation and apoptosis. We proposed that Aβ and Aβ-induced neurotoxicity may be responsible for delirium and its long-term effects, and we have carried out a series of experiments designed to assess the effects of perioperative factors on apoptosis and Aβ generation. In our previous studies, we found that inhalation isoflurane (25) can induce apoptosis and increase Aβ generation. Because caspase-3 activation is one of the final steps in cell apoptosis, we have used caspase-3 activation to represent apoptosis in the current studies as in our previous studies (25).

We first assessed the effects of different concentrations of isoflurane on caspase-3 activation in H4-APP-FL cells. In this experiment, we found that, at 6 hours, 1% isoflurane treatment did not induce caspase-3 activation in H4-APP-FL cells. At 6 hours, 2% isoflurane treatment caused caspase-3 activation in H4-APP-FL cells. These findings not only confirm our previous results but also suggest that isoflurane induces apoptosis in a dose-dependent manner. Future experiments will be required to determine whether isoflurane will also induce apoptosis in a time-dependent manner.

Isoflurane has been shown to enhance Aβ oligomerization and cytotoxicity (24). Recently, we revealed that isoflurane can cause apoptosis and increase Aβ generation (25). Aβ oligomerization and fibrillar aggregation have been reported to be responsible for neurotoxicity (26,30–38); we therefore set out to assess whether isoflurane-induced apoptosis is dependent on Aβ oligomerization and fibrillar aggregation. Earlier studies showed that Congo red can inhibit cytotoxicity and Aβ fibrillar aggregation (26). Here, we found that Congo red inhibited isoflurane-induced apoptosis in H4-APP-FL cells. These findings suggest that isoflurane-induced apoptosis is dependent on Aβ fibrillar aggregation. In future studies, we will assess whether other Aβ oligomerization inhibitors (38) can likewise attenuate isoflurane-induced apoptosis.

Interestingly, we also found that the same concentration of Congo red can inhibit 100 nM STS-induced caspase-3 activation in H4-APP-FL cells. These findings suggest that Congo red can also inhibit more generalized cellular apoptosis. Future studies should include searching for Aβ oligomerization and fibrillar aggregation inhibitors that can specifically inhibit isoflurane-induced apoptosis, to further test our hypothesis that isoflurane-induced apoptosis is dependent on Aβ oligomerization and fibrillar aggregation. These findings provide the pathophysiologic basis linking the more acute process of delirium following anesthesia with the longer term consequences of dementia, through the acute isoflurane-induced generation of well-documented mechanisms of AD.

Apoptosis has been reported to play a role in neurodegenerative disease, including AD (12,27). Clinically applicable concentrations of isoflurane can induce cellular apoptosis in a dose-dependent manner, as well as affect APP processing and Aβ metabolism (25). Moreover, Aβ oligomerization and fibrillar aggregation may promote neurotoxicity following isoflurane anesthesia. The Aβ fibrillar aggregation inhibitor Congo red can inhibit both isoflurane- and STS-induced apoptosis. These results indicate that general anesthesia might precipitate and facilitate the progression of postoperative cognitive disorders, ranging from delirium to dementia. Collectively, our findings further suggest that inhibiting Aβ oligomerization and fibrillar aggregation might represent potential therapeutic strategies for prevention of both short-term and longer term postoperative cognitive dysfunction. The effects of STS on Aβ oligomerization are largely unknown. Future studies should include systemically assessing the effects of isoflurane and STS on apoptosis, Aβ oligomerization and generation and/or accumulation, as well as disposition and amount of amyloid in vitro and in vivo (both wild-type and AD transgenic mice). Moreover, future studies to examine the epidemiologic association of isoflurane anesthesia and AD, along with studies to translate these findings into the clinical setting are urgently needed.

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