Inhaled Anesthetic Enhancement of Amyloid-β Oligomerization and Cytotoxicity

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Background: The majority of surgical patients receive inhaled anesthetics, principally small haloalkanes and haloethers. Long-term cognitive problems occur in the elderly subsequent to anesthesia and surgery, and previous surgery might also be a risk factor for neurodegenerative disorders like Alzheimer and Parkinson disease. The authors hypothesize that inhaled anesthetics contribute to these effects through a durable enhancement of peptide oligomerization.

Methods: Light scattering, filtration assays, electron microscopy, fluorescence spectroscopy and size-exclusion chromatography was used to characterize the concentration-dependent effects of halothane, isoflurane, propofol, and ethanol on amyloid β peptide oligomerization. Pheochromocytoma cells were used to characterize cytotoxicity of amyloid oligomers with and without the above anesthetics.

Results: Halothane and isoflurane enhanced amyloid β oligomerization rates and pheochromocytoma cytotoxicity in vitro through a preference for binding small oligomeric species. Ethanol and propofol inhibited oligimerization at low concentration but enhanced modestly at very high concentration. Neither ethanol nor propofol enhanced amyloid β toxicity in pheochromocytoma cells.

Conclusions: Inhaled anesthetics enhance oligomerization and cytotoxicity of Alzheimer disease-associated peptides. In addition to the possibility of a general mechanism for anesthetic neurotoxicity, these results call for further evaluation of the interaction between neurodegenerative disorders, dementia, and inhalational anesthesia.

WORLDWIDE, an estimated 100 million patients undergo surgery every year, and the majority receives inhaled general anesthetics, principally small haloalkanes and haloethers. Although inhaled anesthetics are associated with low mortality, this has been achieved through better training and monitoring rather than through the development of intrinsically safer compounds. It is becoming increasingly clear, for example, that persistent postoperative cognitive problems, the mechanism of which is not yet clear, occur in the elderly.1–3 Also, recent reports suggest that neurodegenerative disorders, such as Alzheimer and Parkinson diseases, might be accelerated by anesthesia and surgery.4–6 For example, three separate studies produced odds ratios of between 1.2 and 1.6 for the association between previous surgery and Alzheimer disease, although none was sufficiently powered to demonstrate statistical significance. If larger studies confirm this trend, a 20 to 60% increase in Alzheimer disease as a result of surgery and anesthesia is, on the other hand, of considerable societal significance. Potential mechanisms for either postoperative cognitive disorder or neurodegeneration are numerous, ranging from N-methyl-D-aspartate-mediated excitotoxicity7,8 to oxidant stress9 or suppression of cholinergic signaling.10 We provide evidence for a novel biophysical mechanism: enhancement of protein oligomerization.

Although still contentious, the presumed mechanism for most neurodegenerative disorders is an uncontrolled oligomerization (microaggregation) of normally present protein or peptide, such as the amyloid β peptides of Alzheimer disease11,12 or α-synuclein of Parkinson disease.13 In the case of Alzheimer disease, small amyloid β peptides of 35 to 42 amino acids are released after proteolytic cleavage of the amyloid precursor protein (a type I membrane protein whose function is still unknown) and, depending on the environment, cleavage site, and many other factors, these unstable, hydrophobic peptides self-associate coincident with a change in secondary structure from α-helix to β sheet. Many oligomeric forms of these peptides are populated as the complexes grow in size to ultimately achieve the mature fibril, an enormous macromolecular complex of characteristic dimension and secondary structure. The mechanism linking oligomerization and cellular dysfunction or toxicity is not understood, but recent evidence points to the intermediate-sized oligomers, as opposed to the mature fibrils,14,15 as the toxic species. Although the molecular detail underlying assembly and growth of amyloid β oligomers has eluded structural approaches, it must rely on at least two properties of the peptide monomer: disorder and hydrophobicity. Disorder provides the entropic freedom to sample conformations necessary for self-association, usually β-sheet, and the necessary free energy for oligomerization derives from the hydrophobic effect. Once formed, small oligomers
adjust to maximize hydrogen bonding, resulting in the characteristic cross β-structure and continued accretion. The combination of hydrophobic interactions and hydrogen bonds represents sufficient stabilizing interactions to assure a fairly durable complex, although evidence of reversibility has emerged. If the rate-limiting step is the formation of small oligomers, then enhancement can be expected from compounds that bind preferentially to these oligomers or perhaps even the larger oligomers/fibrils.

The inhaled anesthetics are small, relatively featureless hydrophobic molecules that bind principally in internal protein cavities. In some cases, these gases can enhance the formation of protein oligomers, the basis for which is likely twofold. First, the anesthetic molecules favor protein intermediates with enlarged cavities and thus cause destabilization and enhance disorder in some proteins. Second, hydrophobic cavities of suitable volume are often formed at oligomeric interfaces. Although cavity formation is energetically unfavorable, it is apparently compensated for by numerous other hydrophobic contacts in the assembled oligomer. On occupying these interfacial cavities, the anesthetic molecules will restore van der Waals contacts and thus provide additional free energy for oligomerization. Inhaled anesthetics should, therefore, favor amyloid β and similar peptide oligomerization if cavities exist at the intermolecular interfaces. And the stable, insoluble nature of the fibrillar oligomer suggests that the predicted enhancement of kinetics should result in a stable pool of oligomerized material rather than only a transient effect. Thus, a brief anesthetic exposure could translate into a durable effect on the distribution of molecular populations.

Materials and Methods

Amyloid β Peptide Synthesis

Amyloid β1-40 (Aβ40) and amyloid β1-42 (Aβ42) peptides were synthesized on an Applied Biosystems Model 433A (Perkin Elmer, Foster City, CA) using Fmoc (9-fluorenlymethoxycarbonyl) chemistry. Fmoc-Ala-Wang resin was used for synthesis of peptides (Advanced Chemtech, Louisville, KY), and other chemicals were obtained from Applied Biosystems (Perkin Elmer). Trifluoroacetyl acid 2.5%, anisole 5%, thioanisole 5%, 1,2-obtained from Applied Biosystems (Perkin Elmer, Foster City, CA) using Fmoc (9-fluorenlymethoxycarbonyl) chemistry. Fmoc-Ala-Wang resin was used for synthesis of peptides (Advanced Chemtech, Louisville, KY), and other chemicals were obtained from Applied Biosystems (Perkin Elmer). Trifluoroacetyl acid 2.5%, anisole 5%, thioanisole 5%, 1,2,3-Ethanedithiol 2.5%, and water 5% was used to cleave peptides from resin and remove the side chain protecting groups. The resulting peptides were precipitated in cold diethyl ether and were then extracted by centrifugation. The dried peptide pellet was dissolved in 88% formic acid, filtered through nylon acrodiscs 0.2 μm (Gelman Sciences, Ann Arbor, MI), and then purified by high pressure liquid chromatography (System Gold; Beckman Coulter, Inc., Fullerton, CA) using diphenyl columns (Grace Vydac, Hesperia, CA) with water/acetonitril/0.1% trifluoroacetyl acid gradients. Peptide identity was confirmed by Matrix-Assisted Laser Desorption-Ionization-Mass Spectroscopy.

Oligomerization Assays

Aβ40 and Aβ42 were prepared as dimethyl sulfoxide stock solutions (~5 μM), added directly into phosphate buffer with or without presolubilized anesthetics, and then loaded into fluorescence cuvettes for the light scattering assay for oligomerization. With excitation and emission wavelengths both set to 400 nm, oligomerization was monitored as an increase in emission (actually scattering). For the filtration assay for oligomerization, the material was incubated at 37°C for 24-48 h in Teflon-sealed microvials, and then spun through Amicon™ 100 kDa cutoff filters (Millipore Corp., Bedford, MA). Remaining “soluble” peptide concentration in the filtrate was determined by dye binding (BioRad Laboratories, Hercules, CA). In this assay, a decrease in the filtrate peptide concentration indicates a greater degree of oligomerization. Thioflavin-T (25 μM) binding was measured in aliquots removed from incubation stocks (in gas-tight Hamilton syringes; Hamilton, Reno, NV) as the fluorescence yield at 480 nm with excitation at 350 nm. Fluorescence assay was also used to measure reversibility of the anesthetic-induced oligomerization.

Size Exclusion Chromatography

To determine the anesthetic-favored oligomer, amyloid (β1-40; a less hydrophobic form of the peptide that oligomerizes more slowly but is still found in Alzheimer plaque) was incubated in pH 7 phosphate buffer and 0.01% NaN₃ (sodium azide), containing either nothing, 4 mM halothane, 300 mM ethanol, or 60 μM propofol in 300 μl Teflon™ sealed microvials (Agilent Technologies, Palo Alto, CA). These were allowed to incubate for various periods of time at room temperature, and then samples were centrifuged at 10,000 g for 10 min before loading (50 μl) onto a Superdex™ 70 column (Amersham Biosciences, Piscataway, NJ). Samples were eluted at 0.5 ml/min with continuous 215 nm monitoring for 40 min. Standards were injected for molecular weight calibration purposes.

Cell Culture

Rat pheochromocytoma cells were maintained in Gibco™ Roswell Park Memorial Institute medium 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% horse serum, 5% fetal calf serum, and antibiotics (penicillin/streptomycin), 10 mM HEPES, and 1 mM sodium pyruvate. Monolayer cultures at a density of 0.1 x 10⁵ to 0.3 x 10⁵ cells/cm² were incubated in plastic flasks precoated with 0.01% poly-L-ornithine in 95% air, 5% CO₂ humidified atmosphere at 37°C. The culture medium was changed every 48 h. Pheochromocytoma cells were exposed to inhalational anesthetics in a gas tight chamber (Bellco Glass, Inc, Vineland, NJ) inside the

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culture incubator, with air and 5% CO₂ passing through a calibrated vaporizer. Humidification was achieved with water evaporation inside the gas-tight chamber. Gas phase concentrations were verified with infrared spectroscopy of the effluent gas. Pheochromocytoma cells cultured in 24-well plates at a density of 2.5 × 10⁵ were exposed to 15 μM Aβ₄₂ or its equivalent vehicle. Control wells received no treatment. An identically prepared plate was exposed to either 0.8 and 1.5% halothane or 1.2 and 2.5% isoflurane (~1 and 2 minimum alveolar concentration) in the inner chamber of the same incubator for 6 h and then removed and incubated side by side with the nonexposed plate for an additional 72 h. For the propofol and ethanol studies, wells were evenly divided between those receiving 10 μM propofol (final concentration using neat propofol) and those receiving 100 mM ethanol. Both concentrations were between 1 and 2 ED₅₀ (effective dose to produce anesthesia in 50% of the population) and incubated with and without Aβ₄₂ for 72 h. Media was aspirated, and assays for lactate dehydrogenase were performed (see next paragraph). Data were expressed as means of percentage of sham control ± SEM from at least three wells from three experiments. Analysis of variance followed by the Tukey-Kramer multiple comparison test was used to determine significance of the individual conditions.

Lactate Dehydrogenase Assay
A colorimetric lactate dehydrogenase (LDH) release assay (Promega Corp, Madison, WI) was used. Briefly, 50-μl aliquots of media from each well were transferred to clean 96-well plates, incubated with substrate for 30 min at room temperature; then incubation was stopped and absorbance was measured at 490 nm. Data are normalized to release in untreated wells.

Results

Oligomerization Assays
The kinetics of amyloid β₁₋₄₂ (peptide oligomerization in vitro in the presence of halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) were monitored by light scattering. Halothane produced a concentration-dependent enhancement of the oligomerization rate (fig. 1). Even drug concentrations achieved in routine clinical anesthesia (<1.0 mM) significantly accelerate oligomerization, and the sedimented material from these experiments is fibrillar, similar to that found in Alzheimer senile plaque (fig. 2). In other experiments, we verified that a similar kinetic enhancement occurs with the more soluble amyloid β₁₋₄₀ peptide, although on a longer time scale, as expected (data not shown).

To determine if the accelerated kinetics result in a larger ultimate pool of oligomerized material or rather represents a transient kinetic enhancement, we used filtration assays after more prolonged incubations. Halothane and another, more commonly used inhaled anesthetic, isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether) produced a significantly enlarged population of Aβ₄₂ fibrils even after several days of incubation.

Fig. 1. Halothane enhances oligomerization of ~50 μM amyloid β₁₋₄₂ peptide as reflected by scattering at 400 nm. Scale is arbitrary but identical within experiments.

Fig. 2. Digital image from a transmission electron microscope of negative stained amyloid β fibrils assembled in vitro, incubated in pH 7 buffer for 48 h at 37°C, layered and dried on formvar and carbon coated 300 mesh copper grids, and then stained with 2% aqueous uranyl acetate. Fibrils are visualized as both clumped and elongated filaments. The fibrils are approximately 8 nm in diameter. Magnification bar = 100 nm.
growth (fig. 3). A control protein (human serum albumin) with known halothane binding sites was unaffected by even saturating halothane concentrations in any of these oligomerization assays. Another hydrophobic general anesthetic compound, propofol (diisopropylphenol), enhanced oligomerization only at very high concentration (≈100-fold clinical concentrations), and ethanol, a sedative/hypnotic compound of societal importance, caused a smaller enhancement at very high concentration (>1M). At lower, more clinically relevant concentrations, both propofol and ethanol slightly inhibited oligomerization.

Fluorescence
Thioflavin-T fluorescence of Aβ42 samples was clearly enhanced in a time-dependent manner in the presence of halothane (fig. 4), consistent with the scattering and filtration experiments. Halothane was eliminated from some samples after incubation for only 4 h. Persistent differences between control and halothane samples were detectable for at least 3 days (fig. 5). Addition of halothane buffer to samples grown under control conditions had no effect on thioflavin-T fluorescence.

Size Exclusion Chromatography
In an attempt to determine whether anesthetics promote aggregate formation via a favorable interaction with intermediate oligomers, we performed size exclusion chromatography of Aβ40 incubated with anesthetics for several days to allow achievement of a relatively stable state. If only the mature fibril is favored, the population of intermediate oligomers should be reduced or unchanged. On the other hand, if the intermediates are favored, their population should be enhanced. Fig. 6 shows that in the control samples after several days of incubation, the dominant peak remained at approximately a dimer, but a small population of oligomers that eluted at the void volume (>100 kDa molecular weight) was consistently observed. These are smaller than the mature fibrils, which cannot enter the column. Halothane clearly enhanced this population of oligomers, especially the "protofibrils" at the void volume. Ethanol promoted smaller oligomers and propofol was intermediate; neither had an effect as large as halothane. This is the approximate rank order observed in the above filtration assay.
Cytotoxicity

Incubation of pheochromocytoma cells with any of the anesthetic drugs produced only small and insignificant changes in LDH release (Fig. 7A). On the other hand, 15 μM Aβ42 peptide alone produced a small but significant increase in LDH after 72 h of incubation. When either halothane or isoflurane were added to Aβ42 peptide, there was a significant further increase in LDH release at both 1 and 2 minimum alveolar concentration, somewhat more than would be expected from simple additivity. In the propofol and ethanol experiments, Aβ42 peptide caused the same enhancement of LDH release seen in the inhaled anesthetic experiments (fig. 7B), but there was no additional effect of either propofol or ethanol.

Discussion

These data demonstrate that inhaled anesthetics enhance oligomerization and cytotoxicity of amyloid peptides, an effect that is detectable in these in vitro and cell culture assays even at clinical concentrations of these drugs. When combined with the slow reversibility of oligomer formation, these in vitro data suggest that inhaled anesthetics might induce a long-lasting increase in toxic forms of amyloid β in vivo. The probable mo-

Fig. 6. Size exclusion chromatography. Elution profile of amyloid β40 incubated for 4 days at room temperature with buffer only (control, solid line) or with 4 mM halothane (dotted line). The broad dominant peak at 30 min (apparent molecular weight of ~9 kDa) with a downstream shoulder suggests a monomer-dimer mixture of amyloid β40 as the preferred conformation under these solution conditions. Distinct peaks at 20 min (~20 kDa) and 12 min (>100 kDa) suggests that higher order oligomers are assembled from progressively larger oligomers rather than a simple accretion from monomers or dimers.

Fig. 7. (A) Effects of 15 μM amyloid β42 (Aβ42) peptide with and without halothane and isoflurane at 1 and 2 minimum alveolar concentration on lactate dehydrogenase release over 72 h in pheochromocytoma cells. Bars represent the mean ± SEM (>3 wells, three experiments) magnitude of change from untreated control wells. *P < 0.05 as compared with control; †P < 0.05 as compared with control and the equivalent anesthetic concentration alone; ‡P < 0.05 as compared with control, the equivalent anesthetic concentration, and the Aβ42 peptide alone (analysis of variance and post hoc Tukey-Kramer testing). (B) The analogous results for propofol and ethanol.
Lecithin is the presence of the presence of intercellular hy-
drocarbon cavities in the oligomerized conformers, fea-
tures that are known to be favored anesthetic binding sites.\textsuperscript{17} The size exclusion data suggest that these fea-
tures are present in the small “protofibrillar” oligomers; occupancy by anesthetics are expected to stabilize this kinetic intermediate, both increasing their population and leading to an enhanced rate of larger aggregate formation. The smaller effect of ethanol and propofol point toward hydrophobic regions of the complexes being the “catalytic” binding domain for anesthetics, as these alcohols are expected to bind more superficially on the protein structure. The differential effects on protein oligomerization of ethanol versus halothane have been observed in other proteins\textsuperscript{11} and are of interest in view of recent results that suggest light to moderate ethanol consumption may reduce the risk of dementia in humans.\textsuperscript{26}

The addition of inhaled anesthetics to the $\Delta\beta_{14}^{42}$ appeared to produce greater pheochromocytoma cytotoxicity than either treatment alone. This was especially apparent in the isoflurane-treated cells because the anesthetic alone may have decreased LDH release slightly. Similar potentiation was noted in the 1 minimum alveolar concentration halothane experiments but not in the 2 minimum alveolar concentration experiments, an observation we cannot yet explain. It is not currently possible to relate this degree of cytotoxicity to an organ or organ system effect. Subtle as these changes appear to be in these immortalized cells, similar degrees of cytotoxicity in the intact brain could have considerably more functional impact or, on the other hand, remain undetected as a reduction in “reserve.” It will be important to design experiments using the various animal models of neurodegenerative disorders to further test the validity of this aggregation mechanism of anesthetic cytotoxicity.

The anesthetic concentrations used in a few of these experiments were high relative to those that produce anesthesia in animals, primarily for experimental signal-to-noise reasons. For example, 5 mM halothane is about 15 times that necessary to prevent movement in response to pain in most animals. Several features suggest that our results are nonetheless relevant to clinical situations. First, aside for the alcohol agents (propofol, ethanol) there is no evidence for a biphasic nature of the effect, and our assays detected enhanced oligomerization at clinical concentrations (fig. 1). Second, the magnitude of change in amyloid $\beta$ oligomerization kinetics or equilibria necessary for an important clinical effect is unclear. Anticipating a large effect seems unreasonable, as the clinical correlate should have been less subtle than it appears to be. Third, the slow reversibility, or memory effect (fig. 6) of the anesthetics on oligomerization is anticipated to translate a small transient perturbation into a long-lasting stimulus to inflammatory cascades and other mechanisms of cellular dysfunction. Finally, the use of clinical concentrations in an otherwise robust cell line clearly suggests that a biologically relevant interaction between amyloid beta and the inhaled anesthetics exist in roughly the same rank order as the effects on oligomerization.

Taken together, these observations hint at an interaction between inhaled anesthetics and the pathogenesis of neurodegenerative disorders. A potential interaction has been addressed for Alzheimer disease in four different clinical studies, but limited power prevented positive correlations despite all having positive risk ratios. It is important to stress that the mechanism proposed herein is not expected to cause Alzheimer disease but rather to accelerate the underlying pathogenesis and symptomatology. Validation of these predictions may be possible in some of the current animal models, but because none recapitulate all aspects of Alzheimer disease and related disorders and because the effect of anesthesia is likely to be subtle, it will be important to carefully test this hypothesis in large clinical studies.

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