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Production of a novel neuromelanin at the sevoflurane–water interface

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Abstract

Postoperative cognitive dysfunction (POCD) occurs in the elderly following surgery that requires inhaled anesthetics. The molecular mechanism associated with this process is unknown. This study examined the possible role of serotonin, a neurotransmitter involved in cognition. We observed that sevoflurane, a common inhaled anesthetic, formed a separate phase in water similar to that of chloroform. Additionally, sevoflurane sequestered acrolein, which is a lipid peroxidation product associated with aging and is elevated in the elderly brain. The enhanced partitioning of acrolein increased the focal concentration and hence reactivity to serotonin which preferentially occurred at the sevoflurane–water interface. The resulting product exhibited unique properties similar to catecholamine-derived neuromelanin.

Keywords: Sevoflurane; Acrolein; Serotonin; Neuromelanin

A major problem in anesthesia is the rather high incidence of postoperative cognitive dysfunction (POCD) in the elderly that persists for a variable amount of time [1,2] and may even be associated with the onset of Alzheimer's [3,4] and Parkinson's disease [5]. POCD, which is particularly evident following cardiac surgery, is observed in middle-aged patients with increasing incidence in the elderly. The molecular mechanism of this process remains to be fully elucidated.

Serotonin signaling may be a target for the sustained cognitive effects of inhaled anesthetics [6]. Serotonin, which is synthesized from tryptophan [7], is a major neurotransmitter found throughout the CNS. Serotonergic neurons are clustered in areas of the brainstem, particularly in the midline, with axonal projections reaching almost every region of the brain. There is a strong concentration of serotonergic neurons in the Raphe nucleus. Serotonin is implicated in regulating mood and cognition [8]. While the role of serotonin in POCD is unknown, we propose that the reactivity of serotonin to age-related oxidative products may be enhanced by inhaled anesthetics.

Lipid peroxidation occurs following trauma, during inflammation and as a general consequence of aging. Acrolein ($H_2C{=}CH{-}CHO$), a byproduct of lipid peroxidation, contains reactive functional groups. The reaction of carbonyl and vinyl groups with nucleophilic centers, such as nitrogens on proteins and amino acids, results in the formation of stable covalent adducts.

This study examined the effect of acrolein on serotonin with the unique approach of utilizing interface chemistry afforded by the immiscibility of sevoflurane in water. Additionally, we present evidence of a novel serotonin-derived neuromelanin, which is currently thought to be synthesized from catecholamines. Sevoflurane forms a separate phase in water [9], suggesting that the effects of sevoflurane with aqueous components may be due to reaction or interaction at the sevoflurane-water interface. Therefore, we created a sevoflurane interface to test this hypothesis.

Materials and methods

Sevoflurane, obtained from Abbott Laboratories (North Chicago, IL), was layered with nitrogen gas after each use and stored at room temperature. Indole, serotonin and 2,4-dinitrophenylhydrazine (DNPH) were purchased from Sigma (St. Louis, MO) and acrolein from Alfa Aesar

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(Ward Hill, MA). All other chemicals were of reagent grade. Indole and DNPH were dissolved in ethanol and HCl (1N), respectively.

Serotonin (1–8 mM) or indole (5 mM) was incubated with acrolein (0–32 mM) in a sodium phosphate buffer (pH 7.4) for various times at room temperature prior to analysis of fluorescence emission (serotonin: ex/em, 315 nm/336 nm; indole: ex/em, 297 nm/344 nm; 2.5 nm slit widths; scan speed, 600 nm/min) using a Perkin Elmer LS50B luminescence spectrometer (Waltham, MA). Values are given in relative fluorescence units (rfu).

Sevoflurane is not miscible in water, forming a two-phase system. Aqueous solutions of acrolein (10 mM) were mixed vigorously with sevoflurane (0–250 μL) prior to analysis of acrolein concentration in the aqueous phase. Acrolein concentration was determined with the carbonyl reagent, DNPH, measuring absorbance at 370 nm [10] using a GE Healthcare Ultrospec 4000 UV/Vis spectrophotometer (Waukesha, WI). Extinction coefficients were experimentally derived. The sevoflurane/water partition coefficient for acrolein, defined as $\log([\text{acrolein}]_{\text{sevoflurane}}/[\text{acrolein}]_{\text{water}})$, was determined using a two-phase system with sevoflurane (2 mL of 7.6 M), water (2 mL) and acrolein (200 μL of 1.0 M) that was mixed and kept at room temperature for 15 min prior to analysis of acrolein concentration in the water phase as described above.

A two-phase system was created in which the lower phase was composed of sevoflurane and the upper phase was an aqueous mixture containing serotonin. Sevoflurane (4 mL) and acrolein (1000 μL of 1 M) were added to a 22 mL reagent bottle shielded from light and mixed thoroughly. Serotonin (15 mL of 74 mM) was carefully added over the sevoflurane solution and kept unstirred at room temperature for 48 h. Photographs were taken at 24 and 48 h using a Fujifilm finePIX S1 Pro camera with a Nikon 60 mm Macro lens at settings of 1/90 s (shutter speed) and f16.

The amount of melanoid substance was tested as a function of distance from the sevoflurane–water interface. A two-phase system consisted of an upper phase (4 mL, 125 mM serotonin) and a lower sevoflurane phase (2 mL, 88 mM acrolein) that was kept unstirred at room temperature for 1–3 days prior to removal of aliquots (20 μL) of the upper phase at recorded distances from the interface. Melanoid product was determined in dilute aqueous solutions (30:1) by measuring absorbances at 450 nm.

The melanoid product of acrolein-modified serotonin was examined by absorbance spectroscopy using an Ultrospec 4000. Absorbance spectra were recorded over 200–650 nm range (2500 nm/min; 1.0 nm step) following various experimental conditions, and difference spectra were calculated following normalization of spectra. We tested the electrical conductance properties of the melanoid product using a digital 600amp Ideal Industries clampmeter, model 61-766 (Sycamore, IL).

Results and discussion

We initially observed that serotonin (1mM) reacted with acrolein (4 mM) in the absence of sevoflurane as evidenced by decreased serotonin fluorescence (control: 257 ± 0.7; plus acrolein: 190 ± 0.9rfu; ex, 315 nm; em, 336 nm). Higher concentrations of acrolein (32 mM) decreased serotonin (8 mM) emission by 92%. There was also an acrolein-induced red-shift in the wavelength of peak emission (data not shown). Additionally, acrolein (10 mM) reacted with indole (control: 180 ± 4.4; plus acrolein: 136 ± 3.8 rfu; ex, 297 nm; em, 344 nm), the base structure of serotonin, suggesting that both ring and non-ring nucleophilic nitrogens are susceptible to modification by acrolein. These observations represent the unique finding that acrolein, which is age-associated [11], may impair serotonin signaling directly via chemical modification even in the absence of inhaled anesthetics.

We were interested in examining the effects of sevoflurane on the reactivity of acrolein to serotonin. Since sevoflurane forms a separate phase in water [9], the effects of sevoflurane on aqueous acrolein concentration were first investigated. We observed that sevoflurane sequestered acrolein (Fig. 1), resulting in the removal of acrolein from the aqueous environment and in the concentrating of this reactive substance in the sevoflurane phase. The sevoflurane/water partition coefficient of acrolein was 0.91, a logarithmic relationship that represented a greater than 8-fold distribution of acrolein into the sevoflurane phase. We hypothesized that the sequestering of acrolein may create conditions that favor interface chemistry.

At the sevoflurane–water interface, lower-phase acrolein reacted with upper-phase serotonin creating a brilliant color (Fig. 2). The color developed over time, became concentrated at the interface and resisted diffusion through the vessel suggesting that the product was quite large. The melanoid product was yellowish after 24 h and became increasingly reddish-brown over time. None of the melanoid product entered the sevoflurane phase. Even after vigorous mixing no color was visible in the lower sevoflurane phase (data not shown), suggesting that the product is not significantly hydrophobic.

The melanoid product, which was created at the sevoflurane–water interface, formed a gradient that was less concentrated with increased distance from the interface (Fig. 3). The presence of the gradient suggested that the serotonin-derived material did not diffuse readily and that the resulting melanoid product has a high molecular weight. Interestingly, the curve which is represented by the open circles (Fig. 3) exhibited deflection points, suggesting that at the very least there are two populations of melanoid products formed that differ in size. The extent of melanoid product formation was dependent upon the concentration of reactants, incubation time and temperature. Even at low serotonin concentration (10 μM; 10 mM acrolein for 8 days at room temperature), we observed melanoid production (data not shown). Fig. 3

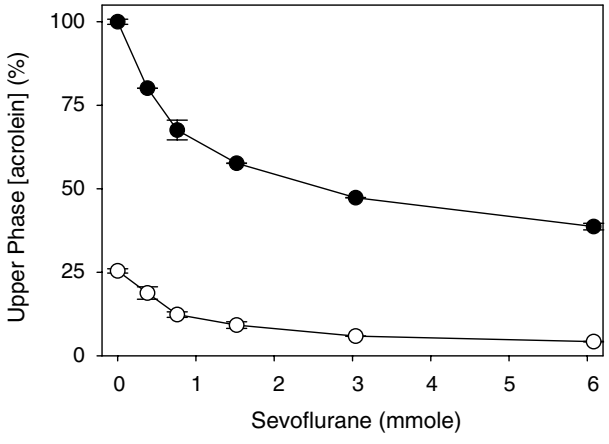

Fig. 1. Sevoflurane sequesters acrolein. Solutions of acrolein (closed circles: 10 mM; open circles: 1 mM) were mixed with small aliquots of sevoflurane resulting in the formation of two phases, and then the upper aqueous phase was tested for acrolein concentration.

Fig. 2. Visible formation of melanoid product at the sevoflurane–water interface. An upper serotonin-containing (74 mM) phase was layered over a lower sevoflurane phase containing sequestered acrolein (<200 mM), kept at room temperature, shielded from light and photographed at 24 h (LEFT panel) and 48 h (RIGHT panel). Reaction of serotonin with acrolein occurred at the interface forming a melanoid product.

(inset) presents the visible spectrum of this acrolein-modified (500 mM for 75 min at 37 °C) serotonin (25 mM) product.

We propose that this melanoid product may represent a novel neuromelanin. Interestingly, structural analogs to serotonin such as tryptophan-derivatives [12,13], tryptamine-derivatives [14], melatonin [15] and 5,6-dihydroxyindole [16] form melanin in the presence of peroxidase and hydrogen peroxide. Our findings corroborate these studies, suggesting that neuromelanin may be derived from compounds other than tyrosine. Furthermore, the current study suggests that neuromelanin may also be produced nonenzymatically. Neuromelanin is found in pigmented neurons located predominately in the following nuclei: substantia nigra, locus ceruleus, dorsal motor nucleus of the vagus nerve and the Raphe nucleus. The process of pigmentation occurs predominately during adult maturation. Non-neuronal melanin is produced via the enzyme tyrosinase, which is absent from the brain. Our findings suggest that melanin may be produced in the absence of enzymes by non-tyrosine precursors.

The melanoid product spectrophotometrically resembles the UV component of serotonin (Fig. 4) with some interesting observations in the difference spectrum (Fig. 4, inset). The melanoid product in these experiments was obtained by extracting an aliquot from the sevoflurane–water interface (serotonin, 125 mM; acrolein, 88 mM; 6 days at 24 °C) and performing two rounds of centrifugation (8700*g* through a 10 kDa filter) and dilution of the unfiltered portion prior to obtaining the UV melanoid spectrum (total dilution 1:3750). The control spectrum was obtained by a 1:120 dilution of a 13.6 mM serotonin solution. Decreased absorbance at 220 nm may suggest delocalization of the π bonds, which may arise from side-by-side alignment of indole rings. Increased absorbance at 236 nm suggests formation of a new ring structure presumably derived from acrolein Schiff base addition to the primary amine or a Michael addition to the indole nitrogen. Increased absorbance at 318 nm may suggest a novel arrangement of multiple ring structures that become increasingly in close proximity with one another. The reddish-brown material (acrolein, 88 mM; serotonin, 125 mM; 6 days at room tem-

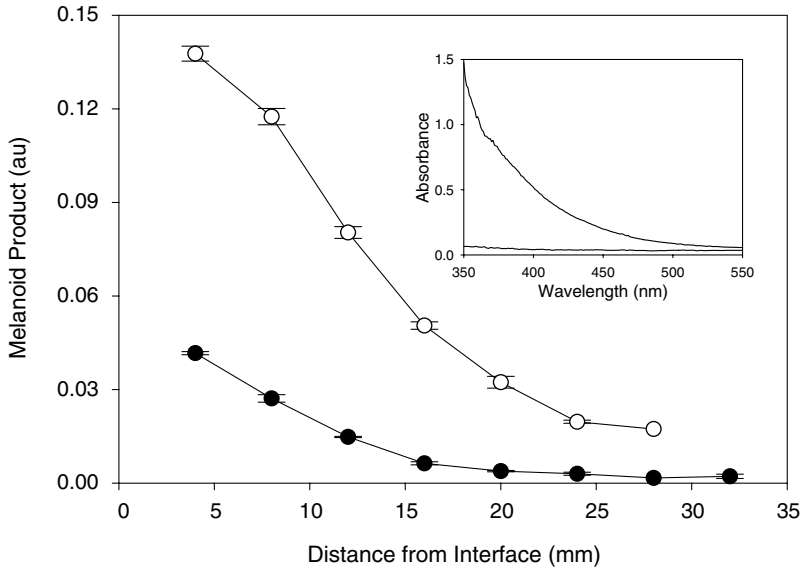

Fig. 3. Gradient of melanoid product from the sevoflurane-water interface. A two-phase system was created containing a sevoflurane lower phase and a serotonin upper phase and incubated for 1 day (closed circles) and 3 days (open circles) prior to removing aliquots at precise distances from the interface and testing for melanoid product using absorbance at 450 nm. INSET: Visible spectrum of melanoid product (upper tracing) against unmodified serotonin (lower tracing).

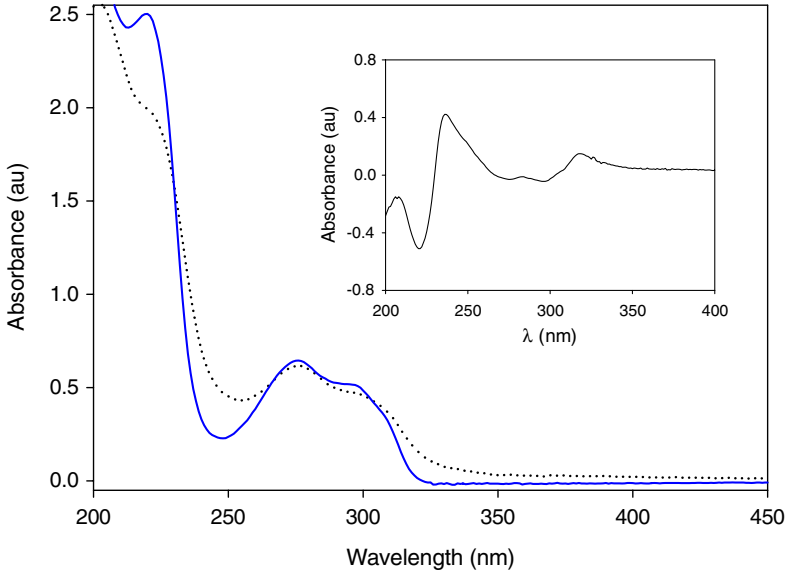

Fig. 4. Absorbance spectra of serotonin and serotonin-derived melanoid. Serotonin spectrum (solid line) was obtained and normalized to the spectrum of the melanoid product (dotted line). INSET: A difference spectrum was calculated exhibiting melanoid-specific increases at 236 and 318 nm and a decrease at 220 nm.

perature) is thought to be a polymer composed of a minimum of 100 serotonin molecules as evidenced by the filtration of the melanoid product through a 300 kDa filter but not a 10 kDa filter (data not shown).

These findings suggest that inhaled anesthetics may act to concentrate lipid peroxidative products, such as acrolein, and increase acrolein's reactivity to nucleophilic agents such as serotonin. The Meyer-Overton correlation suggests that inhaled anesthetics cluster at membrane regions, and it is at these locations that we propose inhaled anesthetics would collect lipid fragments, like acrolein. Consequently, there may be an increased concentration of acrolein at the membrane surface, which is the site of neurotransmitter action, providing an environment where compounds such as serotonin may react with acrolein.

The observation that acrolein chemically modifies serotonin (Figs. 2–4) is a novel finding. The resulting product is reddish-brown in color. Additionally, the acrolein-modified serotonin polymerizes to form a melanoid structure with a molecular weight that is between 10 and 300 kDa consistent with 20 kDa structures previously described [17]. Our melanoid product exhibited conductance properties (70-fold decrease in resistivity compared to tap water), a property of melanin [18]. The literature suggests that neuromelanin products may play a role in degenerative events [19–21]. The production of the serotonin-derived melanoid occurs progressively over time (Figs. 2 and 3). We propose that this polymeric aggregate may act as a trapping agent for free serotonin causing depletion, suggesting a mechanism for POCD. Our model suggests that the pre-formed melanoid may persist. The reactive edge of this polymerizing matrix may, for an undetermined length of time, continue to scavenge free serotonin, thus decreasing its availability for cognitive functions.

In summary, we observed that acrolein reacted with serotonin and indole. Additionally, sevoflurane sequestered acrolein, created an interface with water and allowed acrolein to react with serotonin at the sevoflurane–water interface forming a melanoid product that may represent a novel neuromelanin. Our model suggests a molecular mechanism for POCD.

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References

[1] T. Johnson, T. Monk, L.S. Rasmussen, H. Abildstrom, P. Houx, K. Korttila, H.M. Kuipers, C.D. Hanning, V.D. Siersma, D. Kristensen, J. Canet, M.T. Ibanaz, J.T. Moller, Postoperative cognitive dysfunction in middle-aged patients, Anesthesiology 96 (2002) 1351–1357.
[2] M.L. Ancelin, G. de Roquefeuil, B. Ledesert, F. Bonnel, J.C. Cheminal, K. Ritchie, Exposure to anaesthetic agents, cognitive functioning and depressive symptomatology in the elderly, Br. J. Psychiatry. 178 (2001) 360–366.
[3] N. Bohnen, M.A. Warner, E. Kokmen, C.M. Beard, L.T. Kurland, Alzheimer's disease and cumulative exposure to anesthesia: a case-control study, J. Am. Geriatr. Soc. 42 (1994) 198–201.
[4] N. Bohnen, M.A. Warner, E. Kokmen, L.T. Kurland, Early and midlife exposure to anesthesia and age of onset of Alzheimer's disease, Int. J. Neurosci. 77 (1994) 181–185.
[5] S. Muravchick, D.S. Smith, Parkinsonian symptoms during emergence from general anesthesia, Anesthesiology 82 (1995) 305–307.
[6] R.A. Whittington, L. Virag, Isoflurane decreases extracellular serotonin in the mouse hippocampus, Anesth. Analg. 103 (2006) 92–98.

[7] J.G. Hensler, Serotonin, in: G.J. Siegel, R.W. Albers, S. Brady, D.L. Price (Eds.), Basic Neurochemistry, seventh ed., Elsevier Academic Press, San Diego, CA, 2006, pp. 227–248.
[8] J.A. Schmitt, M. Wingen, J.G. Ramaekers, E.A. Evers, W.J. Riedel, Serotonin and human cognitive performance, Curr. Pharm. Des. 12 (2006) 2473–2486.
[9] T.A. Swearengin, E.E. Fibuch, N.W. Seidler, Sevoflurane modulates the activity of glyceraldehyde 3-phosphate dehydrogenase, J. Enzyme Inhib. Med. Chem. 21 (2006) 575–579.
[10] R. Fields, H.B.F. Dixon, Micro method for determination of reactive carbonyl groups in proteins and peptides, using 2,4-dinitrophenylhydrazine, Biochem. J. 121 (1971) 587–589.
[11] H.F. Poon, V. Calabrese, G. Scapagnini, D.A. Butterfield, Free radicals and brain aging, Clin. Geriatr. Med. 20 (2004) 329–359.
[12] G. Allegri, S. Vogliardi, A. Bertazzo, C.V. Costa, R. Seraglia, P. Traldi, Involvement of 5-hydroxytryptophan in melanogenesis, Adv. Exp. Med. Biol. 527 (2003) 723–730.
[13] A. Smaniotto, S. Comai, A. Bertazzo, C.V. Costa, G. Allegri, R. Seraglia, P. Traldi, A mass spectrometric investigation on the possible role of tryptophan and 7-hydroxytryptophan in melanogenesis, J. Mass Spectrom. 41 (2006) 921–930.
[14] A. Bertazzo, D. Favretto, C.V. Costa, G. Allegri, P. Traldi, Melanogenesis from 5-hydroxytryptamine, 5,6- and 5,7-dihydroxytryptamines. An in vitro study using MALDI-TOF, Adv. Exp. Med. Biol. 467 (1999) 779–787.
[15] A. Rizzi, S. Comai, A. Bertazzo, C.V. Costa, G. Allegri, P. Traldi, An investigation on the possible role of melatonin in melanogenesis, J. Mass Spectrom. 41 (2006) 517–526.
[16] M. d'Ischia, A. Napolitano, G. Prota, Peroxidase as an alternative to tyrosinase in the oxidative polymerization of 5,6-dihydroxyindoles to melanin(s), Biochim. Biophys. Acta 1073 (1991) 423–430.
[17] S. Aime, M. Fasano, E. Terreno, C.J. Groombridge, NMR studies of melanins: characterization of a soluble melanin free acid from Sepia ink, Pigment Cell Res. 4 (1991) 216–221.
[18] L. Zeise, B.L. Murr, M.R. Chedekel, Melanin standard method: particle description, Pigment Cell Res. 5 (1992) 132–142.
[19] G. Prota, M. D'Ischia, A. Napolitano, The chemistry of melanins and related metabolites, in: J.J. Nordlund et al. (Eds.), The Pigmentary System, Oxford University Press, 1988.
[20] J.N. Keller, Age-related neuropathology, cognitive decline, and Alzheimer's disease, Ageing Res. Rev. 5 (2006) 1–13.
[21] A. Borit, L.J. Rubinstein, H. Urich, The striatonigral degenerations. Putaminal pigments and nosology, Brain 98 (1975) 101–112.