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Effect of Age on the Profile of Alkanes in Normal Human Breath

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Ethane and pentane in breath are markers of oxidative stress, produced by ROS-mediated lipid peroxidation of n-3 and n-6 polyunsaturated fatty acids (PUFAs), but little is known about other n-alkanes in normal human breath. We investigated the spectrum of alkanes in normal human alveolar breath, and their variation with age. Fifty normal humans were studied (age range 23–75, median 35). Volatile organic compounds (VOCs) in alveolar breath were captured on sorbent traps and assayed by gas chromatography and mass spectroscopy. Alveolar gradients (concentration in breath minus concentration in ambient room air) of alkanes were determined. C4–C20 alkanes were observed in breath and room air. Their mean alveolar gradients were negative from C4 to C12 and positive from C13 to C20. The mean alveolar gradients of four alkanes (C5–C8) were significantly less negative in the older subjects ($p < 0.05$). There were no significant differences between males and females. Normal human breath contained a spectrum of alkanes which may include new markers of oxidative stress. The mean rate of clearance (via cytochrome p450) exceeded the mean rate of synthesis (by ROS-mediated oxidative stress) for C4–C12 alkanes, while synthesis was greater than clearance for C13–C20 alkanes. The elevated alkane profile in older subjects was consistent with an age-related increase in oxidative stress, though an age-related decline in alkane clearance rate may have contributed.

Keywords: Oxidative stress, alkane, breath, reactive oxygen free species, volatile organic compounds, cytochrome p450, free radical, human

Abbreviations: ROS, reactive oxygen species; PUFAs, polyunsaturated fatty acids; VOCs, volatile organic compounds; BCA, breath collection apparatus; ATD, automated thermal desorption; GC, gas chromatography; MS, mass spectroscopy; SD, standard deviation; RMV, respiratory minute volume

Oxygen is a paradoxical element: it supports aerobic life, yet it is also highly toxic. Aerobic organisms generate most of their energy by reducing molecular oxygen to water, with the addition of four electrons.[1] This process also generates reactive oxygen species (ROS) which are highly toxic by-products. ROS inflict a constant barrage of peroxidative damage upon proteins, DNA, lipid

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membranes and other biological molecules.[2–4] This process has been implicated in cellular aging as well as cellular damage in several pathological processes.[5–7] ROS are constantly manufactured in the body and cleared by antioxidant scavenging and enzymic degradation; the damage inflicted by uncleared ROS is termed oxidative stress.

A non-invasive marker of oxidative stress in humans would be clinically useful because it could indicate the intensity of a pathological process as well as the efficacy of a therapeutic intervention. A number of such markers have been proposed, including ethane and pentane in the breath.[2–4] The rationale of breath testing is that ROS degrade biological membranes by lipid peroxidation, converting polyunsaturated fatty acids (PUFAs) to alkanes which are excreted through the lungs as volatile organic compounds (VOCs). Breath pentane has been reported as a marker of increased oxidative stress in diseases as diverse as breast cancer,[8] heart transplant rejection,[9] acute myocardial infarction,[10] schizophrenia,[11] rheumatoid arthritis[12] and bronchial asthma.[13]

Information about breath VOC markers of oxidative stress is lacking in two main areas: First, studies of human breath alkanes have focused near-exclusively on ethane and pentane, and there are comparatively few reports of other alkanes in normal human breath. We have observed decane and undecane in the breath of patients with lung cancer[14] and propane, butane, hexane, heptane and octane have been observed in the breath of animals.[3]

Second, few studies have accounted for alkanes in the inspired ambient air, where they appear to be near-universal contaminants. Cailleux and Allain questioned whether pentane was a normal constituent of human breath, because its concentrations in breath and room air are frequently similar.[15] We have proposed that this problem may be resolved by determination of the alveolar gradient of a VOC, the difference between its concentration in the breath and in the ambient air.[16,17] Kinetic analysis has demonstrated that the alveolar gradient varies with the difference between the rate of synthesis of a VOC and its rate of clearance from the body.[18] Hence the polarity of the alveolar gradient indicates which of the two processes is predominant: if the alveolar gradient is positive, the rate of synthesis of a VOC is greater than the rate of clearance, and vice versa if the alveolar gradient is negative.

We employed a highly sensitive assay in order to measure the concentrations of alkanes in breath and in air, and to determine whether the alveolar gradients of individual alkanes vary with carbon chain length, age and sex in normal humans.

MATERIALS AND METHODS

Breath Collection Apparatus (BCA) and Assay

The BCA is a portable microprocessor-controlled device with a heated breath reservoir which prevents condensation of water. Alveolar breath was pumped from the breath reservoir through a sorbent trap which captured the VOCs on activated carbon. Sorbent traps contained 200 mg Carbotrap C (20/40 mesh) and 200 mg Carbopack B (60/80 mesh) (Supelco, Inc., Bellefonte, PA). The volume of the breath sample could be varied via a panel-mounted timer and flow meter, and the geometry of the system ensured that the sample comprised alveolar breath virtually uncontaminated by dead-space air. VOCs were desorbed in the laboratory with an automated thermal desorber (ATD 400, Perkin Elmer, Norwalk, CT, USA) which heated the sorbent trap to 300°C. A stream of helium flushed the desorbed VOCs onto a second smaller sorbent trap maintained at 0°C. The cold trap was then heated to 300°C, and the volatilized concentrated sample of breath VOCs was separated by gas chromatography (GC), and identified and quantified by mass spectroscopy (MS). Full details of the method have been described elsewhere.[18,19]

Collection of a Breath Sample

Two 1.0 l samples were collected: one of breath, and one of background room air. Subjects wore a nose clip while inspiring and expiring through a disposable mouthpiece. They encountered very little resistance to expiration because the mouthpiece opened into a wide bore tube (1.0 inch, 2.4 cm dia) which was open to the atmosphere at its distal end. The only resistance to inspiration and expiration was provided by light one-way flap valves in the mouthpiece. No subjects in this study complained of any difficulty in donating a breath specimen. The low-resistance of the breath collection apparatus ensured that breath samples could be collected without discomfort, even for the elderly or patients with respiratory disease. The collection period was 2.0 min at 0.5 l/min.

Human Subjects

Breath samples were collected from 50 normal volunteers comprising 27 males (mean age 38.8 year, SD = 12.8) and 23 females (mean age 38.65 year, SD = 11.4). All had fasted from the previous midnight in order to minimize any potential confounding effects of a recent meal and were collected between 7:00 am and 12:00 noon. Subjects generally sat for approximately 30 min prior to the collections of breath and air in order to allow time for equilibration between VOCs in room air and in the blood. Human research was approved by the institutional review board of St. Vincent's Medical Center, Staten Island, NY. Details of the human research have been described elsewhere.[18]

Analysis of Data

Molar concentrations in breath and air were read from standard curves constructed by loading known quantities of alkanes and internal standard onto sorbent traps for analysis by ATD/GC/MS in the same fashion as the breath samples. Alkane standards were gaseous (C4–C7, Scott Specialty Gases, Plumsteadville, PA 18949) or in chloroform solution (C8–C20, Sigma Chemical Co., St. Louis, MO 63178); internal standard (0.25 ml 2 ppm 1-bromo-4-fluoro-benzene, Supelco) was added via the ATD standard injection accessory. Alveolar gradient was determined as $C_{\text{alveolar breath}} - C_{\text{room air}}$ where C = concentration of VOC (mol/l). In each subject, the alkane profile was determined by plotting alveolar gradient as a function of carbon chain length. These profiles were compared in males and females, and older and younger subjects.

RESULTS

The mean concentration of each alkane in breath and air (including those with zero concentration), frequency of occurrence and alveolar gradient are shown in Figure 1. The profile of mean alveolar gradients of alkanes was negative from C4 to C12, and positive from C13 to C20. The median age was used to split the group into older and younger halves (older half: range 35–75, mean = 47.56, SD = 11.18; younger half: range 23–35, mean = 29.88, SD = 3.23, $p < 0.0001$). The mean alveolar gradients of four alkanes (C5–C8) were significantly higher in the older subjects ($p < 0.05$) (Figure 2). There were no significant differences between the alkane profiles in males and females. Smokers comprised only 5/50 subjects and were not analyzed separately (three were in the younger half, two were in the older half).

DISCUSSION

In the past, studies of breath markers of oxidative stress have emphasized the roles of pentane and ethane, possibly because they are comparatively easy to detect by GC. However, the conversion of PUFAs to alkanes by oxidative stress is a generic pathway which is not restricted to ethane and pentane; animal studies of oxidative stress have also employed propane, butane, hexane, heptane and octane as markers.[2,3]

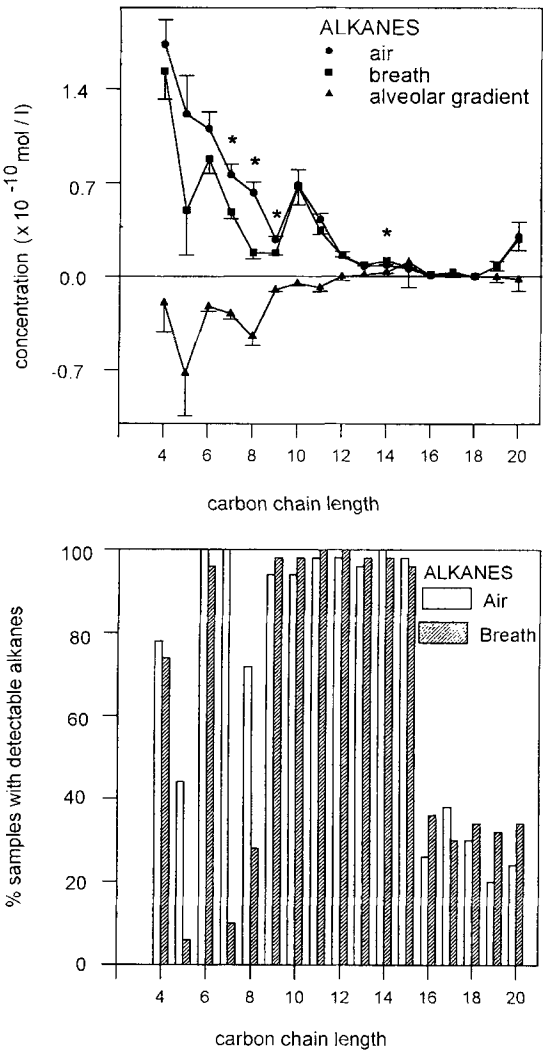

FIGURE 1 Alkanes in breath and air. (a) The upper panel shows the mean concentrations of alkanes in breath and air, their alveolar gradients (concentration in breath minus concentration in air), and their variation with carbon chain length. Asterisks indicate significant differences between concentrations in breath and air ($p < 0.05$). (b) The lower panel shows the frequency distributions of their presence in samples of breath and air.

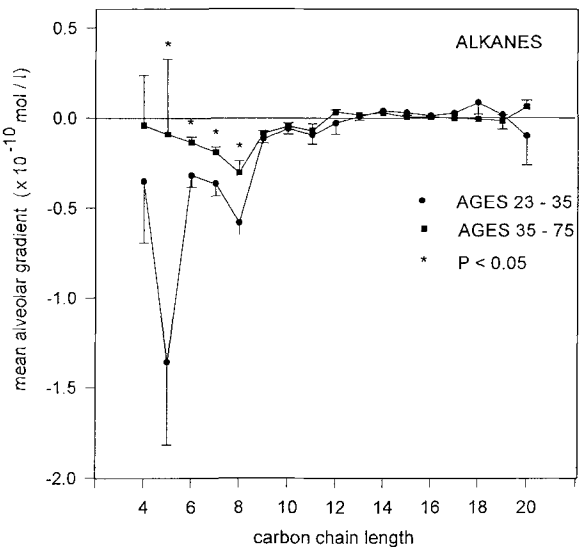

FIGURE 2 Effect of age on the breath alkane profile. The profile of alveolar gradients is shown for the younger and older half of the normal subjects. The significant increases in alkanes in the older subjects were consistent with increased oxidative stress, though reduced clearance of alkanes may have contributed.

This study identified 17 n-alkanes (C4–C20) in room air and in normal human breath, a larger number than has previously been reported. Apart from pentane, these alkanes may comprise new markers of oxidative stress in humans. Their mean alveolar gradients were negative from C4 to C12, and positive from C13 to C20 (Figure 2). This accorded with our earlier observation that the mean alveolar gradient of breath pentane was negative in normal humans.[17] The physiologic significance of the alveolar gradient profile may be inferred from kinetic analysis:

$$\text{alveolar gradient} = \frac{(R_{\text{synthesis}} - R_{\text{clearance}})}{\text{RMV}}$$

where R = rate of synthesis or clearance of VOC (mol/min) and RMV = respiratory minute volume (l/min).[18] The breath alkane profile demonstrates that in normal humans, the mean rate of clearance was greater than the mean rate of synthesis for C4–C12 alkanes, while the opposite was true for C13–C20 alkanes.

The breath alkane profile was significantly less negative in the older subjects. This finding was consistent with previous reports of increased breath pentane excretion with age in healthy normal humans.[20,21] A similar age-related change in breath ethane, butane and pentane occurs in

rats.[22] The physiologic basis of the elevated alkane profile is consistent with an age-related increase in oxidative stress.

Since the alveolar gradient varies with the difference between the rates of synthesis and clearance, it is also possible that reduced clearance of alkanes may have contributed to this observation. Alkanes are metabolized to alkyl alcohols, mainly via the hepatic cytochrome p450 pathway.[23,24] The total cytochrome p450 content of human liver declines with age,[25] which may manifest as a clinically significant reduction in drug clearance rate.[26,27] However, this is not true of all cytochrome p450 hydroxylation enzymes: there is no age-related decline in the activity of hepatic microsomal hydroxylation of alprazolam[28] or of cutaneous aryl hydrocarbon hydroxylase.[29] Further studies are required to determine the contribution, if any, of declining cytochrome p450 activity to the age-related changes in the breath alkane profile.

The greatest difference between frequency of occurrence of an alkane in room air and breath was observed in heptane (Figure 2). It was detected in all samples of room air but in only 10% of alveolar breath samples. Inspired heptane appeared to be cleared from the body with high efficiency by metabolism and excretion, thereby reducing its concentration to undetectable levels in the pulmonary artery and the alveolar breath of 90% of the normal subjects. Similarly, pentane was observed in more room air samples than in breath samples, providing evidence that pentane was energetically cleared from the body, probably via the cytochrome p450 pathway in the liver. The marked negative alveolar gradient of pentane is consistent with our previous observations,[17] and supports the conclusion that pentane is cleared more rapidly than it is synthesized in most normal humans.

The source of alkanes in room air is not known; they may have been derived from the breath of other humans, other biological sources (such as animals and plants) or non-biological sources, such as industrial or automotive emissions. Further studies will be required to determine the origins of room air alkanes and whether this is characteristic of room air at other geographic sites. Experience in our laboratory and elsewhere indicates that pentane can commonly be detected as a contaminant of room air when a sufficiently sensitive assay is employed.[15,17] A similar spectrum of alkanes has been reported in the cabin air of the space shuttle.[30] The origin of methylalkanes observed in the breath is also unknown; one possible source may be methylation of alkanes by microsomal isoenzymes of the cytochrome p450 system.[31]

The study of alkanes in human breath has been complicated by methodological difficulties with the assay, which must be specific and clearly distinguish different VOCs from one another. Previous studies have been questioned because breath pentane assays were apparently contaminated by isoprene, the most abundant VOC in human breath.[32] However, we have found that with careful choice of GC column and analytical conditions, it is possible to separate more than 200 VOCs in individual samples of human breath,[18] and without co-elution of pentane and isoprene.[17]

Other researchers have attempted to resolve the problem of VOCs in background air by supplying their test animals or human subjects with purified air to breathe. We elected not to do this for three main reasons: First, we have found even the most highly purified breathing air from commercial sources to be contaminated with picomolar to nanomolar quantities of alkanes when analyzed by the methods described above. Second, even if truly alkane-free breathing air were available, it would subject the person breathing it to an abnormal and unphysiological stress, because normal humans breathing normal air constantly inspire ambient VOCs. Third, the presence of a VOC in inspired air makes it possible to determine its alveolar gradient, which varies with the kinetic difference between its rate of synthesis and its rate of metabolism. This opens a unique new window onto metabolic activity, and provides

an opportunity to obtain new information that would be otherwise unavailable.

We conclude that normal human breath contains a wider spectrum of alkanes than has previously been reported, and these compounds may include new markers of oxidative stress. We are currently evaluating these alkanes in a number of different disorders, including lung cancer,[14] heart transplant rejection and ischemic heart disease. The breath alkane profile might provide a clinically useful new marker of oxidative stress in disease and aging.

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