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Gas chromatographic method using photoionization detection for the determination of breath pentane

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Abstract

Lipid peroxidation is thought to be an important event in the pathogenesis of atherosclerosis. It has been suggested that pentane, which can be formed during the oxidation of ω-6 fatty acids, is a marker of lipid peroxidation. Previous studies have reported elevated breath pentane and serum markers of lipid peroxidation in smokers. However, chromatographic separation of pentane from isoprene in virtually all of these studies was incomplete and the methods used did not resolve pentane into its isomers, *n*-pentane and isopentane. Additionally, most current methods are complicated, requiring trapping and concentrating steps to obtain adequate sensitivity prior to hydrocarbon analysis. The purpose of the current study was to develop a gas chromatographic system to analyze breath pentane, that addresses the above technical problems and that would provide a simple in vivo method for measuring lipid. *n*-Pentane and isopentane standards were easily separated from isoprene with a Al_2O_3/KCl capillary column contained in a portable gas chromatograph equipped with a photoionization detector. The analysis of repeated measures showed a low coefficient of variation for measurements of *n*-pentane (10%) and isopentane (9%). We measured breath pentane in 27 subjects (15 smokers, 12 non-smokers). There were no significant difference between the baseline and 4 week interval measurements of *n*-pentane for smokers both before and after cigarette smoking. The within-subject variability data showed that the assay is highly reproducible for both low and high pentane levels in smokers. Smokers were found to have higher levels of both *n*-pentane and isopentane than non-smokers ($P<0.001$). In addition, smokers had further significant elevation of pentane levels 10 min after smoking ($P<0.001$), which returned to baseline by 1 h. These studies demonstrate that measurement of breath pentane, using a gas chromatograph with a photoionization detector, is simple and reproducible. Additionally, these results suggest that pentane elevation associated with smoking is secondary to the oxidant effects of cigarette smoke and an important temporal relationship exists between cigarette smoking and breath sample analysis.

Keywords: Lipid peroxidation; Pentane

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1. Introduction

Epidemiologic, animal and human studies support the concept that elevated serum levels of low-density lipoprotein (LDL) increase the risk for atherosclerotic cardiovascular disease [1,2]. However, an understanding of the basic mechanisms that underlie the atherogenicity of LDL is incomplete and currently evolving. Recent studies suggest that oxidation of LDL is an early event in the atherogenic process [3]. Lipoprotein oxidation begins with oxidation of polyunsaturated fatty acids forming lipid peroxides which ultimately decompose to monohydroperoxides, aldehydes such as malondialdehyde, ketones and volatile hydrocarbons. The byproducts of oxidized LDL, such as malondialdehyde, can bind to apolipoprotein B-100 forming aldehyde–protein adducts that change the configuration of the protein moiety of LDL leading to recognition by scavenger receptors. The modified LDL is more avidly incorporated into macrophages than LDL which leads to the formation of foam cells and atherosclerosis [2].

Investigators have developed various in vitro methods to measure the products of lipid peroxidation [4,5]. None of these techniques, however, can be used in vivo and most of them require lengthy analytical procedures. The growing interest in the role of lipid peroxidation in atherogenesis has stimulated the search for simple markers of in vivo lipid peroxidation. In the 1970's, the volatile hydrocarbons ethane and pentane were first reported to be measurable products of in vivo lipid peroxidation [6–8]. Ethane is formed from the peroxidation of ω-3 fatty acids, such as linolenic acid, while pentane is produced from peroxidation of ω-6 fatty acids, such as linoleic and arachidonic acid. These two volatile products of fatty acid oxidation are detected in the breath and have been demonstrated to reflect in vivo lipid peroxidation [9,10].

Cigarette smokers are at risk for increased lipid peroxidation since inhaled cigarette smoke contains many oxidants and also stimulates the release of oxidants from activated pulmonary macrophages [11,12]. Previous studies have reported elevated breath pentane and serum markers of lipid peroxidation associated with smoking [13,14]. However, chromatographic separation of breath pentane from isoprene in virtually all of these studies was incomplete [15,16] and the methods used did not resolve pentane into its isomers, *n*-pentane and isopentane. Although there is substantial evidence that pentane is a product of lipid peroxidation [6,7,16–18], the significance of isopentane is less clear. Therefore, separation of pentane into its isomers is important in order to determine the relative amount of *n*-pentane expired in the breath. Additionally, most current methods are complicated, requiring trapping and concentrating steps to obtain adequate sensitivity prior to hydrocarbon analysis. The purpose of the current study was to develop a gas chromatographic system to analyze breath pentane that addresses the above technical problems and that would provide a simple in vivo method for measuring lipid peroxidation. In addition, we determine the temporal relationship between smoking and levels of breath pentane, as well as the contribution of pentane in cigarette smoke to measured levels in breath. Our results suggest that breath pentane can be efficiently quantified using a portable gas chromatograph with a photoionization detector, and is acutely and chronically elevated in response to cigarette smoking.

2. Experimental

2.1. Gases

n-Pentane and isopentane calibration standards were obtained from Scott (Troy, MI, USA). Hydrocarbon free air was obtained from Acetylene Products (Indianapolis, IN, USA). The ultra-zero compressed-air carrier gas was obtained from Mattheson (Joliette, IL, USA).

2.2. Chromatographic system

The gas chromatographic system consisted of a portable gas chromatograph Model 10S 70 (Photovac International, Deer Park, NY, USA) together with a Al_2O_3/KCl capillary column 10 cm×0.53 mm I.D. (Chrompack Corporation, Netherlands). Gases are detected with a photoionization unit that emits photons from an ultraviolet lamp that ionize sample molecules in the carrier gas stream. Permanent gases (N_2, O_2, CO_2) including water vapor, are not ionized. An electrical signal is generated after ioniza-

tion that is proportional to the quantity of gas present and based on a pentane calibration curve. The results are expressed in parts per billion (ppb). The chromatographic analyses were performed at a temperature of 30°C using an isothermal oven at a flow-rate of 8 ml/min. Ultra zero compressed air was used as the carrier gas. Samples are directly transferred from tedlar collection bags to the chromatographic column via a 2-ml sampling loop. Care should be taken to attach the tedlar bag rapidly to the chromatographic machine.

2.3. Pentane collection

Healthy cigarette smokers ($n=15$) and non-smokers ($n=12$) were recruited for assessment of breath pentane. None of the subjects used prescription medication or had a history of chronic disease. Breath samples were collected after subjects breathed for 1 min through a mouthpiece connected to a one-way multiport valve from a Tedlar bag containing hydrocarbon free air (HCFRA). The exhaled air was discarded. Thus hydrocarbon-containing atmospheric air was largely eliminated from the lungs. After the HCFRA washout, one exhaled breath was collected into a 1-l Tedlar bag for pentane measurement. The nares was closed with a clip throughout the sampling process to prevent contamination of the collected sample with room air. *n*-Pentane, isopentane and total pentane (total pentane=*n*-pentane+isopentane) levels were measured in the breath of each subject, in duplicate.

Seven cigarette smokers underwent an assessment of the temporal relationship between cigarette smoking and breath pentane levels. These subjects were asked to abstain from eating and smoking overnight. Breath samples were collected, in the above described fashion, the next morning for analysis. Subjects then smoked one cigarette over 2 min. Breath samples were obtained for pentane analysis 10 min and 1 h after smoking.

Eight other smokers were asked to provide breath samples, after having abstained from smoking for at least 1 h prior to breath collection. No eating restrictions were imposed on these subjects. Breath pentane levels of these eight smokers were then compared to those of 12 non-smokers. In addition, the 1 h post smoking *n*-pentane levels for both groups of smokers were combined ($n=15$) and compared to the non-smokers ($n=12$).

2.4. Assay validation by gas chromatography mass spectrometry

Breath samples and pentane standards were introduced directly into a Hewlett-Packard 5890 Series II gas chromatograph equipped with an HP 5971 mass selective detector. Sample injection was accomplished via a six-port sample valve fitted with a 1-ml gas sample loop. Separations were performed with a Chrompack 50 m×0.32 mm I.D. PLOT fused-silica capillary column coated with Al_2O_3/KCl in the temperature programmed mode. The oven temperature profile consisted of a 40°C initial temperature held for 3 min and then followed by a 10°C/min temperature ramp to a final temperature of 160°C which was held for 5 min. The carrier gas was helium at a flow-rate of approximately 0.6 ml/min. The mass spectrometer was operated in the selected ion mode with the multiplier set at +400 relative to the autotune valve. Ions *m/e* 27, 41, 42, 57 and 72 were monitored during the chromatographic analysis. Peak identification was based on comparison of retention times and ion ratios of standard reference materials to those observed in breath samples.

2.5. Statistical methods

The variability of repeated measures was determined by analyzing pentane standard at 45 min intervals throughout a 24-h period ($n=32$) and results expressed as the coefficient of variation (C.V.). The within subject variability for both smokers ($n=11$) and non-smokers ($n=8$), was determined by measuring one breath sample for pentane levels at baseline and four week intervals. The variability was calculated using the mean difference and the standard deviation of these differences. A Pearson correlation coefficient was used to determine if a correlation existed between the two measurements and a paired *t*-test to determine if there was a significant change between the first and fourth week of the study.

Data are given as means±S.D., except for breath pentane levels which were skewed to the right in the smokers; therefore, medians and 75th percentiles

have been presented. The Mann–Whitney U-test was used to compare the breath pentane levels in smokers and non-smokers while the Friedman test was used to look at the temporal relationship of cigarette smoking and breath pentane production. Statistical comparisons between smokers and non-smokers for all other variables were based on the unpaired *t*-test.

3. Results

3.1. Subject demographics

Twenty-seven subjects were enrolled in the study. There were 5 men and 10 women in the smoking group and 9 men and 3 women in the non-smoking group. The mean age of smokers was 37.1 years and for non-smokers 36.6 years. There were 12 Caucasians and 3 African–Americans in the smoking group and 10 Caucasians, 1 African–American and 1 Asian in the non-smoking group. None of the subjects had diabetes, hypertension, a history of myocardial infarction or known pulmonary disease. Four subjects in the smoking, and one subject in the non-smoking group had a family history of coronary artery disease. One of the smokers and two of the non-smokers used multivitamins. None of the subjects used prescription medications or supplemental antioxidants. All smokers smoked at least 1/2 pack of cigarettes per day and there was no restriction on the total number of cigarettes smoked.

3.2. Validation of measurements

Previous methodologies for assessing breath pentane have utilized gas chromatography for hydrocarbon separation followed by flame ionization for detection and quantification. Because of the higher sensitivity of photoionization, we selected this methodology for our studies. An Al_2O_3/KCl encapsulated wide bore capillary column was used to separate *n*-pentane and isopentane isomers.

Recently, Kohlmuller et al. [15] questioned the validity of *n*-pentane analysis with commonly used GC columns combined with a flame ionization detector. They demonstrated that columns used in previous studies did not separate pentane from isoprene and suggested that isoprene may account for much of the 'pentane' measured in breath. As shown in Fig. 1, standard gas chromatography combined with mass spectrometry (GC–MS) identified both *n*-pentane and isopentane in the breath. Also, the *n*-pentane, isopentane and isoprene gases present in a smokers breath sample could be easily distinguished with GC–MS using an Al_2O_3/KCl column (Fig. 1). Furthermore, the Al_2O_3/KCl Column used with the photoionization GC in this study easily separated isoprene from both *n*-pentane and isopentane standards due to the longer retention time of isoprene (Fig. 2).

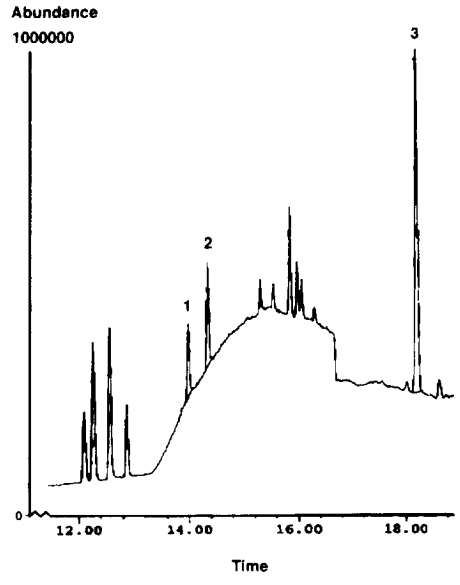

Fig. 1. GC–MS profile, using an AL_2O_3/KCl column, of a breath sample from a smoking study subject after smoking one cigarette. Peaks: 1=isopentane; 2=*n*-pentane; 3=isoprene. Identification was based on comparison to known standards.

3.3. Reproducibility and within subject variability

The analysis of 32 repeated measures of *n*-pentane and isopentane showed a low coefficient of variance (Table 1). The coefficient of variance for *n*-pentane was 10.3% and isopentane was 9.4%. The within-subject variability data showed that the assay is highly reproducible for both low and high pentane levels in smokers (Table 2). There was no significant difference between the baseline and 4 week interval measurements of *n*-pentane for smokers both before

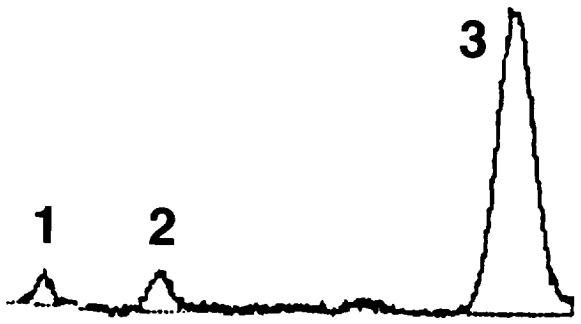

Fig. 2. GC–PID chromatogram showing isopentane (1), *n*-pentane (2) and isoprene (3) using an AL_2O_3/KCl column.

Table 1
Repeated measures analysis

	Mean± S.D.	C.V.	Range
n-Pentane	5.75±0.59	10.3%	4.8–6.7
Isopentane	8.62±0.81	9.4%	7.4–10.0

Mean *n*-pentane and isopentane levels±standard deviation in parts per billion. C.V. indicates coefficient of variation of 32 repeated measurements.

and 10 min after cigarette smoking. A positive correlation was also noted between the baseline and 4 week measurements for *n*-pentane and isopentane for smokers both before and after cigarette smoking. Also, there was no significant difference for non-smokers *n*-pentane and isopentane levels at baseline and four weeks.

3.4. Effects of inhaled pentane on pentane exhalation

Cigarette smoke contains over 4000 compounds, one of which is pentane. Previous studies have not evaluated the possibility that elevated pentane levels in smokers may be due to retained pentane from cigarette smoke. In order to assess the potential effects of inhaled and retained pentane on subsequent measurements of breath pentane, 7 subjects (5 non-smokers, 2 smokers) were asked to take 10 'puffs' (i.e. 100 cc/puff) of air from a 1-l Tedlar bag containing varying concentrations of *n*-pentane ranging from 16 to 23 000 parts per billion (ppb). The number of 'puffs' corresponds to the estimated average inhalation of a typical cigarette and 20 000 ppb of pentane is the highest reported level of pentane in cigarette smoke [20]. After pentane inhalation, subjects breathed room air and underwent breath pentane collection after 10 min with results as summarized in Table 3. No significant effect of pentane inhalation was observed on subsequent breath pentane measurements. This suggests that pentane from cigarette smoke is retained only briefly in the lungs and will not contaminate measurements of breath pentane levels collected at least 10 min after cigarette smoking.

3.5. Pentane levels

The temporal relationship of cigarette smoking to breath pentane levels was investigated in seven consecutive subjects. The initial breath sample of these smokers was obtained after overnight abstinence from smoking and food. At 10 min after smoking one cigarette, *n*-pentane and isopentane levels increased markedly above baseline measurements ($P<0.001$). Both *n*-pentane and isopentane levels returned to baseline levels at 1 h after smoking. Fig. 3 shows a representative chromatogram of

Table 2
Reproducibility of assay

Smokers		Baseline	Week 4	Paired *t*-test	Correlation
Pre-smoking ($n=11$)	*n*-Pentane	4.1±6.2	3.4±3.8	$P=0.62$	$r=0.64$
	Isopentane	4.9±3.0	3.3±2.3	$P=0.02$	$r=0.74$
Post-smoking ($n=9$)[a]	*n*-Pentane	22.9±6.9	25.0±15.6	$P=0.59$	$r=0.79$
	Isopentane	17.4±6.0	16.8±9.8	$P=0.82$	$r=0.67$
Non-smokers ($n=8$)	*n*-Pentane	1.5±1.2	1.4±1.1	$P=0.77$	$r=0.58$
	Isopentane	3.2±1.7	3.3±2.2	$P=0.90$	$r=0.29$

Mean *n*-pentane and isopentane levels±standard deviation. All data presented as parts per billion.
[a] 10 min after smoking.

Table 3
Breath pentane levels before and 10 min after inhalation of *n*-pentane

Pentane concentration[a]	Isopentane		*n*-Pentane		Total pentane	
	Pre	Post	Pre	Post	Pre	Post
16.5	1.5	1.1	2.4	1.3	3.9	2.4
21.8	3.9	1.9	0.3	2.0	4.2	3.9
36.7	1.4	1.5	0.3	1.3	1.7	2.8
40.6	0.5	1.0	6.0	5.9	6.5	6.9
48.3	1.7	1.8	1.8	1.5	3.5	3.3
225	3.2	2.8	2.6	0.4	5.8	3.4
350	2.4	2.3	5.2	4.4	7.6	6.7
23 500	10.0	5.7	1.8	0.9	11.8	6.6
Average	3.0	2.3	2.6	2.2	5.6	4.5

[a] Indicates pentane inhalation concentration. All data presented as parts per billion.

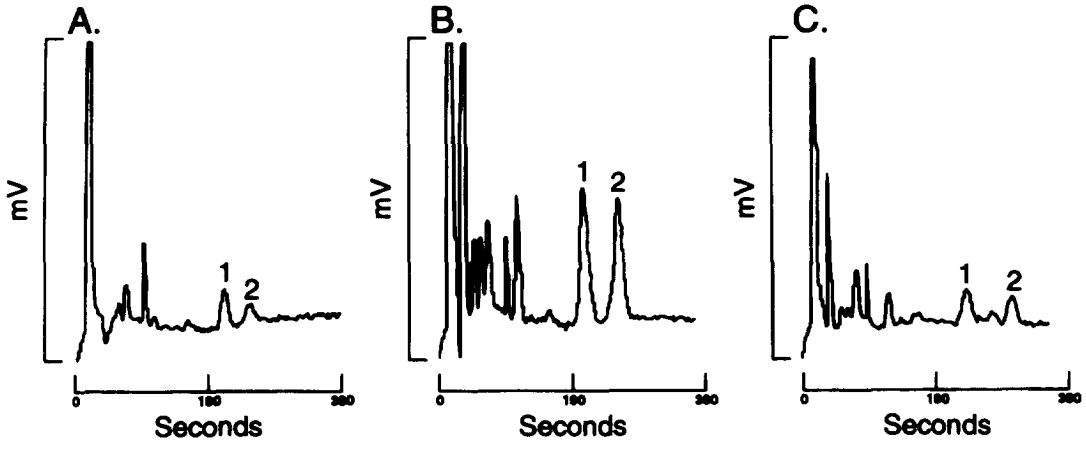

Fig. 3. Representative chromatogram of a breath sample after an overnight smoking abstinence (A), 10 min (B) and 1 h (C) after smoking one cigarette using the PID gas chromatograph. Peaks: 1=isopentane; 2=*n*-pentane.

one subject's breath pre and post cigarette smoking. Isoprene is not included in this figure as it is retained on the column much longer than isopentane or n-pentane. No significant difference in *n*-pentane and isopentane levels were detected between baseline and 1 h after smoking (Table 4).

In order to assess the feasibility of random breath pentane measurement, eight cigarette smokers were asked to give breath samples after a 1-h smoking abstinence. The 1-h smoking abstinence *n*-pentane and isopentane levels were significantly higher ($P<0.001$) in smokers ($n=8$) compared to non-smokers ($n=12$), (Table 5).

Further comparisons were also made between smokers and non-smokers. All three mean pentane

Table 4
Temporal relationship of cigarette smoking and pentane levels in seven smokers

		Mean	S.E.M.	*P*-value
n-Pentane	Baseline	7.4	1.36	<0.001
	10 min	28.7	3.09	
	1 h	5.0	0.83	
Isopentane	Baseline	5.0	0.58	<0.001
	10 min	19.2	1.61	
	1 h	3.9	0.67	
Total pentane	Baseline	12.4	1.77	<0.001
	10 min	47.9	4.38	
	1 h	8.8	1.47	

All data are in parts per billion. All differences were statistically significant using repeated measures ANOVA.

Table 5
Pentane levels in eight smokers and twelve non-smokers

		Median	Range	*P*-value
n-Pentane	Smokers	5.1	1.1–54.5	<0.001
	Non-smokers	1.4	1.0–3.4	
Isopentane	Smokers	5.9	1.5–32.4	<0.001
	Non-smokers	1.9	1.2–3.7	
Total pentane	Smokers	11.8	3.0–66.4	<0.001
	Non-smokers	3.6	2.3–6.0	

All data are in parts per billion. All differences between smokers and non-smokers were statistically significant using the Mann–Whitney U-test.

levels from the smoking group that abstained from smoking overnight (i.e. baseline, 10 min after and 1 h after smoking) were higher than mean levels of the non-smoking control group ($P<0.001$). Because pentane levels returned back to pre-smoking levels after 1 h, the 1 h post smoking pentane levels from the group that abstained from smoking overnight were combined with the randomly obtained pentane levels of smokers. The mean *n*-pentane levels (8.3 ppb) of this combined smoking group ($n=15$) were also significantly higher ($P<0.001$) than the mean *n*-pentane levels (1.6 ppb) of the non-smoking group (data not shown). Ten min after cigarette smoking, mean *n*-pentane levels were 15 fold higher than non-smokers *n*-pentane levels (Table 4).

4. Discussion

Flame ionization techniques have been used in most prior studies of breath pentane, but require larger samples to reach adequate sensitivity thereby necessitating additional measures to concentrate the breath sample prior to separation by gas chromatography [20–22]. Other investigators have shown photoionization detectors to be more sensitive than flame ionization detectors for light hydrocarbon analysis [23]. The present study demonstrates that detection and quantification of pentane isomers in breath by photoionization detection after separation by gas chromatography is simple, rapid and reproducible; and may be a useful method for measuring lipid peroxidation in vivo.

Previous studies have only reported levels of total pentane present in exhaled breath. It is likely that the pentane measured was actually a mixture of *n*-pentane and isopentane, which were readily separated by the capillary columns containing Al_2O_3/KCl reported here. *n*-Pentane arises from ω-6 fatty acids during decomposition of lipid hydroperoxides. Isopentane may arise by simple isomerization of pentane or by decomposition of substituted lipid hydroperoxides. In either case, the changes in pentane with cigarette smoking were paralleled by similar changes in isopentane, suggesting a similar origin of the two.

Recently, Kohlmuller et al. [15] demonstrated that many GC columns (e.g. alumina, etc.) frequently used to separate pentane from other gases uniformly, failed to resolve pentane from isoprene. This is an important distinction as isoprene is normally present in greater quantities than *n*-pentane in both breath and cigarette smoke. As we have shown in this study, capillary Al_2O_3/KCl columns easily separate pentane from isoprene and, thus contamination of pentane measurements with isoprene is not a problem.

The present study demonstrates that smokers have higher levels of *n*-pentane and isopentane compared to non-smokers. In addition, pentane levels markedly increase after smoking and decrease to baseline 1 h after smoking; although smokers continue to exhale higher levels of pentane than nonsmokers even after 1 h or more of abstinence. Thus, smoking is associated with both acute and chronic elevation of breath pentane.

The source of pentane production that occurs immediately after smoking is unclear. Cigarette smoke contains significant quantities of pentane. Studies of hydrocarbon content in cigarette smoke report values ranging from 200 to 20 000 ppb in a single 40 cc 'puff' [14,19]. Pentane from inhaled smoke could become trapped in alveoli and exhaled over several minutes and thereby falsely elevate pentane measurements. It is unknown whether pentane associated with cigarette particulate matter could be trapped in the alveolar space for a longer period of time than 'pure' pentane. Our studies suggest, however, that inhaled pentane from cigarette smoke is not the source of exhaled pentane. Rather, the latter is generated in response to cigarette smoking.

Smokers are subjected to oxidant stress from free radicals present in cigarette smoke [11,12]. Free radicals are found in both the tar and gas phases of the smoke. For example, a quinone–hydroquinone complex, capable of reducing molecular oxygen to superoxide radicals, is present in the tar phase. The gas phase of cigarette smoke contains highly reactive oxygen- and carbon-centered radicals. In vitro studies of gas phase oxidants from cigarette smoke have demonstrated increased lipid peroxidation and modification of lipoproteins in human plasma [24]. These radicals and associated products from free radical reactions may increase ω-6 fatty acid oxidation in both lung tissue and alveolar capillary blood. Thus, the acute increase in pentane after smoking may result from a local free radical effect on pulmonary polyunsaturated fatty acids, causing release of pentane into the respiratory tract.

Cigarette smoking also induces an inflammatory response in the lung. Both leukocytosis and elevation of acute phase reactants reportedly occur with chronic smoking [25]. This inflammatory response results in the accumulation of alveolar macrophages and neutrophils [26]. These phagocytes generate increased amounts of reactive oxygen species such as hydrogen peroxide, superoxide radicals and hydroxyl radicals [12,27], which renders polyunsaturated fatty acid containing particles, such as LDL more susceptible to peroxidative modification [28]. Although the ultimate disposition of these reactive species is not known, the oxidative modification of alveolar cell membrane lipids and serum particles rich in ω-6 fatty acids may result in increased chronic pentane production and elimination in the breath.

Another possible source of chronic pentane production in smokers could be release of stored pentane from adipose tissue as pentane is lipophilic. Pentane release from adipose tissue would depend upon the adipose–blood–air partition coefficients. The partition coefficients for pentane indicate that pentane tends to diffuse from blood to air at body temperature [9,29]. Thus, most pentane contained in cigarette smoke or generated from lipid peroxidation is likely eliminated in the breath and not stored in adipose tissue.

The metabolic clearance of pentane should also be considered in determining its source of elimination in smokers. Pentane is rapidly cleared from the blood primarily by the liver's cytochrome P-450 system and clearance is slowed with ethanol consumption [9]. Our subjects abstained from alcohol consumption prior to testing and thus the latter is an unlikely source of increased pentane in smokers. Also, none of the subjects used medications that could alter cytochrome P-450 metabolism. The effect of cigarette smoke on the liver's P-450 system is negligible and so decreased liver metabolism of pentane due to cigarette smoking is unlikely.

In conclusion, this study describes a sensitive method for measuring breath pentane and is the first to investigate in detail the temporal relationship between cigarette smoking and pentane exhalation. Lipid peroxidation, measured by breath pentane, is elevated acutely and chronically after smoking. Pentane elevation associated with smoking may be due to the oxidant effects of smoking. The temporal relationship between cigarette smoking and pentane should be considered when measuring breath pentane.

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