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Larsson, Lennart; Szponar, Bogumila; Pehrson, Christina

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PO Box 117
221 00 Lund
+46 46-222 00 00

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Tobacco smoking increases dramatically air concentrations of endotoxin

Abstract We used a mass spectrometry-based assay for identifying the endotoxin (lipopolysaccharide, LPS) marker (*R*)-3-hydroxytetradecanoic acid in cigarette smoke particles and found that smoking involved inhalation of 17.4 pmol of endotoxin per each smoked cigarette. Indoor exposure to environmental tobacco smoke (ETS) entailed inhalation of 12.1 pmol of LPS/m^3 air, an amount that was 120 times higher than the levels found in smoke-free indoor air. Endotoxin is one of the most potent inflammatory agents known, hence our results may help to explain the high prevalence of respiratory disorders among smokers, and they may also draw attention to a hitherto unknown or neglected risk factor of ETS.

L. Larsson, B. Szponar, C. Pehrson
Department of Medical Microbiology, Dermatology and Infection, Lund University, Lund, Sweden

Key words: Endotoxin; Tobacco smoke; Indoor air; Chemical markers; 3-Hydroxy fatty acids; Respiratory disorders among smokers.

Lennart Larsson
Lund University, Department of Medical Microbiology, Dermatology and Infection, Sölvegatan 23, 223 62 Lund, Sweden
Tel.: +46 46 177298
Fax: +46 46 189117
e-mail: lennart.larsson@mmb.lu.se

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Practical Implications
Endotoxin represents a largely neglected risk factor of smoking and passive smoking and this knowledge may lead to a reduced smoking.

Introduction

Lipopolysaccharides (LPS), or endotoxin, are macromolecules found in the outer membrane of gram-negative bacteria. Inhaled purified endotoxin has been shown to cause inflammation in the airways leading to compromising of lung function (Thorn, 2001). Occupational endotoxin exposure has in numerous studies been shown to mimic these effects (Douwes et al., 2003) and the comparatively much lower levels of endotoxin in home environments have been found to correlate with the severity of asthmatics' symptoms (Michel, 2003) and the prevalence of wheeze in infants (Park et al., 2001). Recent research suggests that early life endotoxin exposure may induce immune polarization toward a Th1 cytokine profile that may reduce risk of atopic diseases later in life (Braun-Fahrländer et al., 2002).

Chronic bronchitis is common in individuals exposed to occupational dust containing high concentrations of endotoxin, and it is also prevalent among smokers. This knowledge led Hasday et al. (1999) to investigate whether cigarette smoke contains endotoxin. Indeed, they found that substances in both tobacco and cigarette smoke showed activity in the *Limulus* amebocyte lysate (LAL) assay. The lipid A region of LPS contains (*R*)-3-hydroxy fatty acids (3-OH FAs) typically of 10–18 carbon chain lengths (Rietschel, 1976). In the present study, we used gas chromatography-tandem mass spectrometry (GC-MSMS) to analyze these acids in smoke from active smoking and in environmental tobacco smoke (ETS; from passive smoking) originating from commercially available cigarettes.

Materials and methods

Samples

We used a reference mixture of 3-OH FAs of 10, 12, 13, 14, 16, and 18 carbon chain lengths (Larodan AB, Malmö, Sweden) to assist in the identification and quantification of 3-OH FAs in the tobacco and the

tobacco smoke, and 3-OH FAs of 12, 13, and 14 carbon chain lengths when analyzing chiral configuration.

Samples (5–10 mg) of tobacco from commercially available cigarettes and the smoke from cigarettes in the same packages were analyzed.

The smoke generated in experiments involved both ETS ('passive smoking') and simulation of active smoking. To study ETS, we sampled airborne particles in an unventilated, 25-m^3 laboratory room (without any air-conditioning) with ongoing smoking of cigarettes. One cigarette was smoked every 30 min during 7 h (total 15 cigarettes). Sampling was achieved by pumping the air (12 l/min) through PVC filters (0.8 μm pore size, 37 mm I.D.; Poretics Corp., Livermore, CA, USA) that were positioned in the breathing zone (1.5 m above floor level); as a control, sampling was performed analogously in the same room but without cigarette smoking. To simulate active smoking, we connected a burning cigarette to vacuum via a 10-cm length of tubing and then created two 2-s puffs (vacuum) per min, which finished the cigarette in 8–10 min. Smoke transferred through the tubing was collected on the same type of filter as in the ETS experiments, and the filters were analyzed for 3-OH FAs. As a control, sampling was made analogously but without lightening the cigarettes.

Sample preparation and analysis

All samples (tobacco, filters) were analyzed for 3-OH FAs as methyl ester trimethylsilyl (TMS) derivatives as described elsewhere (Saraf and Larsson, 1996). In brief, samples were heated in acid methanol overnight and then subjected to various extraction and purification steps before the TMS derivatization and analysis. 3-OH Tridecanoic acid (3-OH $C_{13:0}$, 50 ng) was added as an internal standard. The amounts of LPS were calculated by dividing the number of moles of 3-OH $C_{14:0}$ by a factor of 4 (Saraf and Larsson, 1996).

In samples of tobacco and filters containing particles collected during the active smoking simulations, 3-OH FAs of 12–14 carbon chain lengths were also analyzed for chiral configuration. In brief, samples were heated in methanol and then the hydroxylated fatty acid esters were subjected to etherification using trimethylsilyldiazomethane. The preparations were hydrolyzed, and the free carboxylic acids were derivatized with a chiral reagent, (*S*)-phenylethylamine. Details on the preparation of the (*S*)-phenylethylamide methoxy derivatives are provided elsewhere (Gradowska and Larsson, 1994).

The GC-MS analyses were performed using an ion trap type of instrument where both ionization and mass analysis occur in the same chamber. The GC-MS instrument (Saturn 2000, Varian, Walnut Creek, CA, USA) was equipped with a fused-silica capillary column (CP-Sil 8CB-MS, 0.25 μm film thickness, 30 m × 0.25 mm I.D., Varian); splitless injection was applied. Helium was used as the carrier gas. The temperature of the column was programmed to rise from 90 to 280°C, at a rate of 20°C/min; the temperature of the injector, transfer line, and ion trap was held at 220, 280, and 180°C, respectively. The methyl ester TMS derivatives were analyzed by using GC-MSMS as described (Saraf and Larsson, 1996) whereas the (*S*)-phenylethylamide methoxy derivatives were analyzed by using selected ion storage covering the regions between *m*/*z* 100–125 and *m*/*z* 320–375.

Results

Figure 1 shows the results of GC-MSMS analysis of filters from ETS experiments. The internal standard was not added in this illustrated example. In the absence of tobacco smoke (controls), the filters contained only very small amounts of 3-OH FAs (close to the detection limit), and the 3-OH FA pattern was dominated by 3-OH $C_{16:0}$ followed by 3-OH $C_{18:0}$, 3-OH $C_{14:0}$ and 3-OH $C_{12:0}$. This pattern was disturbed by ETS, which resulted in a strong predominance of

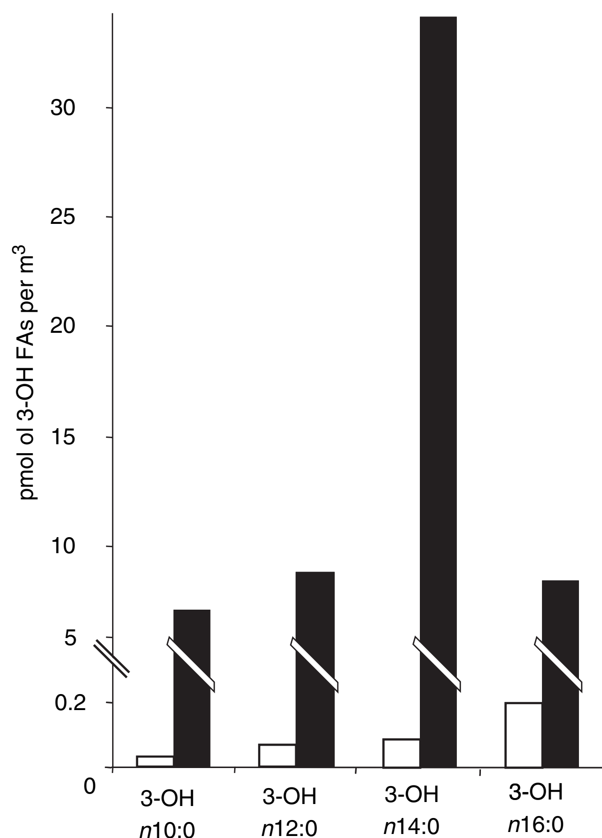

Fig. 1 3-Hydroxy fatty acids of 10–16 carbon chain lengths collected on filters by 7 h of air pumping in an unventilated room in the absence (white bars) and the presence (black bars) of cigarette smoke

3-OH $C_{14:0}$. The amount of LPS was 12.1 pmol/m^3 in the illustrated case of ETS, a level that was 120 times higher than that observed in the absence of tobacco smoke.

3-OH $C_{14:0}$ was also the dominating 3-OH FA in the particles collected on filters during active smoking, and, in the case depicted in Figure 2, smoke from one cigarette contained 17.4 pmol of LPS.

By applying (*S*)-phenylethylamide methoxy derivatization, we found that the detected 3-OH $C_{14:0}$ had (*R*)-configuration; its identity was confirmed by comparing retention time and mass spectrum with those of 3-OH $C_{14:0}$ in a reference mixture. Figure 2 shows that the retention times and spectra are identical, both exhibiting abundant ions of *m*/*z* 105, *m*/*z* 120, and *m*/*z* 362 ($M^+ + 1$).

The cigarette contained 18.8 pmol of LPS per mg of tobacco (14.2 nmol per cigarette); 3-OH $C_{14:0}$ dominated followed by 3-OH $C_{12:0}$ and 3-OH $C_{10:0}$ (data not shown). Thus, the amount of LPS inhaled during active smoking (17.4 pmol/cigarette) represented only 0.12% of the total of LPS in a cigarette.

Discussion

Endotoxin is ubiquitous in indoor environments and may originate from materials (soil, dust, plants, and water) brought in from outdoors, various indoor

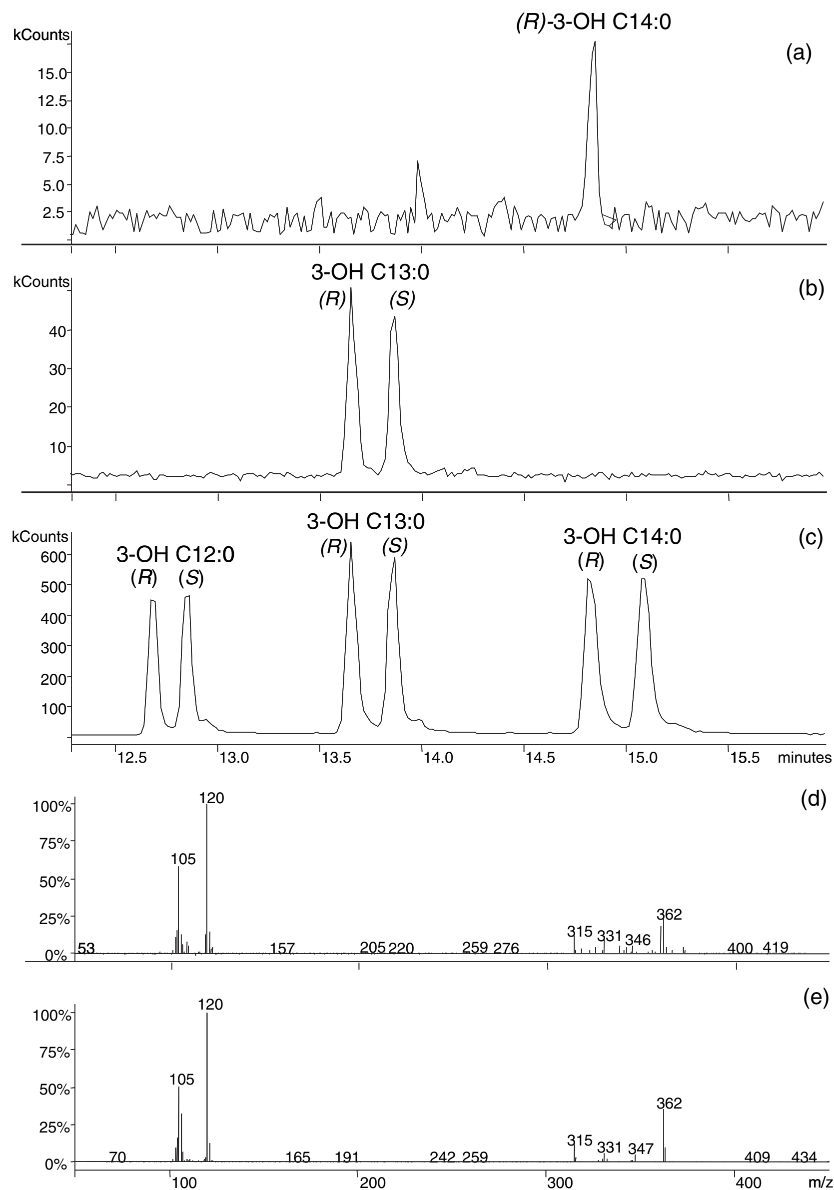

Fig. 2 GC-MS tracings of (*S*)-phenylethylamide methoxy derivatives of 3-hydroxy fatty acids collected on filters during simulated active smoking of six cigarettes (a); the internal standard 3-OH $C_{13:0}$ in the studied sample (b); and 3-OH $C_{12:0}$, 3-OH $C_{13:0}$ and 3-OH $C_{14:0}$ in an external standard mixture (c). Also shown are full MS spectra of the (*S*)-phenylethylamide methoxy derivative of (*R*)-3-OH $C_{14:0}$ in the tobacco smoke (d) and external standard mixture (e)

activities such as washing and cooking, and high humidity caused by water leakage or use of inadequately dried building materials. Endotoxin is one of the most potent inflammatory mediators known. Indeed, Hasday et al. (1999) found that both tobacco and tobacco smoke from commercially available cigarettes contain LAL-reactive material; however, as the LAL assay used may be activated not only by endotoxin but also by other compounds, most notably by glucan (a substance present in most fungi and many plants), the samples were analyzed both before and after being heated in alkaline solution (carried out to deactivate the endotoxin). By this way, both glucan and endotoxin activity were identified in the studied tobacco and smoke particles. With the chemical-analytical method used in the present study we were able to demonstrate unequivocally that high levels of LPS are inhaled during active cigarette smoking and, more importantly, that ETS may involve inhalation of amounts of endotoxin that are dramatically greater than those existing in indoor environments free from tobacco smoke. The findings of the present study and the study by Hasday et al. (1999) may help to explain the high prevalence of respiratory disorders (broncho-alveolar neutrophilia, airway obstruction, bronchial hyperresponsiveness) among smokers. We did not attempt to compare the endotoxin content in smoke from different brands of tobacco, or to determine whether different ways of producing or storing tobacco products affects the levels of endotoxin in the smoke or in the tobacco itself. 3-OH FA analysis represents a direct and very useful method for monitoring tobacco-smoke-associated endotoxin in air. Questions that we will soon address concern whether significant amounts of endotoxin originating from tobacco smoke adhere on particles of airborne house dust, and how ventilation affects concentrations of such endotoxin in indoor air.

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