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IDENTIFICATION OF HEPTANAL AND NONANAL IN BRONCHOALVEOLAR LAVAGE FROM RATS EXPOSED TO LOW LEVELS OF OZONE

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Heptanal and nonanal are identified from *in vitro* studies as potential biomarkers of exposure to ozone, the former resulting from ozonation of palmitoleic acid and the latter from oleic acid. An analytical method is developed based on derivatization using O-pentafluorobenzylhydroxylamine HCl and gas chromatography. These molecules also are present in the lung lavage of Sprague-Dawley rats exposed to 1.3 ppm ozone for 10 hr. These results suggest aldehydes may be useful dosimeters for ozone and indicate that unsaturated fatty acids in the lung lining fluid layer undergo ozonation *in vivo*.

Because of its exceptionally high reactivity, all biomolecules are potential targets for reaction with inhaled ozone (1-5). However, unsaturated fatty acids (UFA) in the lung lining fluid layer (LLFL) have been suggested to be a first-order target (5,6) because of their accessibility, high concentrations (7) and high reactivity toward ozone (1-3,8). We have previously suggested that Criegee ozonides of UFA might be useful biomarkers and dosimeters for ozone exposure (9,10); they are unique ozonation products but are produced in only about 11% yields in ozonation of model systems (9).

Aldehydes are the main reaction products when UFA undergo ozonation in an aqueous environment (11-14). Because UFA are present both in the LLFL and in cellular membranes (15,16), aldehydes are excellent candidates as ozone dosimeters

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Abbreviations: PFBHA, O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine-hydrochloride; UFA, unsaturated fatty acids; LLFL, lung lining fluid layer; 18:1, oleic acid; 16:1, palmitoleic acid; DTPA, diethylenetriaminepentaacetic acid; SIM, selective ion mode.

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and biomarker molecules. One problem occurs: aldehydes also are produced when unsaturated fatty acids undergo autoxidation, and ozone can initiate autoxidation (17). Thus, the product profiles of both ozonation and autoxidation must be compared in order to identify those aldehydes that are useful biomarkers for ozonation.

Aldehyde products in biological systems have been detected mainly as their 2,4-dinitrophenylhydrazone derivatives using TLC and HPLC (18-20) or by headspace gas chromatography (21,22). The latter technique being particularly useful for short chain volatile aldehydes (up to six carbon-atoms). The detectability limits for aldehydes can reach the 10^{-12} g range by derivatization with O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA) (23,24) using capillary GC/ECD or GC/MS in the selective ion mode.

In this communication we show that nonanal and heptanal could be used as biomarkers for ozone exposures of rat lung lavage and surfactant model solutions *in vitro* and lung lining fluid layer *in vivo*, using the PFBHA derivatization method mentioned above.

MATERIALS AND METHODS

Reagents. Oleic acid (18:1) (99%), palmitoleic acid (16:1)(99%), PFBHA, hydrogen peroxide (30% solution), ascorbic acid, and diethylenetriaminepentaacetic acid (DTPA) were purchase from Sigma Chemical Company (St. Louis, MO); nonanal, heptanal, sodium thiosulfate, decafluorobiphenyl, NaCl and $FeSO_4$ from Aldrich Chemical Company (Milwaukee, WI); sulfuric acid AR Select and Hexane nanograde from Mallinckrodt (Paris, KY); sodium sulfate (anhydrous) from Baker Chemical Company (Phillpsburg, NJ). SURVANTA (a modified bovine surfactant) was a generous gift from Ross Laboratories, Division of Abbott Laboratories (Columbus, OH). All chemicals were used without further purification.

Water used for standard solutions, reagent solutions, blanks and controls was deionized and purified by reversed osmosis (Marcor/Osmonics).

Fatty acid emulsions. Emulsions (2.4-4.2 mM) were prepared by mild sonication of the corresponding fatty acid [palmitoleic (16:1) or oleic (18:1)] in 100 mM Na/K phosphate buffer pH 7.6 [containing 0.1 mM diethylenetriaminepentaacetic acid (DTPA) in the ozonation experiments] in an L&R sonifier. The chelating agent DTPA was added to prevent concurrent autoxidation mediated by transition metals during ozonation.

SURVANTA suspensions. Survanta was diluted by suspending 42 μL of the commercial preparation in 25 mL 0.9% saline in order to resemble the lipid concentration in the rat lung lavage.

Animal exposure protocol. Ninety-day old male Sprague-Dawley specific pathogen-free rats (300-320 g) (Harlan Sprague-Dawley, Houston, TX) were used. Rats were acclimatized in standard cages with access to food (Purina Mills, Inc.) and water for at least five days prior to exposure. Animals were individually caged while being exposed to either filtered air or 1.3 ppm ozone for 10 hours in a 0.25 m^3 whole-body exposure chamber (WBEC) (Air/Dynamics, Inc., Baton Rouge, LA). The air flow rate was adjusted to give 12 chamber volume changes per hour. Ozone was

generated by passing air through a Sander ozonizer (Model 200, Sander Aquarientechnik, AM Osterberg, Germany) and then diluted with filtered room air to the desired concentration. The concentration of the ozone inside the chamber was continuously monitored using an ozone analyzer (Dasibi model 1008-AH, Dasibi Environmental Corp., Glendale, CA), connected to a strip chart recorder. The ozone analyzer was calibrated using an Ozone Calibration Kit (Enmet Analytical, Ann Arbor, MI).

Rat Lung Surfactant. Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg), sacrificed by exsanguination and a thoracotomy performed to expose the lungs. To collect the bronchoalveolar lavage, a cannula was inserted into the trachea and secured using a silk suture, and then 2x10 mL of 37°C 0.9% saline was injected into the lung via a syringe and the tracheal cannula. Each infusion was allowed to remain for 1 min and then retrieved. The saline washes were then centrifuged at 4°C for 15 min at 500 xg to remove cells and other debris and the supernatant was then used as surfactant.

Aqueous ozone solutions. *(Caution: Ozone is an extremely strong oxidizing agent and is highly reactive and highly cytotoxic; avoid direct exposure; excess unreacted ozone must always be absorbed and destroyed in a solution of potassium iodide).* Aqueous ozone solution were prepared by bubbling a stream of 2% v/v ozone in oxygen into water that was acidified with sulfuric acid to pH 2 (25). The concentration of ozone in solution was estimated by measuring the absorbance at 258 nm (ε = 2,900 M^{-1} cm^{-1}) (26). The concentration of ozone in solutions prepared as above ranged between 0.21 and 0.24 mM.

Ozonation. An aqueous ozone solution (2 mL) was added to 2 mL of lipid dispersion in a 20 mL vial. The vials were stoppered after the addition of the ozone solution and thermostated in a shaker bath at 37°C for 15 min.

Autoxidation. Autoxidation of lipid dispersions (2 mL) was initiated with 0.67 mL each of $FeSO_4$ 0.6 mM, H_2O_2 1.0 mM and ascorbic acid 1.5 mM. The vials were thermostated at 37°C for 4 hr.

GC Sample preparation. The method employed was adapted from that described by Glaze, Koga and Cancilla (23) for aldehyde analysis during drinking water treatment. An ozonated or autoxidized lipid dispersion (2 mL) was transferred to a 10 mL screw-capped test tube. Two drops of 0.1 M sodium thiosulfate were added to remove any ozone present in the sample, an aqueous PFBHA solution (0.5 mL, 1.0 mg/mL) was added, and the reaction mixture left at room temperature for two hours. The solution was acidified with two drops of 18 N sulfuric acid, and the PFBHA derivatives were then extracted with 1 mL of hexane (containing 400 µg/L of decafluorobiphenyl as internal standard), by shaking for 30 seconds. The hexane extract was transferred to another test tube with a Pasteur pipet, washed with 5 mL of 0.1N sulfuric acid and then dried over 50 mg of anhydrous sodium sulfate.

GC Analysis:

(i) GC/ECD Analysis: A Hewlett Packard 5890 series II GC, with a ^{63}Ni electron capture detector was used. For the injection a Hewlett Packard 7361A autosampler, connected to a cool on-column injector with electronic pressure control, was used. A HP-5 10mx0.53mmx2.65µm column with a 1mx0.53mm retention gap were used for the separation. Helium (8 mL/minute) was used as a carrier, and Argon/methane as a makeup gas. The chromatographic conditions were as follows: 50°C isothermal for 1 minute, 5°C/minute to 220°C. One microliter of sample was injected.

(ii) GC/MS Analysis: a Hewlett Packard GC model 5890 equipped with a HP-5 12.5mx0.20mmx0.33µm column connected to a mass selective detector model 5970B in the selected ion mode (SIM) for *m/z* 181 and 334. Helium (0.75 mL/minute) was used as a carrier. The chromatographic conditions were the same as those for the GC/ECD. The split was set at 20:1. Five microliters of sample were injected. For

confirmation of the nonanal and heptanal peaks, 15 mL of the rat lung lavage from an exposed rat were reacted with PFBHA; the final extract (3 mL) from the derivatization reaction was concentrated to a final volume of 100 μL, and 5 μL were injected in the GC using SIM set at the characteristic *m/z* values: 337(molecular ion for nonanal derivative), 309 (molecular ion for heptanal derivative), 239, 222, 198, 181, 69, 82 and 41.

RESULTS AND DISCUSSION

Results from *in vitro* exposures. Ozonations and autoxidations were carried out on 16:1, 18:1, Survanta (a useful model of lung lavage fluids), and rat lung lavage to establish the products of both processes. Table 1 shows the results for triplicate experiments. As the Table indicates, heptanal results primarily from ozonation of palmitoleic acid (16:1) and nonanal from oleic (18:1). Nonanal is produced in ozonation but not in autoxidation of 18:1, of rat lung lavage, and of Survanta *in vitro*. (Some nonanal can be detected in autoxidized 18:1, but when the concentration of 18:1 was lowered to levels typically found in rat lung lavage, nonanal lied below

Table 1. Yields of heptanal and nonanal from ozonation and autoxidation *in vitro*

Experiment	Relative areas[a]	
	Heptanal	Nonanal
Blank[b]	0.1 ± 0.08	N.D.[c]
16:1 emulsion[d] ozonation[e]	135.07 ± 4.82	N.D.[f]
16:1 emulsion[d] autoxidation[g]	32.12 ± 1.75	N.D.[c]
16:1 emulsion[d] air control[h]	0.59 ± 0.08	N.D.[c]
18:1 emulsion[d] ozonation[e]	N.D.[f]	79.44 ± 8.68
18:1 emulsion[d] autoxidation[g]	N.D.[f]	5.08 ± 0.32
18:1 emulsion[d] air control[h]	N.D.[f]	0.68 ± 0.36
Survanta[i] ozonation[e]	12.65 ± 0.75	50.53 ± 5.18
Survanta[i] autoxidation[g]	0.50 ± 0.20	0.32 ± 0.15
Survanta[i] air control[h]	N.D.[c]	N.D.[c]
Rat lung lavage[j] ozonation[e]	52.82 ± 2.30	97.18 ± 2.11
Rat lung lavage[j] autoxidation[g]	0.15 ± 0.04	0.04 ± 0.00
Rat lung lavage[j] air control[h]	N.D.[c]	N.D.[c]

(a) Values are means ± SEM for three experiments, relative to the area of the internal standard (decafluorobiphenyl). (b) Blanks are the mean of 12 experiments. (c) N.D. = Not Detected (area < 0.01). (d) 2.3 mg of lipid in 2 mL of 100 mM phosphate buffer, pH 7.6, plus 0.1 mM DTPA. (e) 2 mL of ozone solution (0.2 mM), thermostated at 37°C for 15 minutes. (f) Traces are detected, probably due to impurity fatty acids in the substrate. (g) 0.67 mL each of $FeSO_4$ 0.6 mM, H_2O_2 1.0 mM and ascorbic acid 1.5 mM, thermostated at 37°C for 4 hours. (h) 2 mL of buffer was used in place of the 2 mL of ozone-containing solutions, thermostated at 37°C for 4 hours. (i) 2 mL of a 1:600 Survanta dilution in 0.9% saline. (j) 2 mL of rat lung lavage.

detection limits.) Heptanal is a major product from the ozonation of palmitoleic acid, contrasting sharply with the small yields detected from autoxidation.

Ozone is non-discriminating with unsaturated fatty acids because of its high reactivity. Thus, the nonanal/heptanal ratios produced in ozonations should reflect the ratios of 18:1/16:1 in the biological tissue that undergoes ozonation. In fact, LLFL has a ratio (27) of 18:1 to 16:1 of 1.7:1 and gives a nonanal/heptanal ratio of 1.8:1. Survanta has an 18:1 to 16:1 ratio of 5.2:1 and yields nonanal/heptanal in a ratio of 4.0:1.

Results from *in vivo* exposures. The analysis of the lung lavage taken from rats exposed to 1.3 ± 0.1 ppm of ozone for 10 hours gave heptanal (0.08 ± 0.05 relative units) and nonanal (0.06 ± 0.05), n = 4. Lavage from control rats, which were exposed to air but not to ozone, showed no detectible nonanal or heptanal. The identity of the nonanal and heptanal PFBHA derivatives was confirmed by GC/MS-SIM. In addition, rat lavage samples were spiked with nonanal and heptanal and gave increases in the assigned peak areas.

Rabinowitz and Bassett (28) have reported the detection of acids and dicarboxylic acids from the extraction and oxidative workup (with H_2O_2) of the total lung lipids from rats exposed to ozone. That work indicates that unsaturated fatty acids are targets of ozone in the total lung, and this work indicates that 16:1 and 18:1 specifically are ozonation targets in lung lining fluid layer. In addition, our work suggests that both nonanal and heptanal may prove to be useful biomarkers and dosimeters for ozone exposures.

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REFERENCES

1. Pryor, W.A., Giamalva, D.H., and Church, D.F. (1984) J. Am. Chem. Soc. 106, 7094-7100.
2. Giamalva, D.H., Church, D.F., and Pryor, W.A. (1985) Biochem. Biophys. Res. Commun. 133, 773-779.
3. Giamalva, D.H., Church, D.F., and Pryor, W.A. (1986) J. Am. Chem. Soc. 108, 6646-6651.
4. Giamalva, D.H., Church, D.F., and Pryor, W.A. (1986) J. Am. Chem. Soc. 108, 7678-7681.
5. Pryor, W.A. (1991) Am. J. Clin. Nutr. 53, 702-722.

6. Pryor, W.A. (1992) Free Radical Biol. Med. 12, 83-88.
7. Robertson, B., Van Golde, L.M.G., and Batenburg, J.J. (1984) Pulmonary Surfactant, p.1. Elsevier, Amsterdam.
8. Pryor, W.A., Giamalva, D.H., and Church, D.F. (1985) J. Am. Chem. Soc. 107, 2793-2797.
9. Squadrito, G.L., Rao, U.M., Cueto, R., and Pryor, W.A. (1992) Lipids (in press)
10. Wu, M., Church, D.F., Mahier, T.J., Barker, S.A., and Pryor, W.A. (1992) Lipids 27, 129-135.
11. Pryor, W.A., Das, B., and Church, D.F. (1991) Chem. Res. Toxicol. 4, 341-348.
12. Pryor, W.A., Miki, M., Das, B., and Church, D.F. (1991) Chem. Biol. Interact. 79, 41-52.
13. Pryor, W.A. and Church, D.F. (1991) Free Radical Biol. Med. 11, 41-46.
14. Pryor, W.A. and Church, D.F. (1991) in Oxidative Damage and Repair (K.J.A. Davies, ed.), pp.496-504. Pergamon Press, New York, NY.
15. Menzel, D.B. (1976) in Free Radicals in Biology (W.A. Pryor, ed.), Vol. II, pp.181-200. Academic Press, New York, NY.
16. Neas, L.M., Dockery, D.W., Ware, J.H., Spengler, J.D., Speizer, F.E., and Ferris, B.G.,Jr. (1991) Am. J. Epidemiol. 134, 204-219.
17. Pryor, W.A., Stanley, J.P., Blair, E., and Cullen, G.B. (1976) Arch. Environ. Health 31, 201-210.
18. Esterbauer, H. and Zollner, H. (1989) Free Radical Biol. Med. 7, 197-203.
19. Esterbauer, H. (1982) in Membrane Lipid Oxidation (D.C.H. McBrien and T.F. Slater, eds.), pp.101-128. Academic Press, New York, New York.
20. Esterbauer, H., Zollner, H., and Schaur, R.J. (1989) in Free Radicals, Lipid Peroxidation and Cancer (C.V. Pelfrey, ed.), CRC Press, Boca Raton, Florida.
21. Frankel, E.N., Hu, M.-L., and Tappel, A.L. (1989) Lipids 24, 976-981.
22. Frankel, E.N. and Tappel, A.L. (1991) Lipids 26, 479-484.
23. Glaze, W.H., Koga, M., and Cancilla, D. (1989) Environ. Sci. Technol. 23, 838-847.
24. van Kuijk, F.J.G.M., Thomas, D.W., Stephens, R.J., and Dratz, E.A. (1986) Biochem. Biophys. Res. Commun. 139, 144-149.
25. Giamalva, D.H., Church, D.F., and Pryor, W.A. (1988) Chem. Res. Toxicol. 1, 144-145.
26. Bader, H. and Hoigné, J. (1981) Water Res. 15, 449-456.
27. Harwood, J.L., Desai, R., Hext, P., Tetley, T., and Richards, R. (1975) Biochem. J. 151, 707-714.
28. Rabinowitz, J.L. and Bassett, D.J.P. (1988) Exp. Lung Res. 14, 477-489.