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OCCURRENCE OF CHOLESTEROL 7α- AND 7β-HYDROPEROXIDES IN RAT SKIN AS AGING MARKERS

Naoki Ozawa[1]*, Shinji Yamazaki[1], Koji Chiba[1], Hiroyuki Aoyama[1], Hiroki Tomisawa[1], Mitsuru Tateishi[1], and Tadashi Watabe[2]

[1]Drug Metabolism & Analytical Chemistry Research, Upjohn Pharmaceuticals Limited Tsukuba Research Laboratories, 23 Wadai, Tsukuba, Ibaraki 300-42, Japan

[2]Laboratory of Drug Metabolism and Toxicology, Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan

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Evidence for presence of cholesterol 7α- and 7β-hydroperoxides in rat skin was presented for the first time. The 7-hydroperoxides in rat skin were reduced with sodium borohydride and trimethylsilylated for identification with the authentic compounds by gas chromatography/mass spectrometry. A content of cholesterol 7-hydroperoxides in rat skin, determined by high performance liquid chromatography with a chemiluminescence detector, highly correlated with the age of rats ($r = 0.874$; between 1 and 45 weeks old), indicating that cholesterol 7α- and 7β-hydroperoxides were good markers for aging.

Involvement of active oxygen species (·OH, $O_2^{\cdot-}$, $^1O_2$, etc.) and active oxygen-forming species (lipid hydroperoxides, etc.) in some types of diseases and in toxicities of many environmental pollutants and drugs has been suggested/demonstrated through extensive studies on lipid peroxidation (1-5). Evidence for lipid peroxidation *in vivo* was presented by some studies on determination of lipid hydroperoxides in biological samples (6)

*To whom correspondence should be addressed.

Abbreviations:
Ch 7α-OOH, cholesterol 7α-hydroperoxide; Ch 7β-OOH, cholesterol 7β-hydroperoxide; Ch 7-OOHs, cholesterol 7-hydroperoxides; Sito 7β-OOH, β-sitosterol 7β-hydroperoxide; GC-MS, gas chromatography/mass spectrometry; HPLC, high performance liquid chromatography.

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including studies on determination of hydrocarbons in expired air, which have been considered to form during lipid peroxidation *in vivo* (7). Most frequently used methods for analysis of lipid peroxides in plasma and other tissues & organs are presently colorimetry with thiobarbituric acid (6) and uv-absorptiometry of the extracted lipid fractions (8). In recent attempts to measure lipid hydroperoxides in living body, investigators (9, 10) measured mixtures of phospholipid hydroperoxides in human plasma and some tissues & organs in animals, suggesting relationship between the contents of the hydroperoxides and some diseases. However, these did not give conclusive evidence for the structures of lipid hydroperoxides *in vivo*.

In this paper, we, for the first time, present evidence for existence of cholesterol 7α- and 7β-hydroperoxides (Ch 7α-OOH and Ch 7β-OOH, respectively) in rat skin and demonstrate the close relationship between a content of the hydroperoxides in rat skin and age of the rats.

MATERIALS AND METHODS

Chemicals: Ch 7α-OOH, Ch 7β-OOH, and β-sitosterol 7β-hydroperoxide (Sito 7β-OOH) were synthesized by photooxidation and subsequent isomerization/epimerization (11-13). Cholesterol esterase (EC 3.1.1.13) and microperoxidase (MP-11) were purchased from Sigma Chemical Co., St. Louis, Mo and [1α,2α(n)-^{3}H]cholesterol from Amersham International plc, Amersham, U.K. Other chemicals used were of reagent grade.

Analysis of Ch 7α-OOH and Ch 7β-OOH: Male Sprague-Dawley rats, shaved in their back (about 5 x 5 cm), were anesthetized by diethyl ether to excise the dorsal skin consisting of epidermis and dermis. To the skin (1 g) were added 5 ml of saline containing 0.1% diethylenetriaminepentaacetic acid and 30 ml of chloroform-methanol (2:1, v/v) containing 0.1% butylated hydroxytoluene (BHT), 0.015% 2,5-dimethylfuran (DMF), and Sito 7β-OOH (5 nmol, internal standard). After homogenization and centrifugation, an aliquot (1/20) of the organic layer was evaporated to dryness and was dissolved in 0.2 ml *t*-butanol containing 5% Triton X-100 and then incubated at 37°C for 15 min with cholesterol esterase (10 units) in 1.8 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM disodium ethylenediaminetetraacetate. After hydrolysis, Cholesterol 7-hydroperoxide (Ch 7-OOHs) fraction was extracted with chloroform and was applied to Bond Elut® Sil (Analytichem International, Harbor City, CA). An organic phase obtained by elution of the column with chloroform was evaporated to dryness to apply to Bond Elut® NH2 (Analytichem International). After eluting with ethyl acetate, the eluate was evaporated to dryness. The dried sample was dissolved in mobile phase described below and subjected to high performance liquid chromatograph (HPLC) with normal phase column (mobile phase: hexane/*i*-propanol=100:5) to collect the total fraction of Ch 7α-

OOH and Ch 7β-OOH and Sito 7β-OOH. After evaporation to dryness the collected materials were applied to HPLC with reverse phase column (mobile phase: methanol/water=90:10). The fraction from retention times between 9.0 and 14.0 min containing these three hydroperoxides was then applied to the final HPLC with chiral phase column (mobile phase: methanol/water=85:15) and a chemiluminescence detector {chemiluminescence reagent: isoluminol (10 μg/ml) and microperoxidase (20 μg/ml) in 50 mM borate buffer, pH 9.5; flow rate: 1.5 ml/min} (15, 17).

HPLC system: HPLC conditions used in the assay were as follows: pump (880-PU, JASCO, Tokyo), chemiluminescence detector (825-CL, JASCO), UV detector (875-UV, JASCO), columns (reverse phase: Wakopak Lichrosorb RP18-5, 4.6 x 150 mm, Wako Pure Chemical Industries, Osaka, Japan; normal phase: Wakopak Lichrosorb Si60-5, 4.6 x 150 mm, Wako Pure Chemical Industries; chiral phase: Chiralcel OD, 4.6 x 250 mm, Daicel Chemical Industries, Tokyo), flow rate (1 ml/min).

RESULTS AND DISCUSSION

Ch 7α-OOH and Ch 7β-OOH were obtained from rat skin by HPLC using normal phase, reverse phase, and chiral phase columns as described in MATERIALS AND METHODS. Each isolated hydroperoxide was reduced to the corresponding hydroxy derivative with sodium borohydride in methanol, which was then trimethylsilylated with *N,O*-bis(trimethylsilyl)trifluoroacetamide for gas-chromatography/mass spectrometry (GC-MS) analysis. The derivatives were identified with those of authentic hydroperoxides with respect to their retention times of GC and assignment of mass spectra, in which both hydroperoxide derivatives gave the parent ion peaks at *m/z* 546 and the base ion peaks at *m/z* 456 ([M - trimethylsilanol]$^+$) (Fig. 1).

Ch 7α-OOH and 7β-OOH were quantitatively determined by HPLC with a chemiluminescence detector as described in MATERIALS AND METHODS. When given quantities of Ch 7α-OOH (0.1-10 nmol/g skin) and Ch 7β-OOH (0.5-50 nmol/g skin) were added to the rat skin during the HPLC analysis of these compounds, they showed linear relationships between contents of the hydroperoxides added to the skin and those detected in the skin homogenates (r=0.999). In the assay, antioxidants, i.e. BHT and DMF, were absolutely necessary to prevent autooxidation of cholesterol and cholesterol ester during the assay process. An experiment using [^{3}H]cholesterol showed that 4.2 x

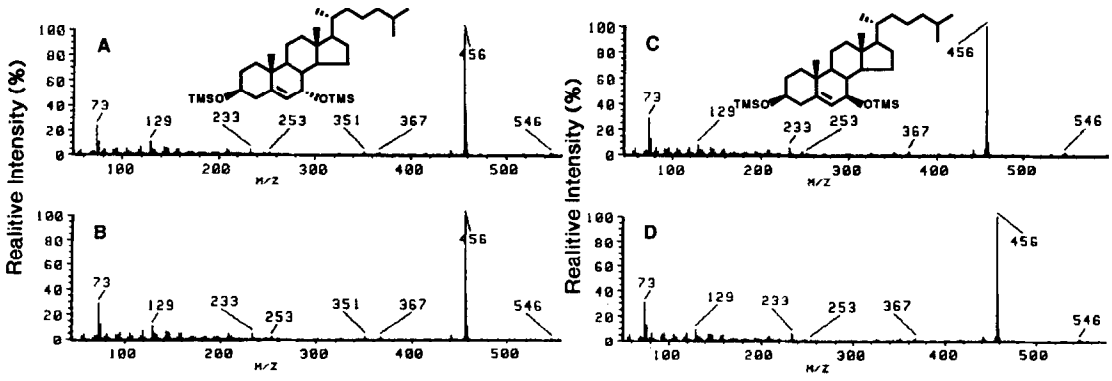

Fig. 1. Mass spectra of derivatives of Ch 7α-OOH and Ch 7β-OOH obtained from rat skin.
GC-MS conditions used were as follows: GC system (5890A, Hewlett Packard Co., Palo Alto, CA, USA), column (DB-17, 0.25 mm x 10 m, J & W Scientific, Folsom, CA, USA), oven temperature (from 200°C to 280°C, 10°C/min), injection port temperature (270°C), flow rate of carrier gas (0.9 ml helium/min), mass spectrometer (5988A, Hewlett Packard), ionizing voltage (70 eV), ionizing current emission (0.3 mA). Under the GC-MS conditions, retention times of derivatives from Ch 7α-OOH and Ch 7β-OOH were 5.7 and 6.5 min, respectively. (A): 7α-hydroxycholesterol-TMS; (B): Derivative from Ch 7α-OOH in rat skin; (C): 7β-hydroxycholesterol-TMS; (D): Derivative from Ch 7β-OOH in rat skin.
TMS: trimethylsilyl.

10^{-3}% and 2.3 x 10^{-3}% of [^{3}H]cholesterol added to rat skin were autooxidized to Ch 7α-OOH and Ch 7β-OOH, respectively, during the assay process in the absence of the antioxidant mixture. However, addition of the antioxidant mixture to extraction solvent (chloroform-methanol) prevented Ch 7α-OOH and Ch 7β-OOH from forming more than the detection limits (9.4 x 10^{-5}%) by autooxidation. Also, antioxidant itself did not alter the value of Ch 7-OOHs suggesting that hydroperoxides did not react with the antioxidants used. By the assay, it was demonstrated that a sum of Ch 7-OOHs well correlated with age of rats (r = 0.874; 1-45 weeks old; Fig. 2). The predominant species of the epimer in the skin was Ch 7β-OOH in all ages of rats used. The ratios of Ch 7β-OOH to Ch 7α-OOH, 4.3-8.0, did not vary to a large extent among the ages.

Relation of active oxygen species to aging has been suggested by studies from diverse aspects (14-19), such as effect of antioxidants in vivo (15), effect of uv-radiation (16), and

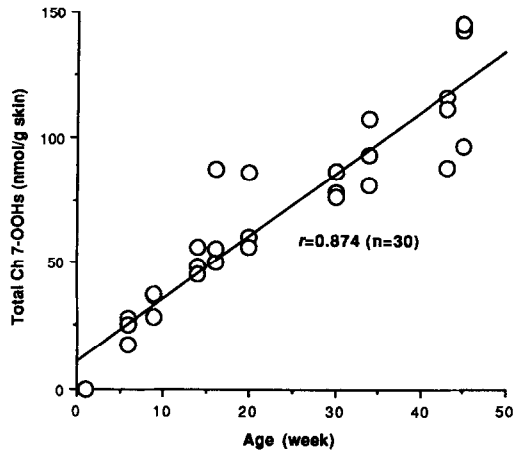

Fig. 2. Correlation between a content of Ch 7α-OOH and Ch 7β-OOH in rat skin and age of rats.

change in enzyme activity of superoxide dismutase (14, 17). One of these studies described the relationship of skin cancer to lipid peroxidation induced by light energy (18) as well as by a cancer promoter, TPA (12-*O*-tetradecanoylphorbol-13-acetate) (19). Whether a content of Ch 7α-OOH and Ch 7β-OOH correlates with skin cancer or not is currently the subject of our further study.

REFERENCES

1. Pryor, W. A. (1978) Photochem. Photobiol., 28, 787-801.
2. Sies, H. Ed. (1985) Oxidative Stress, Academic Press, London.
3. Mason, R. P. (1982) In Free Radicals in Biology, Vol. V, pp. 161-222. Academic Press, New York.
4. Cerutti, P. A. (1985) Science, 227, 375-381.
5. Harman, D. (1981), Proc. Natl. Acad. Sci. USA, 78, 7124-7128.
6. Yagi, K. (1976) Biochem. Med., 15, 212-216.
7. Riely, C. A., Cohen, G., and Lieberman, M. (1974) Science, 183, 208-210.
8. Corongiu, F. P., Banni, S., and Dessi, M. A. (1989) Free Radical Biol. Med., 7, 183-186.
9. Miyazawa, T. (1989) Free Radical Biol. Med., 7, 209-217.
10. Yamamoto, Y., and Niki, E. (1989) Biochem. Biophys. Res. Commun., 165, 988-993.
11. Kulig M. J., and Smith, L. L. (1973) J. Org. Chem., 38, 3639-3642.
12. Lythgoe, B., and Trippett, S. (1959) J. Chem. Soc., 471-472.
13. Teng, J. I., Kulig, M. J., Smith, L. L., Kan, G., and Van Lier, J. E. (1973) J. Org. Chem., 38, 119-123.
14. Reiss, U., and Gershon, D. (1976) Biochem. Biophys. Res. Commun., 73, 255-262.

15. Packer, L., and Smith, J. R. (1974) Proc. Nat. Acad. Sci. USA, 71, 4763-4767.
16. Fuchs, J., Huflejt, M. E., Rothfuss, L. M., Wilson, D. S., Carcamo, G., and Packer, L. (1989) J. Invest. Dermatol., 93, 769-773.
17. Stohss, J., El-Rashidy, F. H., Kobayashi, R. H., Wulf, B. G., and Potter, J. F. (1984) Age, 7, 3-7.
18. Black, H. S., Lenger, W. A., Gerguis, J., and Thornby, J. I. (1985) Cancer Res., 45, 6254-6259.
19. Perchellet J.-P., and Perchellet, E. M. (1989) Free Radical Biol. Med., 7, 377-408.