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Cytotoxicities of Autoxidized Polyunsaturated Fatty Acids toward Cultured Human Umbilical Vein Endothelial Cells[1)]

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Cytotoxicities of autoxidized polyunsaturated fatty acids toward human umbilical vein endothelial cells were examined. Autoxidized linoleic acid is more toxic than linoleic, autoxidized linolenic, or autoxidized arachidonic acid. Autoxidized linolenic acid is less toxic than linolenic acid. Autoxidized arachidonic acid is as toxic as linolenic or arachidonic acid. It was found that major toxic components in autoxidized linoleic acid are linoleic acid hydroperoxides, and that a major component in autoxidized arachidonic acid is (*E*)-4-hydroxy-2-nonenal.

Keywords——cytotoxicity; linoleic acid; linolenic acid; arachidonic acid; lipid peroxide; autoxidation; human umbilical vein endothelial cell

It has been reported that the peroxidation products of polyunsaturated fatty acids, such as hydroperoxides, and the degradation products of the peroxidized compounds, such as unsaturated aldehydes, are toxic in biological systems.[2–5)] However, the cytotoxicity of peroxidation products of polyunsaturated fatty acids is not fully understood. Presumably, the toxic effect of the peroxidation products varies according to types of cells and polyunsaturated fatty acids. In order to compare the toxicities of peroxidized polyunsaturated fatty acids, we attempted to examine their cytotoxicity toward human umbilical vein endothelial cells and to identify their toxic components.

Polyunsaturated fatty acids were autoxidized by the bubbling of oxygen gas at 40 °C in benzene in the presence of cobalt(II) acetate[6)]; the cobalt catalyst was chosen because of its solubility in the solvent. Linoleic acid was slowly autoxidized and the peroxide value (POV) of its autoxidized mixture was 1890 μeq/g after 3 d of autoxidation. High performance liquid chromatographic (HPLC) analysis revealed that in the mixture linoleic acid hydroperoxides were produced in about 15% yield. The POV of autoxidized linolenic acid reached almost the maximum after 3 d of autoxidation (2050 μeq/g), although only small amounts of the hydroperoxides could be detected. Arachidonic acid was rapidly autoxidized and the POV of the autoxidized mixture reached the maximum after 1 d of autoxidation (2660 μeq/g). It was observed that the hydroperoxides in the mixture increased rapidly and then decreased gradually. The carbonyl values (COV) of the autoxidized fatty acids continued to increase during autoxidation. When measured after 3 d of autoxidation, the COV of autoxidized linoleic acid (710 μeq/g) was smaller than those of autoxidized linolenic (1340 μeq/g) and arachidonic acids (1060 μeq/g). The thiobarbituric acid value (TBAV) of the autoxidized fatty acids was measured after 3 d of autoxidation and expressed as equivalent malondialdehyde content (nmol/mg). The TBAV of autoxidized linoleic acid (42 nmol/mg) was much smaller than those of autoxidized linolenic (875 nmol/mg) and arachidonic acids (841 nmol/mg).

The cytotoxicities of the autoxidized polyunsaturated fatty acids toward human umbilical vein endothelial cells were examined. Autoxidized linoleic acid is more toxic than linoleic,

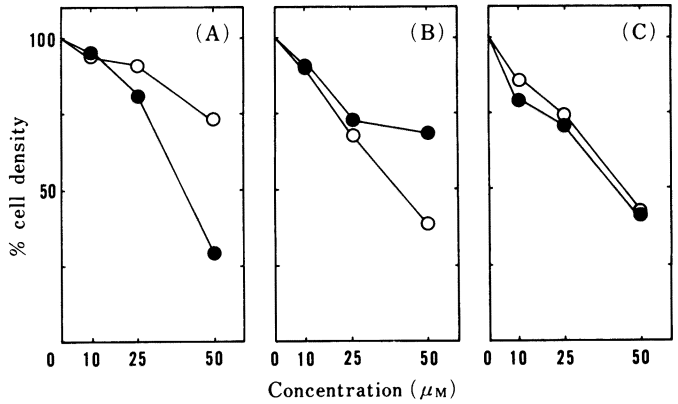

Fig. 1. Cytotoxicities of Polyunsaturated Fatty Acids (○) and Autoxidized Fatty Acids (●) toward Human Endothelial Cells

The cytotoxicities of linoleic and autoxidized linoleic acids (A), linolenic and autoxidized linolenic acids (B), and arachidonic and autoxidized arachidonic acids (C) are shown.

autoxidized linolenic, or autoxidized arachidonic acid (Fig. 1). Interestingly, autoxidized linolenic acid is less toxic than linolenic acid itself (Fig. 1B). Autoxidized arachidonic acid is as toxic as linoleic or arachidonic acid (Fig. 1C).

We tried to separate toxic components in the autoxidation mixtures. Each mixture was divided into six fractions by silica gel column chromatography. The toxic fractions were further fractionated by reverse-phase HPLC and the cytotoxicities of the resulting fractions were monitored. The toxic component of autoxidized linoleic acid was found to be a mixture of the monohydroperoxy derivatives of linoleic acid [50% lethal concentration (LC_{50}), 20 μM], and the toxic component of arachidonic acid to be (*E*)-4-hydroxy-2-nonenal (yield, 0.6%; LC_{50}, 25 μM). The structures were identified from the spectral data and by comparison with authentic samples. No toxic fractions were obtained from autoxidized linolenic acid.

Autoxidized linoleic acid was found to be more cytotoxic than autoxidized linolenic and arachidonic acids. The different cytotoxicities may reflect the difference in the reactivities and stabilities of the primary products, hydroperoxides, and the compositions of the secondary products formed from the hydroperoxides. Hydroperoxides from linolenic and arachidonic acids appear to be too unstable to remain after 3 d of autoxidation. The different cytotoxicities between autoxidized linolenic and arachidonic acids seem to be dependent on the occurrence of toxic secondary products. The HPLC analysis of 2,4-dinitrophenylhydrazones of autoxidized linolenic acid showed that in the autoxidized mixtures, there is no (*E*)-4-hydroxy-2-nonenal, which is a main toxic component in autoxidized arachidonic acid.

Since endothelial cells line the wall of blood vessels as a monolayer, their injury is thought to cause damage to the blood vessels. For example, the onset of atherosclerosis is presumed to be due to repeated injury to endothelial cells.[7] It is possible that the autoxidation of linoleic acid and lipids with linoleic acid moieties is a risk factor of blood vessel damage.

Experimental

Linoleic, linolenic, and arachidonic acids, and heparin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Human umbilical vein endothelial cells were isolated by a modification of the method of Jaffe *et al.* using 0.1% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) in 0.15 M phosphate-buffered saline (pH 7.4).[8] Cells were inoculated in gelatin-precoated plastic dishes and cultivated in MCDB-104 medium supplemented with 69 ng/ml endothelial cell growth factor (ECGF), 10 ng/ml epidermal growth factor (EGF; Collaborative

Research, Walthan, MA, U.S.A.), 100 ng/ml heparin, and 10% fetal bovine serum (FBS; Grand Island Biochemical Co., Grand Island, NY, U.S.A.). ECGF was isolated from newborn bovine brains.[9]

For autoxidation, oxygen gas was bubbled at a flow rate of 100 ml/min at 40 °C for 3 d into a 0.1 M benzene solution of each fatty acid containing a catalytic amount of cobalt(II) acetate.[6] Peroxide value,[10,11] carbonyl value,[12] and thiobarbituric acid value[13] were measured in the usual ways. Reaction products were applied to a silica gel column and eluted stepwise with hexane (benzene in the case of autoxidized arachidonic acid)–diethyl ether (90:10, 80:20, 60:40, 40:60, 20:80, and 0:100, v/v). Furthermore, cytotoxic fractions were separated by HPLC (Shimadzu, LC-6A) using a reverse-phase column (Gasukuro Kogyo Co., Inertsil ODS-5; acetonitrile:water, 40:60—100:0, v/v). The recovery of residual linoleic, linolenic, or arachidonic acid in each 3-d autoxidation mixture was 33.8, 45.5, or 38.7%, respectively.

Cells (*ca.* 1×10^5 cells/cm^2) were treated for 3 h with Earle's solution containing each agent whose cytotoxicity was to be tested. Surviving cells were counted by the trypan blue exclusion test of adhering cells as described previously.[3] Cytotoxicities of polyunsaturated fatty acids and the autoxidized mixtures toward human endothelial cells were expressed as the percentage cell densities of treated cultures compared with the cell densities of control cultures. The cytotoxicities of the polyunsaturated fatty acids and their autoxidized mixtures were examined at the following concentrations: linoleic acid and its autoxidized mixture, 7.0 μg/ml; linolenic acid and its autoxidized mixture, 6.9 μg/ml; arachidonic acid and its autoxidized mixture, 7.6 μg/ml (the concentrations of each fatty acid correspond to 25 μM).

References and Notes

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