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Application of Water-Soluble Radical Initiator, 2,2′-Azobis-[2-(2-imidazolin-2-yl)propane] Dihydrochloride, to a Study of Oxidative Stress

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It is essential to generate free radicals at a controled and constant rate for specific duration and at specific site to study the dynamics of oxidation and also antioxidation. Both hydrophilic and lipophilic azo compounds have been used for such purpose. In the present work, the action of 2,2′-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (AIPH) was examined and compared with those of 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) and 2,2′-azobis[2-methyl-N-(2-hydroxyethyl)-propionamide] (AMHP). The rate constant of free radical formation (ek_d) for AIPH was 2.6×10^{-6}/s at 37°C in PBS (pH 7.4) solution, indicating that AIPH gives 3.8 times more free radicals than AAPH under the same conditions. It was found that the dynamics of oxidation and antioxidation induced by AIPH can be studied satisfactorily in the oxidation in micelles, LDL and erythrocyte suspensions, plasma, and cultured cells. The extent of cell death induced by AIPH and AAPH was directly proportional to the total free radicals formed. Interestingly, it was found that rats would not drink water containing AAPH, but they drank water containing AIPH. The levels of 8-iso-prostaglandin F2α (8-isoPs), 7-hydroxycholesterol (FCOH), lysophosphatidylcholine in the plasma of rats given water containing 50 mM AIPH for 1 month increased compared with those of control rats which drank water without AIPH. It may be concluded that AIPH is useful for kinetic and mechanistic studies on oxidative stress to membranes, lipoproteins, cells, and even animal models.

Keywords: Azo initiator; Antioxidant; Free radical; Lipid peroxidation; AIPH; AAPH

Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; AIPH, 2,2′-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride; AMHP, 2,2′-azobis[2-methyl-N-(2-hydroxyethyl)-propionamide]; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); BSTFA, N,O-bis(trimethylsilyl)trifluoroacetamide; CE, cholesteryl ester; CEOH, cholesteryl ester hydroxide; CEOOH, cholesteryl ester hydroperoxide; CEO(O)H, cholesteryl ester hydroxide and hydroperoxide; EDTA, ethylenediaminetetraacetic acid; FC, free cholesterol; FCOH, 7-hydroxycholesterol; FCOOH, cholesterol 7-hydroperoxide; FCCO, 7-ketocholesterol; 8-isoPs, 8-iso-prostaglandin F2α; MeLOOH, methyl linoleate hydroperoxides; MeO-AMVN, 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile); MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-di-phenyltetrazolium bromide; PBS, phosphate-buffered saline; LDL, low density lipoprotein; PC, phosphatidylcholine; PCOH, phosphatidylcholine hydroxide; PCOOH, phosphatidylcholine hydroperoxide; PCO(O)H, phosphatidylcholine hydroxide and hydroperoxide; PLPC, 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphatidylcholine; PR, pyrogallolsulfonphthalein; SDS, sodium dodecyl sulfate; SIN-1, 3-morpholinosydnonimine; t_{inh}, inhibition period; TMP, tocopherol-mediated peroxidation; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

INTRODUCTION

The role of free radicals in biology has been the subject of extensive studies in relation to oxidative stress and cellular signaling. It is now accepted that at high concentrations, free radicals are hazardous, whereas at moderate concentrations, they play an important role as regulatory mediators in signaling processes. In order to study the dynamics of oxidation and also antioxidation, it is essential to generate free radicals at a controled and constant rate for specific duration and at specific site. The irradiation of γ-ray and ultraviolet has been used for such purpose. The azo compounds have been also successfully utilized in many *in vivo* as well as *in vitro* studies, since the rate and site of free radical formation can be easily controled by choosing

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appropriate azo compound and concentration.[1] Above all, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), also abbreviated as ABAP, and 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) have been often used as a source of free radicals which were formed in hydrophilic and lipophilic compartments, respectively.[2–4] They decompose unimolecularly and thermally without biotransformation to give free carbon-centered radicals, which react with oxygen quite rapidly to yield peroxyl radicals.† The half-lives of AAPH and AMVN at 37°C are 5.6 and 4.2 days, respectively,[1] suggesting that they generate free radicals at a constant rate for the initial several hours. This is advantageous for kinetic analysis.

This, however, means that high concentration of azo compound is required to generate free radicals at a considerable rate and this may cause difficulty under certain circumstances. For example, it is not easy to incorporate high concentration of AMVN into LDL particles and 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN) has been used for such case,[7] since it decomposes much faster than AMVN and less amount of MeO-AMVN is required.

It was found that the intraperitoneal administration of AAPH to mice caused oxidative damage which could be suppressed by antioxidants[8] and that AAPH induced oxidative hemolysis of erythrocytes.[9,10] It has been also reported that AAPH induced apoptosis.[11] Since AAPH is relatively stable, a high concentration of AAPH is often required. Furthermore, it was found that rats would not drink water containing AAPH and they lost body weight. These results prompted us to study other water-soluble azo compounds. In the present work, the action of 2,2′-azobis[2-(2-imidazolin-2-yl)-propane] dihydrochloride (AIPH) was examined and compared with those of AAPH and thermally stable 2,2′-azobis[2-methyl-N-(2-hydroxyethyl)-propionamide] (AMHP). Rats drank water containing either AIPH or AMHP.

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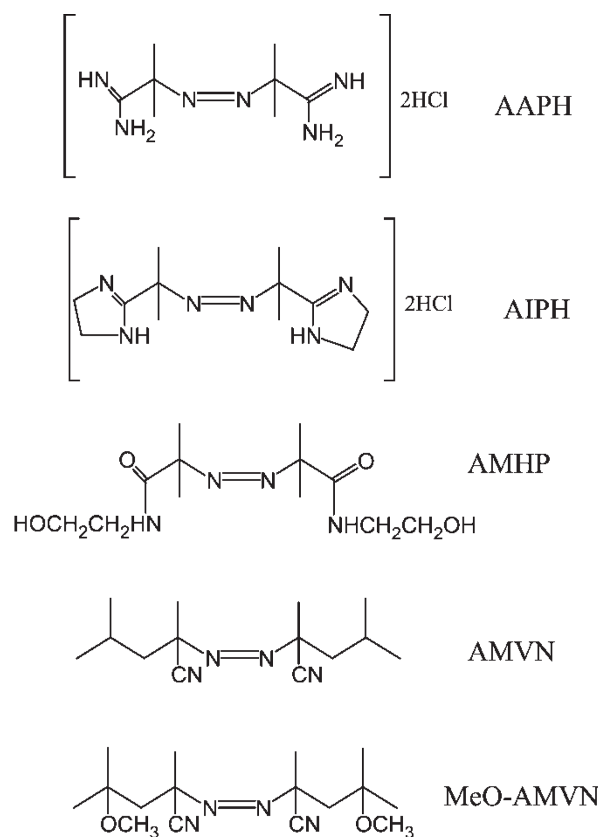

SCHEME 1 Azo compounds used in this study.

MATERIALS AND METHODS

Chemicals

Methyl linoleate (MeLH) was obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan) and used as received. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and pyrogallolsulfonphthalein (PR) purchased from Sigma Aldrich Japan K.K. (Tokyo, Japan) were used as received. 3-[4,5-dimethylthiazol-2-yl]-2,5-di-phenyltetrazolium bromide (MTT) was obtained from Dojindo, Kumamoto, Japan. AMVN and MeO-AMVN used as a lipophilic radical initiator and AIPH, AMHP and AAPH used as a water soluble radical initiator were obtained from Wako Pure Chemical Industries (Osaka, Japan). The chemical structures of these radical initiators are shown in Scheme 1. Authentic 7α and 7β-hydroxycholesterol (FCOH) and 8-iso-prostaglandin F2α (8-isoPs) were purchased from Steraloids Inc. (Newport, RI, USA) and Cayman Chemical Company (MI, USA), respectively. Other materials were those of the highest grade available commercially.

Radical Formation by the Decomposition of AIPH

The decomposition of AIPH was measured by following its disappearance at 37°C in air from the absorption at 356 nm ($\varepsilon = 23.1\,M^{-1}\,cm^{-1}$) with a spectrophotometer (Shimadzu UV-2450) equipped with a thermostated cell. The rates of free radical flux from AAPH and AIPH in PBS (pH = 7.4) solution were measured using PR, a water-soluble dye having a strong absorption at 540 nm, as a radical scavenger. PR decreased at a constant rate in the presence of radical initiator.

†The alkoxyl radicals can be generated from peroxyoxalate[5] and hyponitrite.[6] The di-tert-butylperoxyoxalate is a convenient tert-butoxyl radical source, but care should be taken since it is explosive.

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Oxidation of MeLH Micelles

The oxidation of MeLH micelles induced by radical initiators was performed in aqueous dispersions at 37°C in air. The emulsions were prepared by vigorously mixing MeLH and phosphate-buffered saline (PBS, pH 7.4) containing 0.5 M SDS with a vortex mixer for 2 min. The accumulation of methyl linoleate hydroperoxides (MeLOOH) was followed with a spectrophotometer by absorption at 245 nm. Water-soluble radical initiator and Trolox were added to the aqueous suspensions as an aqueous solution.

Oxidation of Plasma and LDL

After an overnight fast, blood from healthy donor (40 years old male) was collected in ethylene-diaminetetraacetic acid (EDTA)-containing tubes. The samples were placed on ice immediately after the collection. Plasma was obtained by centrifugation at 3000*g* for 10 min at 4°C and used immediately. LDL was separated from human plasma of healthy donors by ultra-centrifugation as described in the literature,[12] within a density cut-off of 1.019–1.063 g/ml, and then dialyzed with cellulose membranes in phosphate-buffered saline (PBS, pH 7.4) containing 100 μM EDTA. The protein concentration of LDL was measured using the bicinchonic acid protein assay reagent (Pierce, Rockford, IL). The oxidation of plasma (1/1 with PBS) and LDL (0.25 mg protein/ml) was carried out at 37°C under air in PBS solution. The oxidation was initiated by an addition of AAPH or AIPH dissolved in PBS solution (1/100 volume to reaction solution). The mixture of oxidized samples was extracted with chloroform/methanol (2/1) by twice as volume as the sample and chloroform layer was injected to an HPLC for α-tocopherol and lipid hydroperoxides analyses. The accumulations of free cholesterol (FC) hydroperoxide (FCOOH), 7-ketocholesterol (FCCO), FCOH, and phosphatidylcholine hydroperoxides (PCOOH) were followed with an HPLC using post-column chemiluminescence detector (CLD-10A, Shimadzu, Japan, for FCOOH and PCOOH analyses) and spectrophotometric detector (SPD-10AV, Shimadzu, Japan) at 210 (for FCOH analysis) and 245 (for FCCO analysis) nm. ODS-2 column (5 μm, 250 × 4.6 mm, GL Science, Japan) was used and methanol/acetonitrile/water (45:46:9 by volume) was delivered as eluent at 1 ml/min and after passage through the UV detector, the elute was mixed with a luminescent reagent in the post-column mixing joint in the chemiluminescence detector (Shimadzu, Japan) at 40°C. The luminescence reagent containing cytochrome C (10 mg) and luminol (2 mg) in 1 l alkaline borate buffer (pH 10) was loaded at the flow rate of 0.5 ml/min. The accumulations of CE hydroperoxide (CEOOH) were also followed with an HPLC by spectrophotometric detector (SPD-10AV, Shimadzu, Japan) at 234 nm. ODS column (LC-18, 5 μm, 250 × 4.6 mm, Supelco, Japan) was used and acetonitrile/isopropyl alcohol/water (44:54:2 by volume) was eluted at 1 ml/min. α-Tocopherol was also measured using an HPLC by an amperometric electrochemical detector (NANOSPACE SI-2, Shiseido, Tokyo, Japan) set at 700 mV, with an ODS column (LC-18, 5 μm, 250 × 4.6 mm, Supelco, Japan) and methanol/tert-butyl alcohol (90/10 by volume) as eluent at 1 ml/min.

Oxidation of Rat Erythrocyte

Male Wister rats (specific pathogen-free, weighing 200–220 g) were purchased from Japan SLC, Inc. (Shizuoka, Japan). Rats were fed a standard laboratory diet (CE-2; Nippon Clea Co.) and maintained under standardized conditions of light (7 am–7 pm), temperature 22°C, and humidity (70%). Rats were sacrificed under anesthesia with sodium pentobarbital (30 mg/kg, ip). Blood was collected from the inferior vena cava using a heparinized syringe, and erythrocyte and plasma were separated by centrifugation (2400*g* for 10 min). The oxidation of rat erythrocyte (10% hematocrit with saline) was induced by either AAPH or AIPH at 37°C under air. The extent of hemolysis was determined by measuring absorbance at 540 nm spectrophotometrically.[13] Experimental protocols were approved by the Animal Welfare, Care and Use Committee in AIST Kansai.

Oxidation of JURKAT Cells in Serum Free Medium

Jurkat E6-1 cells, human T-leukemia (American Tissue Type Collection, Rockville, MD), were cultured in RPMI-1640 containing 100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B and 10% heat-inactivated fetal calf serum at 37°C under an atmosphere of 95% air and 5% CO_2, as described previously.[14] To avoid the effects of antioxidants in serum, the studies on the effects of radical initiators were conducted in the serum free medium containing 5 μg/ml human insulin, 5 μg/ml human transferrin, 92 nM $FeCl_3$, 100 nM sodium selenite and 2.5 mg/ml bovine serum albumin as described previously.[14] For the determination of cell viability, MTT assay was conducted at the indicated times. The cells were incubated with 0.5 mg/ml MTT at 37°C for 2 h. Isopropyl alcohol containing 0.04 N HCl was added to the culture medium (3:2, by volume), and they were mixed by a pipette until the formazan was completely dissolved. The optical density of formazan was measured at

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570 nm using a Multiskan Ascent plate reader (Theromo Labsystems, Helsinki, Finland).

Oxidation of Jurkat Cells in PBS Solution

The oxidation of Jurkat E6-1 cells was also performed as suspensions in PBS solution. Cells were washed with PBS (pH 7.4) twice and then oxidized by AIPH. For the determination of cell viability, DNA intercalating dye propidium iodide (Sigma-Aldrich Co., St. Louis, MO, USA) assay[15] was conducted at the indicated times. Propidium iodide (10 μg/ml) treated cells were analysed with a Cytomics FC500 Flow Cytometry System (Beckman Coulter, Inc., Miami, FL, USA) for excitation at 488 nm argon laser and emission signal was collected at 575 nm. Data were collected from 10,000 events. Propidium iodide negative cells were interpreted as viable cells.

Administration of Azo Compounds to Rats and Analysis of 8-isoPs, FCOH, and Lyso PC

Male Wister rats (specific pathogen-free, weighing 200–220 g) were purchased from Japan SLC, Inc. (Shizuoka, Japan). Rats were fed a standard laboratory diet (CE-2; Nippon Clea Co.) and water containing either AIPH or AMHP. They were maintained under standardized conditions of light (7 am–7 pm), temperature 22°C, and humidity (70%). Rats were sacrificed after 1 month under anesthesia with sodium pentobarbital (30 mg/kg, ip). Blood was collected from the inferior vena cava using a heparinized syringe, and erythrocyte and plasma were separated by centrifugation (2400*g* for 10 min). The plasma was extracted by chloroform and methanol (2/1, by volume) and lyso PC in the chloroform layer was measured by using the thin-layer chromatography (TLC) equipped with flame ionization detection (FID) system (MK-5, Iatron Laboratories, Tokyo, Japan).[16] Samples in chloroform solution (2 μl) were spotted on to sintered silica-gel rods (Chromarod-SIII, Iatron Laboratories, Tokyo, Japan) with a Drummond micro dispenser. The spotted Chromarods were developed in chloroform/methanol/water (45:20:4, by volume) at 25°C for 40 min and then dried with a blower. The Chromarods were developed again in the same eluent at 25°C for 40 min and then dried at 120°C for 10 min. The Chromarods were scanned by FID with a constant hydrogen flow-rate of 160 ml/min.

FCOH and 8-isoPs in rat plasma were also measured as follows. The plasma was extracted with chloroform and methanol (2/1, by volume) and evaporated to dryness under nitrogen. Reduction of lipid hydroperoxides was carried out by the addition of excessive amount of sodium borohydride to methanol solution of lipid extracts followed by the saponification. The reduced sample was suspended in 20% KOH in methanol and pure ether and mixed for 3 h in the dark at 4°C in a shaker. The mixture was neutralized with 20% acetic acid in water and extracted with hexane. The sample was evaporated to dryness under nitrogen and derivatized with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) for 1 h at 60°C and then injected into the gas chromatograph (GC 6890 N, Agilent Technologies Co. Ltd.) equipped with a quadrupole mass spectrometer (5973 Network, Agilent Technologies Co. Ltd.). Fused-silica capillary column (HP-5MS, 5% phenyl methyl siloxane, 30 m × 0.25 mm, Agilent Technologies Co. Ltd.) was used. Helium was used as the carrier gas at a flow rate of 1.2 ml/min. Temperature programming was from 60–280°C at 10°C/min. The injector temperature was 250°C and temperatures of transfer line to mass detector and ion source were 250 and 230°C, respectively. Electron energy was set at 70 eV. The identification of FCOH and 8-isoPs was conducted by their retention times and mass patterns ($m/z = 546, 456$ for FCOH and 571, 481 for 8-isoPs) and ions at 456 and 481 were selected for quantification for FCOH and 8-isoPs, respectively. FC was used as a reference compound for the analysis as its content did not differ significantly in plasma samples used in this study.

Experimental protocols were approved by the Animal Welfare, Care and Use Committee in AIST Kansai.

Statistical Analysis and Reproducibility

The experiments shown in Figs. 1–5 were repeated, in general, for several times and the results were reproducible within experimental error of ±10%. For other experiments, statistical analyses were performed on a Microsoft personal computer by an analysis of variance using Dunnett test for multiple comparisons (ANOVA). Data were expressed as mean values ± SD.

RESULTS

Rate of Radical Formation from AIPH

As shown in Fig. 1(A), AIPH decomposed spontaneously in PBS solution at 37°C and its disappearance rate was constant at least for the initial several hours. The rates of decomposition of AIPH were proportional to the concentrations of AIPH (Fig. 1B), the rate constant for unimolecular decomposition of AIPH (k_d) being $4.9 \times 10^{-6}\ s^{-1}$ at 37°C in PBS (pH 7.4) solution. The decomposition of AIPH was carried out at different pH (4.4, 6.7, 7.5, 8.7, 10) and the rate constant k_d was found to be similar in this pH range (data not shown). The rate of free radical formation from the azo compound depends on the efficacy of

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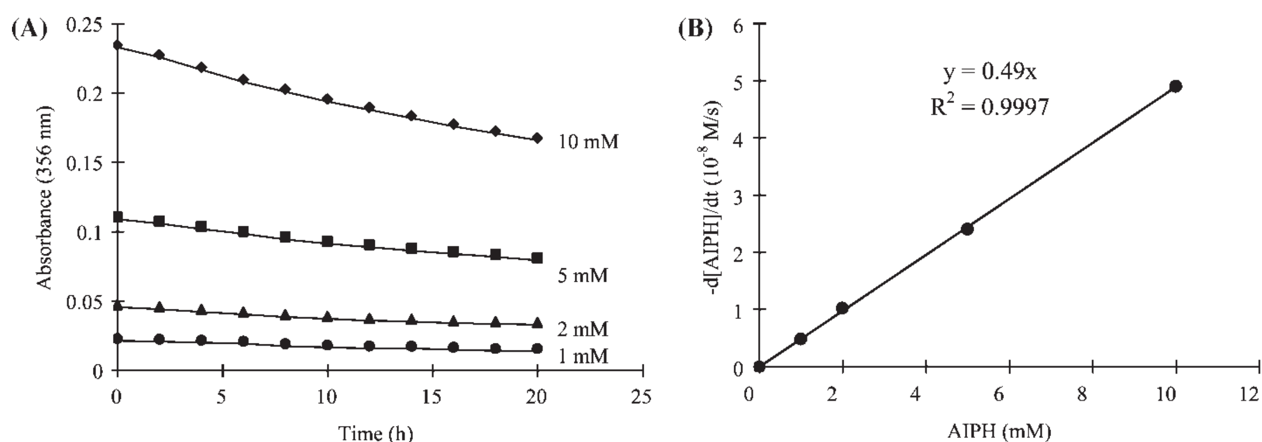

FIGURE 1 (A) Decomposition of AIPH as followed by a decrease in absorption at 356 nm and (B) initial decomposition rates in PBS solution (pH 7.4) at 37°C in air. The rate was calculated by using a molar extinction coefficient 23.1 M^{-1} cm^{-1} measured in this study.

free radical generation (e), that is, the extent of cage reaction, as well as on its rate of decomposition. It has been observed that the efficiency of radical formation depends on the reaction medium, especially on the viscosity.[17–19] The actual rate of free radical flux was measured by using PR as a water-soluble radical scavenger. PR has a strong absorption at 540 nm in PBS solution and its absorption decreased with time by the reaction with the radicals generated from either AAPH or AIPH. The rate of PR consumption was calculated from the slope of Fig. 2(A) and the extinction coefficient (25200/M/s, which was determined by measuring the standard samples). The rate of free radical flux from either AAPH or AIPH is given by Eq. (1):

$$\text{Rate of radical flux} = 2\,ek_d[\text{AAPH or AIPH}] = -n\,d[\text{PR}]/dt \qquad (1)$$

where n is the number of radicals scavenged by one molecule of PR.

The value of ek_d for AAPH was obtained as 6.8×10^{-7}/s at 37°C in PBS (pH 7.4) solution.[1] The value of ek_d for AIPH was obtained as 2.6×10^{-6}/s at 37°C in PBS (pH 7.4) solution from the slope of AIPH relative to that of AAPH, 4.56–1.20 (Fig. 2B),

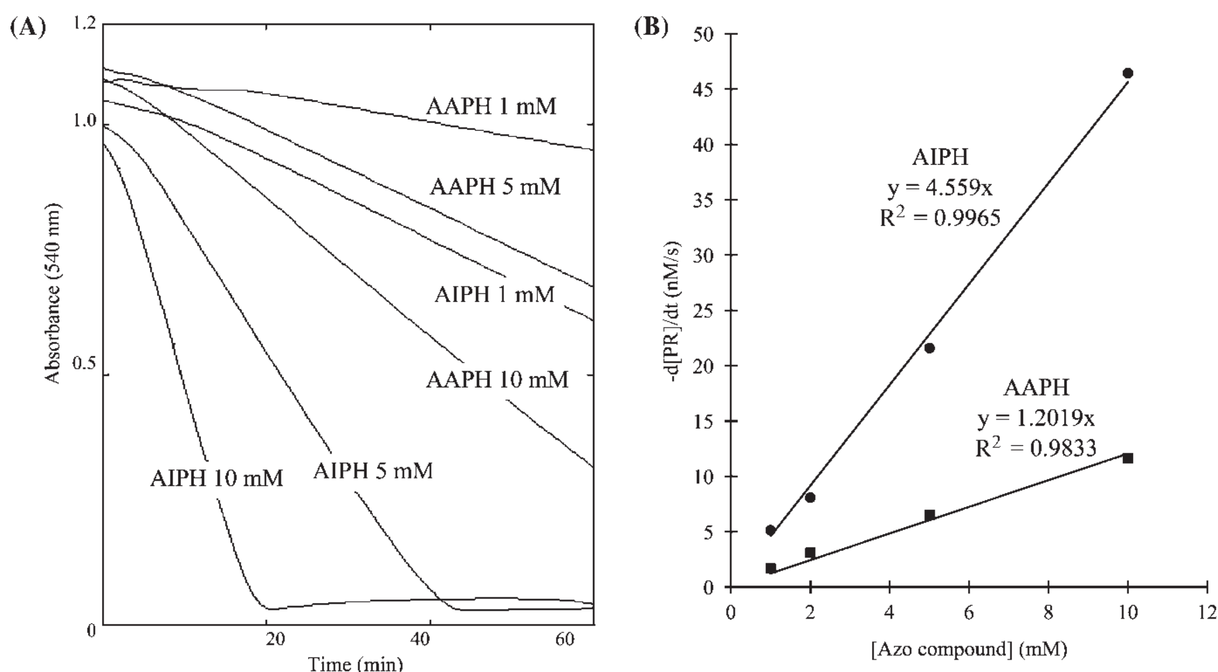

FIGURE 2 (A) Typical results of the consumption of pyrogallolsulfonphthalein (PR) in the reaction with either AIPH or AAPH in PBS solution (pH 7.4) at 37°C in air. Concentrations are shown in the figure. (B) Plots of the consumption rates of PR against the concentrations of azo initiators.

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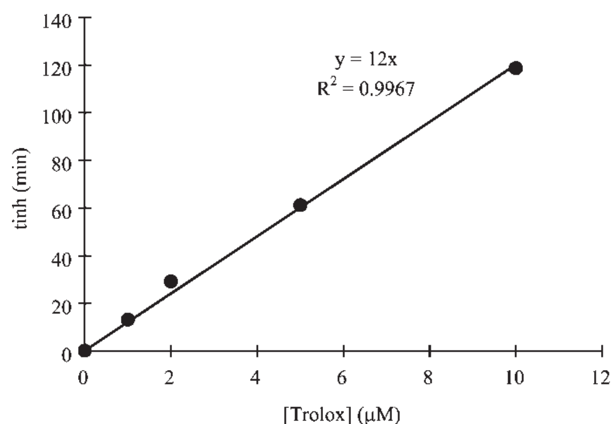

FIGURE 3 Plots of inhibition period (t_{inh}) against concentrations of Trolox in the oxidation of methyl linoleate induced by 1.0 mM AIPH in aqueous suspensions containing 0.5 M SDS (PBS, pH 7.4) at 37°C in air. The oxidation was followed by measuring the absorption at 245 nm.

and the above ek_d for AAPH. Thus, AIPH gives 3.8 times more radicals than AAPH under the same concentrations.

The oxidation of MeLH is known to give four conjugated diene hydroperoxides quantitatively,[20] which can be measured from the strong absorption at 234 nm. The peroxyl radicals were generated by the decomposition of AIPH in the presence of oxygen. Water-soluble vitamin E analog, Trolox, exerted antioxidant effect on the oxidation of MeLH in aqueous dispersions containing 0.5 M SDS (PBS, pH 7.4) induced by peroxyl radicals. As shown in Fig. 3, the inhibition period (t_{inh}) was directly proportional to the Trolox concentration. Since each molecule of Trolox is known to scavenge 2 molecules of radicals, the rate of free radical flux from AIPH is expressed as equation (2):

$$\text{Rate of radical flux} = 2\,ek_d[\text{AIPH}] = 2[\text{Trolox}]/t_{inh} \qquad (2)$$

The value of ek_d for AIPH was obtained from Fig. 3 and Eq. (2) as 1.4×10^{-6}/s at 37°C in aqueous dispersions containing 0.5 M SDS (PBS, pH 7.4), which is smaller than that obtained in PBS solution. Apparently, the difference in ek_d between PBS solution and micelle systems is ascribed to a smaller efficiency of radical formation (e) in more viscous SDS aqueous solution than PBS solution.[18]

Oxidation of Plasma and LDL

A typical example of the results of human plasma oxidation is shown in Fig. 4. This plasma sample obtained from healthy volunteer contained 42.5 μM

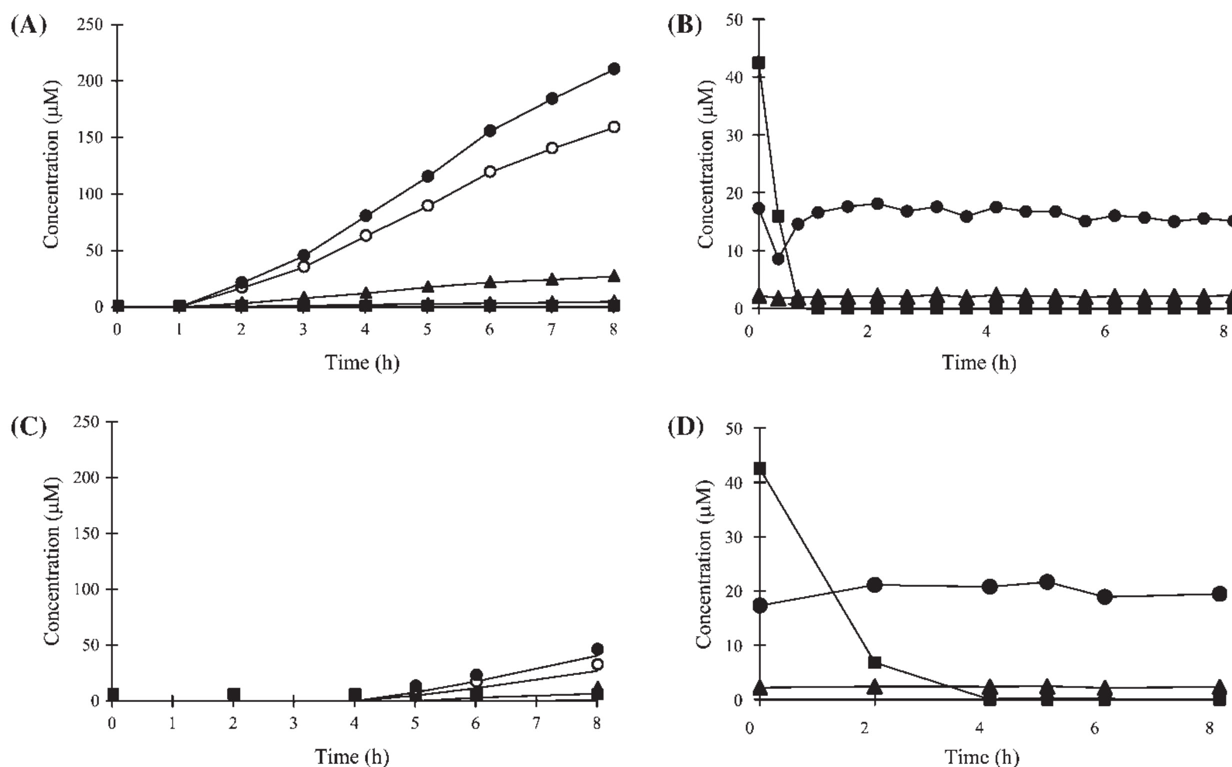

FIGURE 4 Oxidation of human plasma in PBS (pH 7.4) solution (1/1 by volume) induced by either 5 mM AIPH (A and B) or AAPH (C and D) at 37°C in air. Formation of hydroperoxides (A and C) and concentrations of ascorbic acid (square), α-Toc (circle), and γ-Toc (triangle) (B and D) were measured by HPLC as described in "Materials and Methods" section. In Figs. (A) and (C), closed circle, CEOOH + CEOH; open circle, CEOOH, closed triangle, PCOOH + PCOH; open triangle, PCOOH; closed square, cholesterol hydroperoxide. The initial concentrations of vitamin C, α- and γ-tocopherol contained endogenously in the plasma were 42.5, 17.3, and 2.2 μM, respectively.

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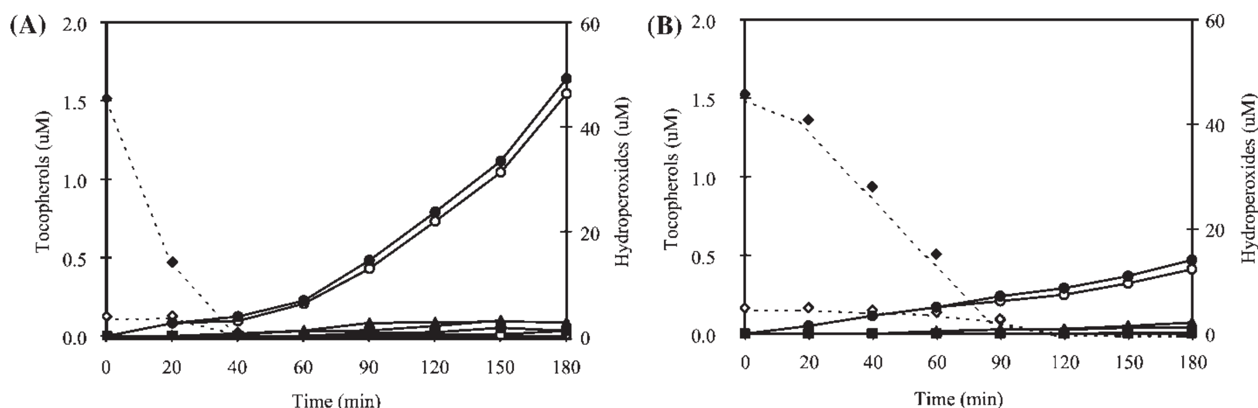

FIGURE 5 Oxidation of human LDL (0.25 mg protein/ml) in PBS (pH 7.4) solution induced by either 1 mM AIPH (A) or AAPH (B) at 37°C in air. Formation of CEOOH, CEOH, PCOOH, PCOH, FCOOH, FCCO, FCOH and concentrations of α- and γ-tocopherol were measured by HPLC as described in "Materials and Methods" section. Solid circle, CEOOH + CEOH; open circle, CEOOH; solid triangle, PCOOH + PCOH; open triangle, PCOOH; solid square, FCCO + FCOH; open square, FCOOH; solid diamond, α-tocopherol; open diamond, γ-tocopherol.

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vitamin C and 17.3 and 2.2 μM α- and γ-tocopherol, respectively. The oxidation was induced by either AIPH (Fig. 4A and B) or AAPH (Fig. 4C and D) at the same concentration. Initially, vitamin C was consumed and depleted within 0.7 and 4 h by AIPH and AAPH, respectively. The ratio of the consumption rate of vitamin C in the oxidation by AAPH to AIPH was 1:4.5, which is close to the ratio of their radical formation (1:3.8) obtained in Fig. 2. The formation of lipid peroxidation products was observed after disappearance of vitamin C. The ratio for the rate of total oxidation products induced by AIPH to that by AAPH was 3.2, which is again close to the ratio of the radical formation by AIPH to AAPH. The consumption of α-tocopherol was small even after depletion of vitamin C, probably because uric acid and other hydrophilic compounds scavenged aqueous radicals derived from AIPH or AAPH efficiently.

The oxidation of isolated LDL induced by the same concentrations of AIPH and AAPH was also carried out (Fig. 5). In this case, α-tocopherol was consumed initially by a rate much faster than that of γ-tocopherol. The rates of consumption of α-tocopherol were 0.87 and 0.14 nM/s with AIPH and AAPH, respectively. If one molecule of α-tocopherol is assumed to scavenge two molecules of radicals, the values for ek_d are obtained as 8.7×10^{-7} and 1.4×10^{-7}/s for AIPH and AAPH, respectively, which are considerably smaller than those from the data in PBS. The concomitant formation of hydroperoxides was observed during the consumption of α-tocopherol in both AIPH and AAPH cases.

Oxidation of Rat Erythrocyte

In order to explore further the application of AIPH to oxidative stress, the oxidation of erythrocyte was carried out. As shown in Fig. 6, AIPH and AAPH (150 mM) induced the hemolysis of erythrocytes, but its extent by AIPH was much larger than that by AAPH at both 1 and 1.5 h. The same extent of hemolysis was observed by 40 mM AIPH and 150 mM AAPH, as presumed from the ratio of their ek_d values.

Oxidation of Jurkat Cells

The effects of AIPH and AAPH on the viability of Jurkat cells cultured in serum-free medium were studied. AIPH and AAPH induced cell death in a concentration-dependent manner, and AIPH was more toxic than AAPH (Fig. 7). AMHP caused no significant cell death (data not shown). Interestingly, the extent of cell death was determined by the amount of free radical formed, independent of AIPH or AAPH (Fig. 8). It can be seen from Fig. 8 that about 1×10^5 cells/ml (20% of 5×10^5 cells/ml) died by 100 nmol/ml radicals formed. Previously, it was observed that 50% of erythrocytes underwent hemolysis when 300 nmol/ml radicals were formed from AAPH in 10% suspension of erythrocytes at 37°C.[9]

Administration of AIPH to Rats and Analysis of 8-isoPs, FCOH, and Lyso PC

In order to examine the effects of long term exposure to free radicals, rats were fed drinking water containing either 50 mM AIPH or 50 mM AMHP for 1 month. Interestingly, rats did not drink water containing AAPH. The reason for rejection of AAPH is not known at present. The body weight of rats given water containing either AIPH or AMHP increased in the same way as control rats which were given water without an azo compound. The concentrations of lyso PC, 8-isoPs ($m/z = 481$), and FCOH ($m/z = 456$) to FC ($m/z = 458$) relative to

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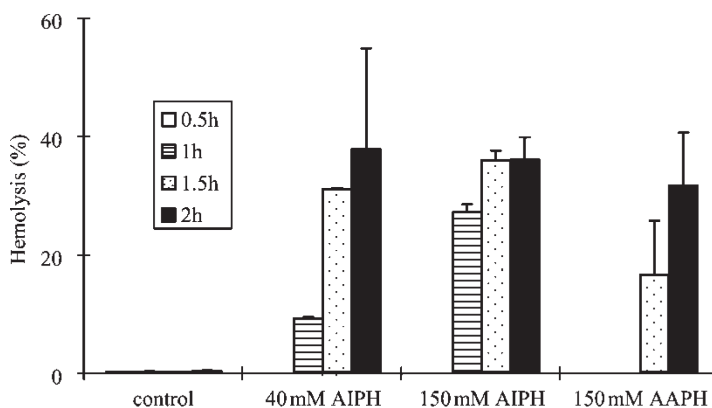

FIGURE 6 Oxidative hemolysis of human erythrocyte (10% hematocrit) in saline induced by either AIPH (A) or AAPH (B) at 37°C in air. Hemolysis of the erythrocyte was followed as described in "Materials and Methods" section.

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controls are summarized in Table I. All these data suggest the increase in the oxidative stress marker by AIPH in drinking water compared with the control.

DISCUSSION

In the present work, the kinetic data of AIPH was measured and compared with the well known azo compound, AAPH. Furthermore, the application of AIPH to several oxidation systems such as micelle, plasma, LDL, and cultured cells was also studied. The rate constant for decomposition of AIPH obtained by this work in PBS solution was in good agreement with that of the previous report.[21] Moreover, the ek_d value of AIPH in solution, micelle, LDL, and plasma were also assessed. The rate constant for decomposition of azo compound is determined primarily by the compound structure and temperature. However, the efficacy of radical production e is apparently quite sensitive to the viscosity.[17] It was previously found from the ESR spin probe study that the viscosity of the medium increased in the order of homogeneous solution $<$ micelle $<$ liposome.[18] In any event, the rate of chain initiation and the kinetic chain length in the oxidation of lipids can be easily controled by varying the concentrations of azo compounds.

The oxidation of plasma and cultured cells has been the subjects of extensive studies including the inhibition by antioxidants. For example, the plasma has been oxidized by either metal ions,[22–25] azo initiator,[22,26–32] or peroxynitrite.[33,34] We have previously reported the susceptibility of plasma lipids to peroxidation induced by either lipid-soluble radical initiator, MeO-AMVN, or SIN-1.[35] It is

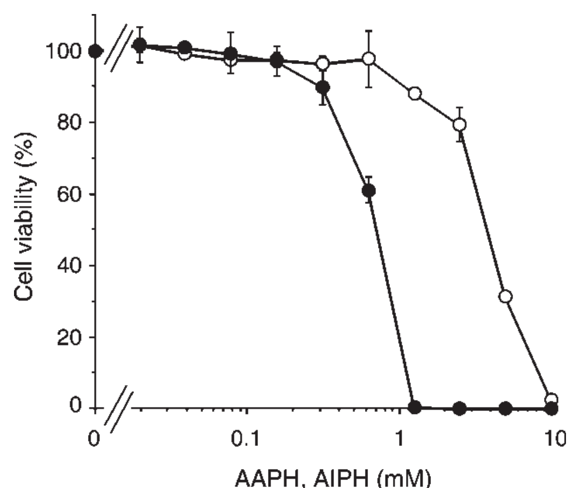

FIGURE 7 Effects of AIPH and AAPH on cultured cell viability. The Jurkat cells (5×10^5 cells/ml) were incubated with various amounts of radical initiators in the serum free medium at 37°C for 24 h, and the cellular viability was measured using MTT, as described in "Materials and Methods" section. The viable cells relative to time 0 (0.643 ± 0.054 at 570 nm) are shown as means ± S.E. ($n = 3$).

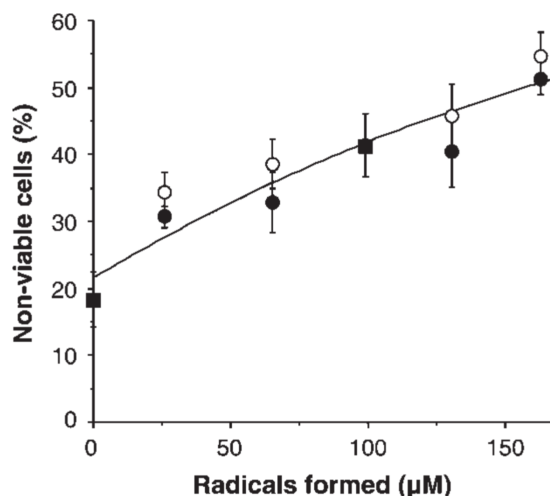

FIGURE 8 Cytotoxicity of free radicals formed from AIPH and AAPH. The Jurkat cells (5×10^5 cells/ml) were incubated with AIPH (0.53, 1.3, 2.0, 2.6, 3.3 mM) or AAPH (2.0, 5.0, 7.6, 10, 12.5 mM) at 37°C for 3 h and the cellular viability was measured using propidium iodide, as described in "Materials and Methods" section. The non-viable cells relative to total cells ($n = 3$) were plotted against the total radicals formed in 3 h from AIPH (open circle) and AAPH (closed circle).

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TABLE I Effects of AIPH and AMHP on the levels of 8-isoPs, FCOH, and Lyso PC in Plasma of Rats, mean ± SD (relative)

	Relative ion level to FC (m/z 458), 10^{-3}		
	8-isoPs (m/z 481)	FCOH (m/z 456)	Lyso PC mM in plasma
AIPH	1.55 ± 1.96 (16)	2.47 ± 1.12* (3.7)	0.30 ± 0.08 (1.1)
AMHP	1.40 ± 0.05** (14)	1.44 ± 0.15** (2.2)	0.27 ± 0.06 (1.0)
Control	0.10 ± 0.06 (1.0)	0.66 ± 0.18 (1.0)	0.27 ± 0.08 (1.0)

Significant differences from controls are shown by stars, *$p < 0.1$, **$p < 0.05$.

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possible to distinguish the actions of hydrophilic and hydrophobic antioxidants toward aqueous and lipophilic radicals separately. For example, as shown in Fig. 4, the amount of free radical formation in aqueous phase from 5.0 mM AIPH in 0.7 h is calculated as 66 μM from the ek_d value (26×10^{-7}/s) at 37°C in PBS (pH 7.4) solution, which is 1.6 times that of the initial vitamin C content (42.5 μM). On the other hand, little consumption of α- and γ-tocopherol was observed during the reaction conducted by this experiment. Considering the existence of other water-soluble antioxidant in plasma such as uric acid, the radicals formed from AIPH in aqueous phase were scavenged by the water-soluble antioxidants almost quantitatively. Interestingly, tocopherols were consumed very slowly during the period while hydroperoxides were formed, suggesting that α-tocopherol acted as a prooxidant due to its phase-transfer and chain-transfer activity, the tocopherol-mediated peroxidation (TMP), as observed by Bowry *et al.*[36] Cholesteryl ester hydroperoxide (CEOOH) and hydroxide (CEOH) were the major products and phosphatidylcholine hydroperoxides (PCOOH) and hydroxide (PCOH) were formed only as minor products. The ratio of CEOOH + CEOH/PCOOH + PCOH was 7.0, which was similar to that previously observed in the plasma oxidation induced by 3-morpholinosydnonimine (SIN-1).[35] LDL is composed of surface layer containing phospholipids and FC and inner core having cholesterol ester and triglycerides with one macromolecule of apolipoprotein B-100. It has been observed that the viscosity of outer monolayer of LDL is high and that the inner core is much more fluid than outer monolayer.[37] As shown in Fig. 5, the ratio of CEOOH + CEOH/PCOOH + PCOH was much larger than 1, which was similar to that observed in the oxidation of plasma. Interestingly, the obvious onset of formation of CEOOH and CEOH was observed after the depletion of α-tocopherol in the case of AIPH (Fig. 5A), which was not the case in AAPH (Fig. 5B). This may be attributed to a smaller TMP at higher rate of chain initiation.[36]

Application of azo compounds to cells and even to whole body is an advantageous method for studying the oxidative stress. Ishisaka *et al.*, reported that AAPH induced apoptosis of HL-60 cells by activation of caspase-3 through lysosomal cysteine proteases.[11] Although the mechanism of Jurkat cell death is unclear at present, it was clearly demonstrated by this study that the cell death occurred depending on the amount of radical flux. This observation is also applicable to the oxidation of erythrocyte. The total amount of lipids contained in the confluent (5×10^5 cells/ml) solution of Jurkat cell was less than 50 μM[38] and that in the erythrocyte solution (10% hematocrit) was much less than 50 μM (unpublished data obtained in our laboratory), resulting in the difficulty of using lipid-soluble azo compound such as AMVN and even MeO-AMVN since they may be incorporated entirely into the lipids.

It is noteworthy that rats did not drink water containing AAPH but they did drink water containing AIPH or AMHP. It is a big advantage to use AIPH in whole body, since long-term accumulation of oxidative stress by radicals will be clarified. Interestingly, it was observed that the administration of AIPH to rats for 1 month increased 8-isoPs, FCOH, and lyso PC which are known as the oxidative stress markers.[39–45]

Taken together, it may be concluded that AIPH, a water-soluble radical initiator, is useful for kinetic and mechanistic studies on oxidative stress to membranes, lipoprotein, cells, and even whole body.

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