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# OXIDATION OF CHOLESTEROL MOIETY OF LOW DENSITY LIPOPROTEIN IN THE PRESENCE OF HUMAN ENDOTHELIAL CELLS OR $CU^{+2}$ IONS: IDENTIFICATION OF MAJOR PRODUCTS AND THEIR EFFECTS

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Summary: Oxidation of lipoproteins is believed to play a key role in atherogenesis. In this study, low density lipoproteins (LDL) was subjected to oxidation in the presence of either human umbilical vein endothelial cells or with $Cu^{+2}$ ions and the major oxides formed were identified. While cholesterol-α-epoxide (C-αEP) was the major product of cholesterol peroxidation in the presence of endothelial cells, cholest-3,5-dien-7-one (CD) predominated in the presence of $Cu^{+2}$ ion. Both steroids were identified by gas chromatography/mass spectrometry. HDL cholesterol was resistant to oxidation. When tested on human skin fibroblasts in culture C-αEP (10 μg/ml) caused marked stimulation of $^{14}C$-oleate incorporation into cholesterol esters, while CD stimulated cholesterol esterification only mildly. These studies show that a) C-αEP is the major peroxidation product of LDL cholesterol moiety in the presence of endothelial cells and b) it causes marked stimulation of cholesterol esterification in cells. C-αEP may play a key role in increasing cholesterol esterification noted in atherogenesis. 

Oxidation of lipoproteins is now believed to play a key role in the pathogenesis of atherosclerosis (1). Lipoproteins modified by oxidation either with transient metals (2) or endothelial cells (3) are readily taken up by the macrophages leading to foam cell formation within the aorta (1). Oxidation of lipoproteins results in the modification of unsaturated fatty acids into a mixture of aldehydes. Previous studies of cholesterooxidation carried out in pure solutions or using crystals noted transformation of

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cholesterol into a variety of toxic oxides notably 7-keto cholesterol, 7-α- and 7-β-hydroxy cholesterol, 25-hydroxy cholesterol, and others (5,6). To date studies on the nature of the cholesterol oxidation products formed from either plasma or individual lipoproteins catalysed by transient metals have been limited (7) and some of the major oxidation products formed remain unidentified. Furthermore, the nature of cholesterol oxidation products formed in the presence of endothelial cells remains unknown. In this communication we have identified the major products of oxidation of cholesterol moiety of low density lipoprotein (LDL) with a) $Cu^{+2}$ ions, and b) with human umbilical vein endothelial cells. While the major cholesterol oxidation product formed with $Cu^{+2}$ was CD, in the presence of endothelial cells cholesterol α epoxide was the major product. Furthermore, we show that while cholesterol α epoxide is a powerful stimulant of cholesterol esterification, in human skin fibroblasts CD is less active.

## MATERIALS AND METHODS

All tissue culture media chemicals, calf serum (CF) were purchased from Gibco (Grand Island, NY). $^{14}C$ oleic acid (spect. act. 50 mCi/mMole) was purchased from Amersham International (Amersham, UK). Human skin fibroblast cells were purchased from American Type Culture Collection (Rockville, MD).

Blood samples were collected from volunteers in EDTA tubes. Plasma was separated by centrifugation at 1500 xg for 15 minutes at 4°C. Plasma LDL and HDL were isolated by ultracentrifugation by the method of Havel et al (8) as described previously (9). Plasma cholesterol levels were measured by enzymatic methods (10) or by gas chromatography (9).

Deliberate lipid peroxidation was carried out by incubating plasma or lipoprotein samples with 10 μM cupric acetate up to 72 hours as described by Triau et al (11). Positive controls for peroxidation effects included those samples containing antioxidant butylated hydroxy toluene (BHT). Aliquots of the samples were taken to measure lipid peroxidation by thiobarbutric acid method of Satoh (12).

Human umbilical vein endothelial cells (HUVEC) were obtained from umbilical cord by collagenase digestion (0.1% collagenase CLS-1; Worthington, Freehold NJ) in M199 containing 0.5% BSWA for 20 min. at 37°C (13). Isolated cells were suspended in growth medium [consisting of medium M199, 15% fetal calf serum (FCS; Gibco Co., Grand Island, NY), endothelial cell growth supplement (ECGS; Sigma) 20 μg/ml, heparin 90 μg/ml, 2 mM L-glutamine], and then plated on 0.5% gelatin-fibronectin (1 $\mu g/cm^2$) coated dishes. Greater than 95% of these cells showed

immunflorescent staining with anti-factor VIII antibody and di-acetylated-LDL-receptor (14). At confluence cells were passed (1:3) using 0.25% trypsin in 0.02% EDTA.

LDL modification by endothelial cells was carried out by incubating 100-200 μg/ml of LDL in lipoprotein deficient media M199 for 24-48 hours. After appropriate time the medium was collected. The cells were washed with phosphate buffered saline and collected with a rubber policeman. Aliquots of cells and media were used for sterol analysis.

$^{14}C$ oleic acid and unlabeled oleic acid were prepared as described by Cayatte et al (15) An ethanol solution of $^{14}C$ oleic acid was evaporated to dryness and resuspended with a 1:1 (v/v) solution containing unlabeled sodium oleate in 95% ethanol and transferred to the culture medium. All experiments used a final constant molar concentration (0.2 mM/ml) of unlabeled sodium oleic acid. The human skin fibroblasts were subcultred by general procedures described earlier (15) in 6 well dish with Dulbecco modified Eagle's minimum essential medium (DMEM) supplemented with penicillin (100 U/ml), streptomycin (100 mg/ml), 1% (v/v) L-glutamine (Gibco Company), 10% (v/v) heat-inactivated (30 min at 65°C) calf serum, and kept in a humidified incubator at 37°C in an atmosphere of 95% air and 5% $CO_2$. After 24 hours medium was replaced with fresh DMEM medium with 5% calf serum. Cells were allowed to grow until approximately 60-70% confluency. At this time cells were washed twice with ice cold sterile phosphate buffer saline (PBS) at pH 7.4 and exposed to 2% calf serum DMEM containing $^{14}C$-labeled oleic acid and cold oleic acid and then incubated with 10 μg/ml of different cholesterol oxides in the humidified incubator for 24 hours.

After the indicated period of time, the culture was terminated and the cells were washed four times each with ice-cold phosphate-buffered saline and the cells removed with trypsin (0.25%). After centrifugation at 900 x g for 7 min. the pellet was brought to a volume of 500 ul in NaCl (0.15M), pH 7.4 and the suspension sonicated for 15 sec in ice. Aliquots were taken for protein determination by Lowry's method as modified by Peterson (16) and for lipid analysis. Lipids were extracted according to Folch et al (17) by adding an appropriate volume of chloroform-methanol (2:1). The organic phase containing the cellular lipids was carefully removed, evaporated to dryness under $M_2$, and resuspended in chloroform-methanol (2:1). The different lipids were separated by thin layer chromatography, and radioactivity was measured by liquid scintillation counting as described (15) previously.

For the extraction of cholesterol oxides, from the medium and cells aliquots of the samples were subjected to mild saponification (18), and steroids were extracted with 10% ethyl ether in petroleum ether and subjected to thin layer chromatography on silica gel G (19) using solvent system benzene:ethyl acetate (2:3, v/v). The steroids were detected by spraying with 10% phosphomolybdic acid in ethanol. In some instances

steroids were chromatographed on silica gel G in the solvent system benzene:ethyl acetate (3:1) which clearly separated monoketo steroids (5β-cholestanone) from the solvent front. The bands were detected by 2,6-dichloroflorscien spray and areas corresponding to standard 5β-cholestanone, cholesterol β-and α-epoxide, 25-hydroxy cholesterol, 7-keto cholesterol, 7α-and 7β-cholesterol were scraped, eluted with chloroform:methanol (2:1) and subjected to gas chromatography/mass spectrometry.

The steroids were initially identified by gas liquid chromatography as described (14) by retention times relative to 5α-cholestane. The sterols were analysed using a Perkin Elmer Sigma I gas chromatograph. A DB-17 column (15 meter, - 1μ film thickness, I.D. 0.53 mm) was used. Chromatographic conditions were: injector: 275°C; detector 280°C, column 248°C; carrier gas helium, 10 ml/min.

The major product of cholesterol oxidation was further identified by gas chromatography/mass spectrometry (GC/MS). The GC (Varian 3400, Varian Associates, Sugarland, TX) was equipped with a capillary column (30 meter, DB-5, 0.25 micron film thickness, 0.25 mm I.D., J&W Scientific Co., Folsom, CA) and interfaced to a MS (Finnigan 8900 Ion Trap Detector, Finnigan MAT, San Jose, CA). The DB-5 column was directly interfaced to the MS, the injector and transfer line were at 260°C and the column was programmed from 120°C to 265°C at 9.9°C/min with a 0.5 minute delay and a 25 minute hold time at 265 °C.

## RESULTS

The nature of the cholesterol oxidation products from LDL and HDL in the presence of $Cu^{+2}$ ions was analyzed by gas-liquid chromatography on 30 meter DB-17 columns (Figure 1A and B). Samples derived from oxidized LDL displayed a major peak with a retention time of 3.33 (relative to 5α-cholestane) and other oxygenated compounds noted previously (5). HDL samples, however, yielded only traces of peaks corresponding to known products of cholesterol oxidation.

The peak derived from LDL with a relative retention time of 3.33 (relative to cholestane) was further purified by thin layer chromatography. This peak migrated as a band in an area corresponding to keto derivatives of cholesterol (5β cholestanone, and $\Delta^4$-cholest-3-one) and gave a retention time close to these ketones upon gas chromatography. This compound was further identified by GC/MS. Mass spectrum of this compound (Fig. 2A) showed a major molecular ion at m/e 382 consistent with elemental composition of $C_{27}$ $H_{42}$ O. The absence of (M-$H_2O$) and (M-$CH_3$-$H_2O$) ions ruled out the presence of hydroxyl group in the molecule. The ions at m/e

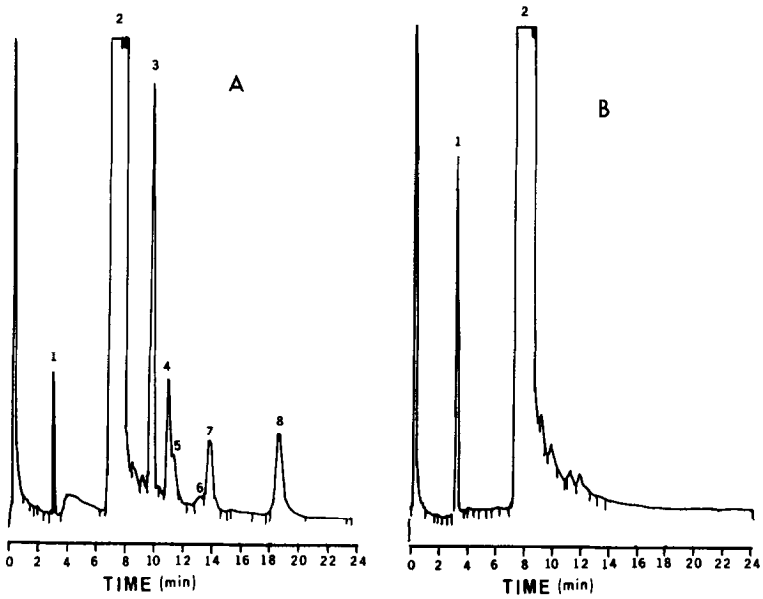


Figure 1. Gas-liquid chromatographic analysis of oxysterols formed from LDL (A) and HDL(B) fractions following oxidation with $Cu^{+2}$ ions. The oxysterols were extracted and analyzed in 30 m DB-17 column as described under Experimental Procedures. Peak identification: 1 = 5α-cholestane (internal standard); 2 = cholesterol; 3 = peak under consideration; 4 = 5β-6β-epoxy cholesterol; 5 = 25 hydroxycholesterol; 6 = 7α-hydroxy cholesterol; and 7 = 7 keto cholesterol.

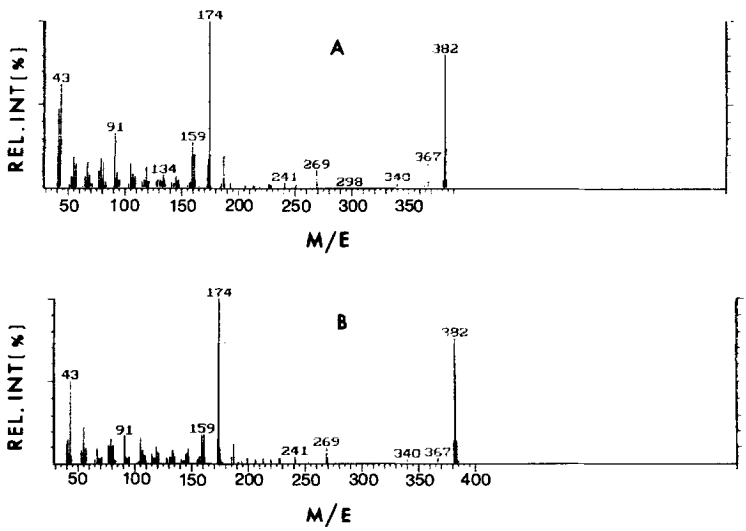


Figure 2. Mass spectrum of standard cholest-3.5-dien-7-one (A) and the compound isolated (peak 3 in Figure 1A) from human plasma LDL after oxidation with $Cu^{+2}$ ions (B). The compound was purified and subjected to GC/MS as described under Experimental Procedures.

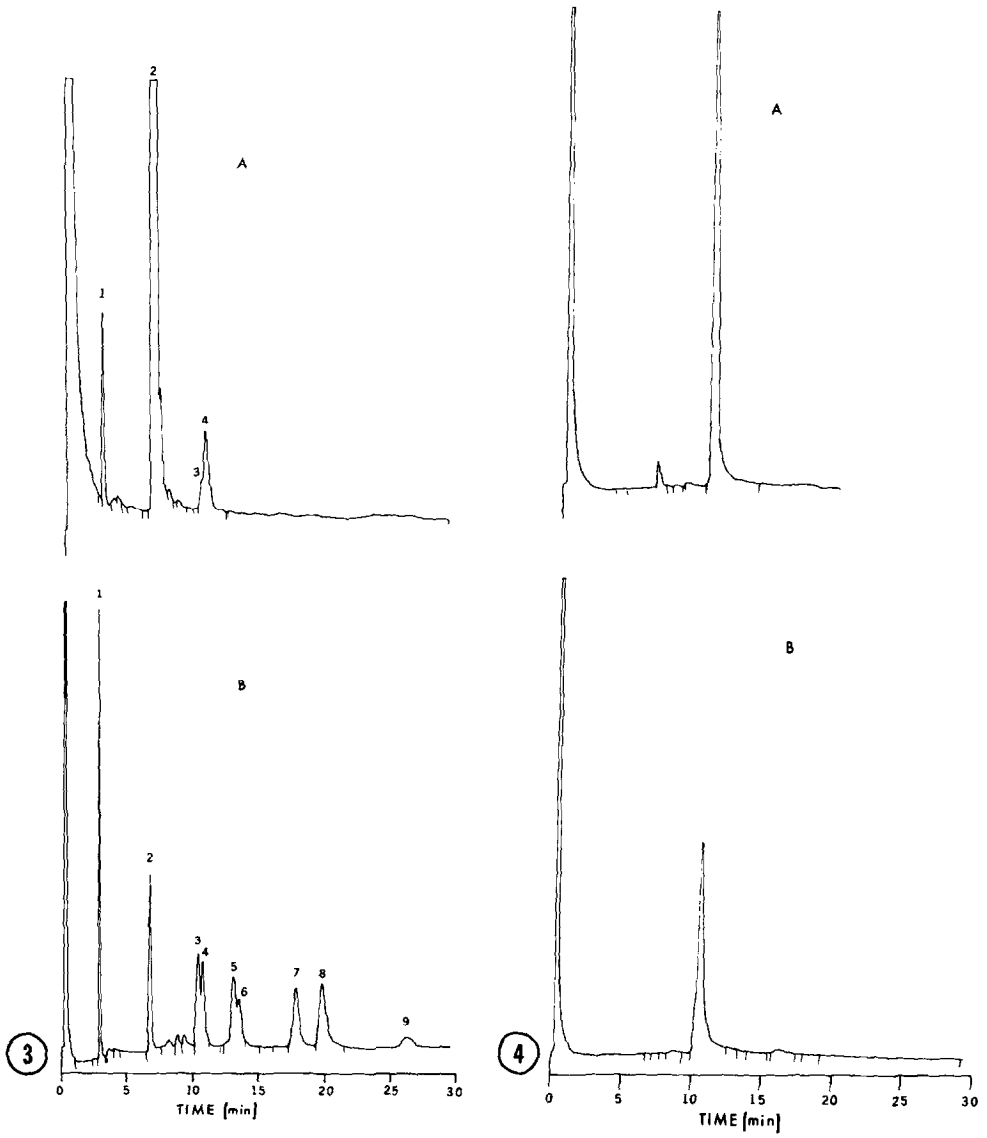


Figure 3. Gas-liquid chromatographic analysis of oxysterols formed from human LDL incubated in the presence of endothelial cells for 48 hours (A). A mixture of standard oxysterols chromotographed under identical condition is also shown (B). The samples were processed and analyzed on a DB-17 column as described under Experimental Procedures. Peak identification: 1 = 5α-cholestane (internal standard); 2 = cholesterol; 3 = 5β-6β-epoxy cholesterol; 4 = 5β, 6α-epoxy cholesterol; 5 = 25-hydroxy cholesterol; 6 = 7α-hydroxy cholesterol; 7 = 7 keto cholesterol; and 8 - 26 hydroxy cholesterol.

Figure 4. Gas liquid chromotographic analysis of 5β, 6α-epoxy cholesterol following purification by TLC (A) and authentic compound (B). Column conditions were described under Figure 1 and in the text.

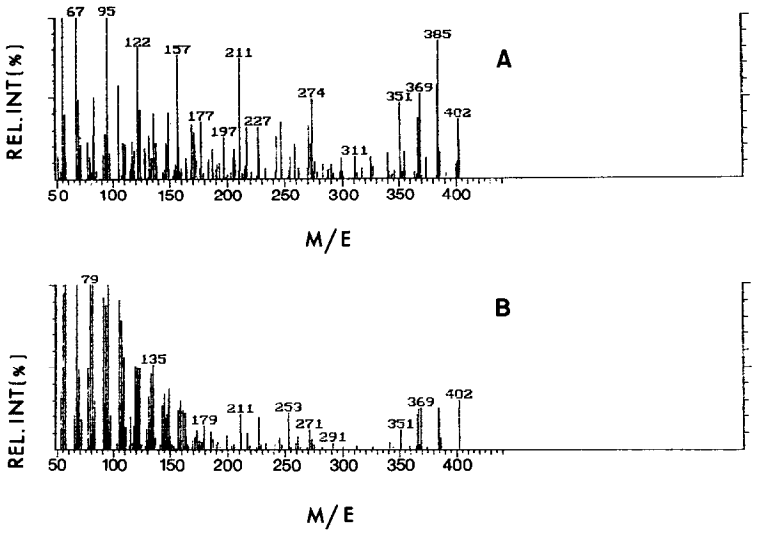


Figure 5. Mass spectrum of standard α-epoxy cholesterol (A) and the compound isolated from human plasma LDL after oxidation with endothelial cells (B). The compound was purified and subjected to GC/MS as described under Experimental Procedures.

269 resulting from the loss of side chain (M-$C_8H_{17}$) indicated the presence of ketone group and two double bonds in the tetracyclic part of the molecule. The mass spectrum corresponded to the spectrum of the authentic CD (Fig. 2B). Therefore, this compound was identified as CD. The other compounds formed corresponded to previously noted oxysterols (cholesterol α and β-epoxide, 7α-hydroxy cholesterol, 25-hydroxy cholesterol 7-keto cholesterol, etc.)

Gas chromatographic analysis of cholesterol oxidation products in the media upon 48 hour incubation of LDL with human endothelial cells is shown in Figure 3A. Only two significant peaks (apart from cholesterol) was noted in these samples when compared to authentic standard mixture of cholesterol oxides (Fig. 3B), and these peaks corresponded to cholesterol α-epoxide and cholesterol β-epoxide. The major peak had a retention time of 4.15 (relative to 5α-cholestane). When this compound was further purified by thin layer chromatography the band migrated in an area corresponding to epoxy derivatives of cholesterol. The purified band upon gas chromatography gave retention time identical to cholesterol α-epoxide (Figs. 4A and B). This compound was further identified by GC/MS. Mass spectrum of this compound (Fig. 5A) showed a molecular ion at m/3 402 consistent with the elemental composition $C_{27}$ $H_{46}$ $O_2$. It gave major peaks m/e 385, m/e 369 (M-$H_2o$-$CH_3$) and m/e 351. The mass spectrum corresponded to the spectrum of authentic cholesterol α-epoxides (Fig.

TABLE 1. INCORPORATION OF $^{14}C$ OLEIC ACID INTO CELLULAR LIPIDS BY HUMAN SKIN FIBROBLASTS: EFFECTS OF CHOLESTEROL OXIDATION PRODUCT

| | $^{14}C$ OLEIC ACID INCORPORATION (CPM/10 UG OF PROTEIN) | | | | |
|---|---|---|---|---|---|
| Groups | Phosphlipids | Diglycerides | Free Fatty Acids | Triglycerides | Cholesterol Ester |
| Control | 17851 ± 1504 | 7327 ± 972 | 6786 ± 1227 | 49898 ± 4462 | 954 ± 136 |
| α-Epoxy Cholesterol | 18767 ±1302 | 8493 ± 877 | 8933 b ± 1835 | 58803 c ± 4277 | 9156 d ± 1305 |
| β-Epoxy Cholesterol | 16445 ± 2098 | 8941 a ± 820 | 9262 b ± 976 | 45148 ± 4789 | 2783 d ± 346 |
| Cholest-3, 5-dien-7-one | 18342 ± 2413 | 8068 ± 621 | 7986 ± 843 | 53610 ± 7843 c | 1293 ± 206 d |

Cells were incubated for 24 hours in 35 mm dish with 2 ml of DMEM supplemented with $^{14}C$ oleic acid (0.25 μc/ml) in the presence of cholesterol oxides (10/μg/ml). Fatty acid incorporation into different lipids was evaluated as described under experimental procedures. Values are expressed as mean ± SE of 5-6 analysis.

a $p < 0.02$ when compared to the control group.

b,c,d $p < 0.05$ when compared to control group.

5B). Therefore this compound was identified as cholesterol α-epoxide. The minor component had a retention time corresponding to cholesterol β-epoxide but could not be identified conclusively. Cholesterol α-epoxide was also the major oxysterol present in the endothelial cell extracts after incubation with LDL.

Further studies were done on the effect of cholesterol α and β-epoxides, and CD on human skin fibroblasts in culture. At a concentration of 10 μg/ml cholesterol α-epoxides caused marked stimuation of $^{14}C$-oleate incorporation into cholesteryl esters (Table 1). Cholesterol-β-epoxide was also potent in the stimulation of cholesterol esterification, but CD had only a mild effect in this respect.

## DISCUSSION

The results of our studies have shown three important points relevant to the possible role of oxidation of cholesterol moiety of lipoproteins in atherogenesis: a) The major oxidation product formed from plasma and LDL cholesterol fraction in the presence of $Cu^{+2}$ ions is CD. The formation of this product far exceeds that of other oxygenated derivatives of cholesterol noted previously (5,6). It is of interest to note that this compound has also been identified in milk and butter earlier (20). Although its origin is not clear it could arise from dehydration of 7-keto cholesterol (5). Although the effect of other oxygenated derivatives of

cholesterol (25-hydroxy, 7-keto, and 24-and 26-hydroxy cholesterol) on cellular growth and metabolism has been investigated (21,22), nothing is known regarding the effect of CD. b) The major product formed from LDL fraction in the presence of human endothelial cells, however, is cholesterol α-epoxide. Some of the other oxygenated products, i.e. 7-α-hydroxy, 7-keto, and 25-hydroxy-cholesterol, were not formed under these conditions. Cholesterol α-epoxide is also formed during cholesterol oxidation in the presence of $Cu^{+2}$ ions, but its concentration is less than that of CD. c) Our studies show that cholesterol α-epoxide is extremely potent in stimulating cholesterol esterification. While β-epoxy cholesterol was also effective in increasing cholesterol esterification, CD, on the other hand, stimulated cholesterol esterification only mildly. Therefore, cholesterol α-epoxide, the major compound formed in the presence of endothetial cells, might have an active role in regulating cholesterol metabolism during atherogenesis. Since cholesterol α epoxide is also a mutagen (23), it may cause permanent changes in aorta.

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