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EFFECT OF CHOLESTEROL α AND β EPOXIDES ON CELL KILLING AND TRANSFORMATION

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Abstract—V-79 Chinese hamster lung fibroblasts and C3H-10T1/2 mouse embryo cells were used to study the toxicity and transformation effects of the 5α,6α-, and 5β,6β-epoxy derivatives of cholesterol. Both epoxides were found to be carcinogenic, and the transformation frequency increased with epoxide concentration and exposure time. The 5β,6β-epoxide caused a higher level of transformation than the α-isomer, and this is consistent with its greater toxicity.

Epoxidation of cholesterol under physiological conditions is well recognized, and both the 5α,6α- and 5β,6β-epoxy isomers‖ have been detected in a variety of tissues (for a review, see Ref. 1). Enzymatic, cytochrome P-450 mediated oxidations yield the α-isomer as the major or sole product [2], whereas auto-oxidation and hydroperoxide-induced oxidations favour the β-isomer with yield up to 10-fold over the α-isomer [3]. Although neither isomer is active in the Ames Salmonella mutagenicity assay [4], cholesterol epoxides have been associated with ultraviolet-induced skin cancer [5], and more recently it has been shown that the α-epoxide is a weak direct-acting mutagen in mammalian cells [6]. Since the 5β,6β-epoxide is not readily available, evaluation of the biological effects have been focused mainly on the 5α,6α-epoxide. Furthermore, commercially available 5α,6α-epoxide is contaminated with up to 5% of the β-isomer, which often is not taken into consideration, particularly in earlier biological studies [1]. For these reasons we prepared the pure 5α,6α- and 5β,6β-epoxides and compared their cytotoxicity and transformation effects on mammalian cells.

MATERIALS AND METHODS

Cholesterol epoxides. Stereoselective epoxidation of cholesterol with *m*-chloroperbenzoic acid in CH_2Cl_2 [7] followed by crystallization from acetone gave pure 5,6α-epoxy-5α-cholestane, m.p. 141° (lit. [7], m.p. 141–143°). The β-isomer was obtained by the method of Levine and Wall [8]. Briefly, cholesterol acetate was converted to 5α-bromo-3β,6β-diacetoxycholestane with acetyl hypobromite followed by decomposition and hydrolysis of the bromosterol via reflux in methanolic sodium hydroxide. Silica gel column chromatography in hexane-ethyl acetate (5–30%) followed by crystallization from methanol gave pure 5,6β-epoxy-5β-cholestane, m.p. 131°. The isomeric epoxides are distinguished by their different mobilities on a high performance liquid chromatographic partisil PAC column (Whatman) eluted with 4% isopropanol in hexane (rel. retention time βα-isomer is 1.2) and the characteristic chemical shifts of the 6β- and 6α-proton doublets in the 1H NMR ($CDCl_3$) spectra (5α,6α-epoxide, 2.90 ppm: J = 3.6 Hz, 6β-H; 5β,6β-epoxide, 3.05 ppm: 1H, d, J = 2.7 Hz, 6α-H) (identical to data reported in Ref. 1, p. 508).

Transformation effects and cytotoxicity. Chinese hamster lung fibroblasts were used for the toxicity studies, and C3H-10T1/2 mouse embryo cells were used to compare the toxicity and transformation effects in these experiments.

The Chinese hamster lung fibroblasts were continuously cultured in exponential growth phase in 25 cm^2 flasks that were maintained in a 37° incubator containing 98% air and 2% CO_2. The medium used was a ratio of 1:1 Dulbecco's modified medium and F12 medium (D.F.) containing 10% fetal calf serum and 0.05% gentamycin. This culture medium supported good cell growth at low serum concentrations for which growth would not occur in Eagle's basal medium. For pH control, the medium contained 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) buffer and 10 mM $NaHCO_3$. This buffer combination resulted in good pH control (pH 7.4) even at high cell densities. Under these conditions, cells had a doubling time of about 10–12 hr.

The C3H mouse embryo cells, developed by Reznikoff *et al.* [9, 10] and designated as 10T1/2 clone 8, were obtained from the American Type Culture Collection. The cells were at passage 7 and

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‖ Systematic names for common sterols given trivial names in the text include: cholesterol, cholest-5-en-3β-ol; 5α,6α-epoxide, 5,6α-epoxy-5α-cholestan-3β-ol; 5β,6β-epoxide, 5,6β-epoxy-5β-cholestan-3β-ol; 3β,5α,6β-triol, 5α-cholestane-3β,5,6β-triol.

cultured to passages 8 and 9, when they were frozen in liquid nitrogen. For the experiments, the cells were thawed at 37° and cultured in D.F. medium containing 10% heat-inactivated fetal calf serum with no antibiotics and having the same pH buffering system as described above. Cells were cultured up to passage 15 in 25 or 75 cm^2 flasks, and higher passage numbers were not used for the experiments. Incubation was carried out under the same conditions as for V79 cells, and under these conditions cells had a population doubling time of about 20 hr.

For experiments, both the V79 cells and the C3H-10T1/2 cells from an exponentially growing culture were trypsinized (0.01% trypsin for 5 min), suspended as single cells, counted and plated into plastic flasks at numbers estimated to give 50–150 survivors for V79 cells in 25 cm^2 flasks and 200–400 survivors for C3H-10T1/2 cells in 75 cm^2 flasks. These flasks were incubated overnight before experimental treatment was started.

The 5α,6α- and 5β,6β-epoxides were dissolved in 95% ethyl alcohol. This solution was inoculated into the cell culture flasks to give the desired concentration of the epoxide at a dilution factor equal to or greater than 100×. These dilutions ensured that the alcohol concentration in the cultures was less than 1%. The cultures were agitated vigorously while the epoxide solutions were added. After the desired exposure time at 37° the solutions were poured from the flasks, the cultures were rinsed with 5 ml of medium, and then 5 ml of medium was added to the 25 cm^2 flasks (V79 cells) and 25 ml was added to the 75 cm^2 flasks (C3H-10T1/2 cells). All procedures were done at 37°. The flasks designated for survival were incubated for 8 and 10 days for the V79 and C3H-10T1/2 cells, respectively, before the cultures were rinsed, fixed, and stained, and the surviving colonies, were counted. For the V79 and C3H-10T1/2 cells, the multiplicity after overnight incubation was 1.9 and 1.1 respectively. The results were not adjusted for the multiplicity factor. The plating efficiencies ranged from 15 to 20% for the C3H-10T1/2 cells and from 70 to 90% for the V79 cells.

For the transformation assay, system 16 or more flasks were used. The medium was changed every 7 days for 6 weeks. The cultures were then fixed and stained (2% Giemsa), and cell transformation was scored using the morphological criteria previously described by Reznikoff *et al.* [9, 10]. Only type II and III transformed foci were scored.

The data were analyzed in two ways. The experimental mean and standard error were calculated directly from the number of replicate flasks for each datum point and are presented as transformation frequency per surviving cell. Since cultures were incubated for 6 weeks and refed weekly, a potential source of error was that transformed cells could become dislodged and form new foci. To avoid this possible problem, the mean transformation frequency and standard error were calculated from Poisson analysis of the number of flasks with no transformant $P(O) = e^{-\lambda}$ as previously described by Han and Elkind [11]. When all flasks contained transformed foci, the calculation was made assuming one flask to be free of transformed foci. This value is given in Table 1 and is preceded with a "greater than" sign.

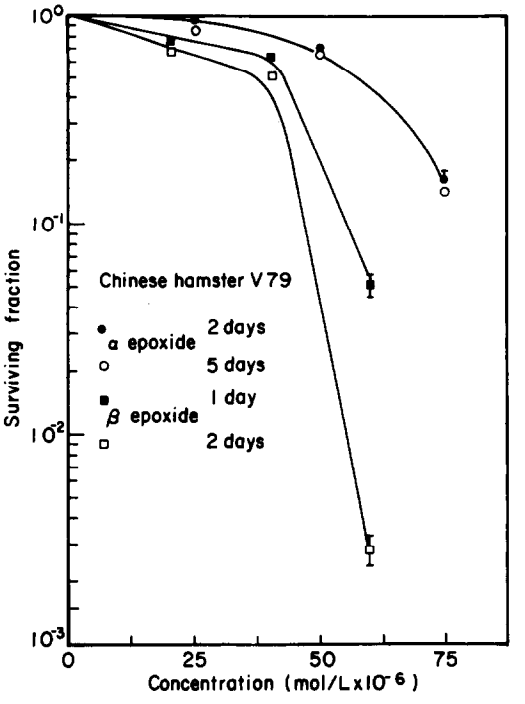

Fig. 1. Survival of V79 cells incubated with 5α,6α-epoxide (α) and 5β,6β-epoxide (β) as a function of epoxide concentration and time. Plating efficiency: 80%.

Standard error of the mean for four or more replicate flasks is shown on the figures when greater than the datum point symbol. Experiments were repeated three times, and the curves shown on the figures were fitted by eye.

For irradiation, an X-ray machine operating at 250 kV and 15 mA with an added 1 mm filter was used. The dose rate was 3.0 Gy/min, and all irradiations were performed at 37°.

RESULTS

The data shown in Fig. 1 indicate that for V79 cells both isomeric epoxides became significantly toxic at concentrations greater than 25 μM for the 2- and 5-day exposure time. The 5β,6β-epoxide was much more toxic than the α-isomer. Toxicity became significant at the 20 μM concentration and increased rapidly at concentrations above 40 μM. Only 1- and 2-day exposure times were used for the β-epoxide because longer exposures resulted in extremely high toxicity, prohibiting the measurement of survival.

Figure 2 shows the survival of C3H-10T1/2 cells after exposure to the isomeric epoxides. These cells were more sensitive than the V79 cells. Even the 25 μM concentration of the 5α,6α-epoxide was significantly toxic for 2- or 3-day exposure times. A 5-day exposure was excessively toxic and could not be measured. As with the V79 cells, the C3H-10T1/2 cells were also more sensitive to treatment with the β-isomer. Even a 1-day treatment with a 20 μM solution of the 5β,6β-epoxide caused significant toxicity.

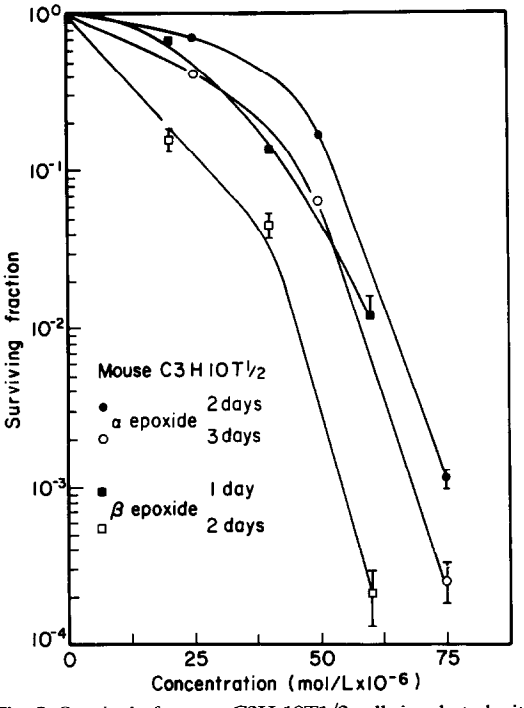

Fig. 2. Survival of mouse C3H-10T1/2 cells incubated with 5α,6α-epoxide (α) and 5β,5β-epoxide (β) as a function of epoxide concentration and incubation time. Plating efficiency: 15–20%.

For the higher concentrations of 50 and 75 μM, toxicity increased very rapidly and could not be measured at concentrations greater than 75 or 60 μM for the 5α,6α- and the 5β,6β-epoxides respectively.

The effects of epoxides on cell transformation are shown in Table 1. The transformation frequency is shown based directly on the number of transformed foci counted or on the Poissonian calculation of P(O) as outlined in Materials and Methods. There is good agreement between these two types of calculation. Two experiments are shown in Table 1. The transformation data show that both epoxides are carcinogenic and that the transformation frequency increased with epoxide concentration and exposure time. The 5β,6β-epoxide caused a higher level of transformation than the α-isomer, and this is consistent with its greater toxicity. The exposure to 1% alcohol for 5 days was only slightly toxic and did not cause transformation.

For comparison, the transformation frequency in response to a 3.5 Gy dose of X-rays is also shown. The exposure to the isomeric epoxides at the highest concentrations for the longest exposure times caused transformation frequencies equivalent to the 3.5 Gy treatment.

DISCUSSION

Sevanian and Peterson [6] showed that the 5α,6α-epoxide is a weak mutagen in Chinese V79 cells. Our

Table 1. Transformation and survival after exposure to 5α,6α- and 5α,6α,-epoxides

Treatment*	Viable cells per flask	% Survival†	Numbered Flasks	Numbered Foci‡	Transformation frequency × 10^{-3} Per viable cell§	Transformation frequency × 10^{-3} Poisson calculation‖	Significance¶ (T-Test)
Experiment 1							
Control	376	100	16	0	< 0.16	< 0.17	
EtOH	493	89.0 (4.5)	16	1	0.13	0.13	NS
X3.5	406	39.6 (2.9)	16	10	1.54 (0.44)	1.71 (0.51)	P < 0.005
α 1-50	369	64.4 (2.3)	16	3	0.51 (0.27)	0.56 (0.31)	P < 0.20
α 2-50	202	26.8 (1.0)	16	5	1.54 (0.74)	0.42 (0.66)	P < 0.05
α 5-50	201	21.4 (0.7)	16	5	1.75 (0.60)	1.86 (0.76)	P < 0.025
β 1-50	100	17.6 (2.7)	16	4	2.50 (1.4)	2.87 (1.3)	P < 0.025
Experiment 2							
Control	409	100	16	0	< 0.15	< 0.16	
EtOH	510	93.8 (2.8)	16	0	< 0.12	< 0.13	NS
X3.5	357	34.3 (6.9)	18	12	1.86 (0.51)	1.94 (0.54)	P < 0.005
α 2-25	386	71.1 (11.6)	16	2	0.32 (0.15)	0.35 (0.23)	NS
α 2-50	166	17.4 (3.1)	16	4	1.51 (0.67)	1.73 (0.81)	P < 0.05
α 2-75	2	0.11 (0.02)	16				
β 1-20	328	70.0 (8.6)	16	3	0.57 (0.31)	0.63 (0.34)	P < 0.20
β 1-40	126	14.3 (2.0)	16	4	1.98 (0.88)	2.28 (1.1)	P < 0.05
β 1-60	199	0.4 (1.9)	18	7	1.95 (0.60)	2.48 (0.83)	P < 0.005

Mouse C3H 10T1/2 cells were plated in 75 cm^2 flasks and incubated overnight prior to exposure to cholesterol epoxides or X-rays. The flasks designated for survival were incubated for 10 days. For the transformation assays the medium was changed every 7 days for 6 weeks. The cultures were then rinsed, fixed and stained, and the surviving colonies were counted. Cell transformation was scored using established morphological criteria [9, 10].

* X3.5: 3.5 Gy dose of X-rays; α: 5α,6α-epoxide; β: 5β,6β-epoxide. The first number after α or β is the exposure time in days and the second number is the concentration in μM.

† Plating efficiencies ranged from 15 to 20%. The numbers in parentheses are standard deviations.

‡ Total number of type II and III transformants was counted.

§ Transformation per surviving cell × 10^{-3}.

‖ Transformation calculated from P(O). See Materials and Methods.

¶ Statistical difference relative to control. NS = not significantly different (P > 0.20).

results with the transformation tests on mouse C3H-10T1/2 cells confirm these observations. Furthermore, we have shown that the 5β,6β-epoxide exhibits substantially higher transformation than the α-isomer. Both isomeric epoxides are widespread in biological material [1], and both epoxides are slowly hydrated to yield 5α-cholestane-3β,5,6β-triol [12]. This conversion product is more than twice as toxic as the 5α,6α-epoxide but only marginally mutagenic [6]. Accordingly, the enhanced cytotoxicity observed after longer epoxide treatment periods could partially result from 3β,5α,6β-triol formation. However, we observed a substantial increase in transformation parallel with higher cytotoxicity, suggesting that also during prolonged incubations the transformation effects result from direct epoxide action. Sevanian and Peterson [6] suggested that tissue with low epoxide hydratase activity may be prone to developing cholesterol epoxide induced mutagenic lesions. Our observation of the enhanced transformation effects of the β-isomer, combined with its ready *in vivo* formation through non-enzymic pathways, suggests that particularly the 5β,6β-epoxide may be involved in potential biological damage associated with cholesterol epoxidation.

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