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Cholesterol-3-beta, 5-alpha, 6-beta-triol induced genotoxicity through reactive oxygen species formation

Y.W. Cheng [a,*], J.J. Kang [b], Y.L. Shih [a], Y.L. Lo [a], C.F. Wang [a]

[a] School of Pharmacy, Taipei Medical University, No. 250, Wu-Shing Street, Taipei 101, Taiwan
[b] Institute of Toxicology, College of Medicine, National Taiwan University, 100 Taipei, Taiwan

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Abstract

The mutagenicity of oxysterols, cholesterol-3β,5α,6β-triol (α-Triol), 7-keto-cholesterol (7-Keto) and cholesterol-5α,6α-epoxide (α-Epox) were examined by the Ames method and chromosome aberration test in this study. Only α-Triol concentration-dependently caused an increase of bacterial revertants in the absence of metabolic activating enzymes (S9), but not 7-keto and α-Epox. The mutagenic effect of α-Triol was reduced by the addition of S9. On the other hand, although α-Triol significantly induced chromosome aberration in CHO-K1 cells with and without S9. However, the addition of S9 reduced the degree of abnormal structure chromosome compared to without S9 mix. Catalase and superoxide dismutase (SOD) inhibited α-Triol induced increase of revertants in *Salmonella typhimurium* and chromosome aberration frequency in CHO cells, suggesting that reactive oxygen species (ROS) might be involved in the genotoxic effect of α-Triol. Treatment with α-Triol increased the ROS production in CHO cells, which could be attenuated by catalase and SOD. Results in this study suggested, for the first time that α-Triol, causes genotoxic effect in an ROS-dependent manner.

Keywords: Oxysterol; Cholesterol-3β,5α,6β-triol; Ames test; Chromosome aberration; Genotoxicity; Reactive oxygen species; Antioxidant

1. Introduction

Oxysterols are oxygenated cholesterol derivatives and constitute a family of compounds with various biological activities (Guardiola et al., 1996). They are formed in the diet during heating (Chien et al., 1998), or are generated in cholesterol-containing products during prolonged storage (Li et al., 1996). They are found in noticeable amounts in powdered milk, cheese, egg products (Addis, 1986) and other meat-containing dishes (Tai et al., 2000). Humans can absorb oxysterols from food into the bloodstream (Emanuel et al., 1991). It has also been shown that oxysterol can be cleared from the plasma rapidly and be widely re-distributed in different parts of the body (Krut et al., 1997; Vine et al., 1997). Oxysterols may be taken up from the plasma by tissues and organs many times more rapidly than cholesterol (Krut et al., 1997). Not only supplied by food, they can also be synthesis in vivo, either by oxidation or by enzymatic reaction (Smith, 1996).

Oxysterols are potent regulatory molecules which can inhibit hydroxymethyl-glutaryl-coenzyme (HMG-CoA reductase) (Parish et al., 1999), and prevent lymphoid cell growth (Larsson and Zetterberg, 1995), altering the membrane fluidity, permeability, stability, and activity of membrane-bound enzymes, and interfering with gap junction communication and modulation of intracellular calcium (Guardiola et al., 1996). In addition,

* Corresponding author. Tel.: +886 2 27361661x6123; fax: +886 2 23783181.
E-mail address: ywcheng@tmu.edu.tw (Y.W. Cheng).

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oxysterols have been shown to exhibit cytotoxicity in a number of cell lines, including smooth muscle cells, fibroblasts and vascular endothelial cells (Guardiola et al., 1996). Induced apoptosis (Lizard et al., 1998; O'Callaghan et al., 2001), and reactive oxygen species (ROS) were reported to involved in this effect (Lizard et al., 1998). Yoon et al. (2004) have suggested that 22-hydroxycholesterol (22-OH) might induce carcinogenesis through induced cyclooxygenase-2 expression in cholangiocytes.

Studies on the role of oxysterols in carcinogenesis and mutagenesis whilisted are largely inconclusive. The aim of this study was to investigate further the induction of genotoxicity of cholesterol-3β,5α,6β-triol (α-Triol), and the possible involvement of ROS in the induction of genotoxicity using two in vitro short-term mutagenicity bioassays, the Ames Salmonella assay and the chromosome aberration test with mammalian cells CHO. We found that α-Triol induced genotoxicity can be attenuated by metabolic detoxification, possibly due to antioxidant enzyme in liver S9, and ROS was involved in this mutagenic effect.

2. Materials and methods

2.1. Chemicals

Cholesterol-3β,5α,6β-triol (α-Triol), 7-keto-cholesterol (7-Keto) and cholesterol-5α,6α-epoxide (α-Epox), Alcolor 1254, and the chemical of positive control for Ames test, 9-aminoacridine, 4-nitroquinoline-*N*-oxide (4-NQO), 2-aminoanthracene(2-AA), mitomycin C (MMC), hydrogen peroxide (H_2O_2), and sodium azide were all obtained from Sigma. Chem. Co. (St. Louis, MO, USA). Salmonella strains were purchased from MOLTOX (Molecular Toxicology, Annapolis, MD, USA).

2.2. Ames Salmonella/microsome test

Mutagenicity was evaluated by using the method of Maron and Ames (1983) with some adaptation (Cheng et al., 2004). The *Salmonella typhimurium* were grown for 14 h at 35 ± 2 °C with continuous shaking. Bacteria were grown to a density of $1–2 \times 10^9$ cells/ml with OD 600 nm absorbance between 0.2 and 0.3. Top agar containing 2 ml of heated agar, 0.1 ml of test chemical, 0.1 ml of bacteria, and 0.5 ml of S9 solution were mixed and added to three different minimal glucose agar plates. All plates were incubated at 37 °C for 48 h, and the number of bacteria colonies was determined. Rat liver S9 used for metabolic activation was prepared according to the method of Maron and Ames (Maron and Ames, 1983) and Matsuoka et al. (1979). Acrolor 1254 (30 mg/kg body weight) was injected into rat to induced liver enzyme.

2.3. Chromosome aberrations

Chinese Hamster Ovary Epithelial Cells (CHO-K1, ATCC: CCL-61) were plated into 6 cm dishes at 5×10^5 cells/plate for 24 h treatment group. After overnight incubation, the cells were treated with ethanol (solvent), mitotomycin C (1 μg/ml), benzo(*a*)pyrene (5 μg/ml), and various concentrations of oxysterol (1, 5, 10 μg/ml) for 3 h with or without S9. SOD (200 U/ml) and catalase (1000 U/ml) were added 30 min before oxysterol treatment. Three hours after the end of the treatment time, colcemid was administrated at 0.1 μg/ml and metaphase chromosomes were prepared as described (Tsutsui et al., 1983). For determination of both chromosome aberrations, 100 metaphases per experimental group were scored. Structural chromosome aberrations observed in each experimental group were classified into seven types: chromosome-type gap (G); chromosome-type break (B); chromosome-type ring (R); chromosome-type dicentric (D); chromatid-type gap (g); chromatid-type break (b); and chromatid-type exchange (e).

2.4. Analysis of ROS production by flow cytometry

Intracellular ROS generation was measured by a flow cytometer with an oxidation-sensitive 2′,7′-dichlorofluorescin diacetate (DCFH-DA) fluoroprobe (Rothe and Valet, 1990). First, 2×10^6 CHO-K1 cells were stained with 20 μg/ml DCFH-DA for 30 min at 37 °C in the dark. Cells were then collected after PBS washing for fluorescence measurement. The level of intracellular ROS was determined with a FACS Calibur™ flow cytometer (Becton Dickinson, San Jose, CA, USA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. For each treatment, 10,000 cells were counted, and the experiment was performed in triplicate.

2.5. Statistical analysis

The data are expressed as the means ± SEM for the number of experiments indicated. Statistical analysis of the data was performed by Student's *t*-test, and $P < 0.05$ was considered as significant different.

3. Results

3.1. Mutagenicity of cholesterol-3β,5α,6β-triol

Our data showed that α-Triol, but not 7-Keto and α-Epox, significantly and concentration-dependently increased colony formation in TA97, TA98, TA100, TA102 and TA1535 (Table 1A). The increasing folds reached the significance of genotoxicity by over three

Table 1
Induction of His^+ revertants in five strains of *Salmonella typhimurium* by treatment of α-triol with and without metabolic activation (S9)

Strains	His^+/plate (−S9)						
	Negative control[a]	Positive control		α-Triol (μg/plate)			
		Positive[b]	H_2O_2[c]	25	50	100	200
(A) Without S9							
TA 97	69 ± 4	440 ± 41***	96 ± 4**	100 ± 6* (1.4)[d]	122 ± 11** (1.8)	130 ± 8*** (1.9)	147 ± 9*** (2.1)
TA 98	23 ± 1	387 ± 42***	42 ± 10**	20 ± 2* (0.9)	24 ± 2 (1.0)	35 ± 5* (1.5)	77 ± 12*** (3.3)
TA 100	121 ± 14	287 ± 28***	136 ± 8*	206 ± 29* (1.7)	162 ± 11 (1.3)	178 ± 21* (1.5)	229 ± 29** (1.9)
TA 102	167 ± 10	1146 ± 120***	1181 ± 361***	182 ± 10 (1.1)	255 ± 18** (1.5)	253 ± 19** (1.5)	253 ± 24* (1.5)
TA 1535	9 ± 1	1541 ± 148***	13 ± 2**	19 ± 1*** (2.1)	28 ± 5*** (3.1)	35 ± 5*** (3.9)	29 ± 2*** (3.2)
(B) With S9							
TA 97	100 ± 8	591 ± 61***	93 ± 4	81 ± 6 (0.8)	74 ± 12 (0.7)	82 ± 7 (0.8)	104 ± 15 (1.0)
TA 98	35 ± 2	3099 ± 387***	28 ± 3	31 ± 3 (0.9)	31 ± 2 (0.9)	28 ± 3 (0.8)	32 ± 3 (0.9)
TA 100	130 ± 11	472 ± 76***	101 ± 6	172 ± 17 (1.3)	103 ± 12 (0.8)	128 ± 22 (1.0)	167 ± 20 (1.3)
TA 102	199 ± 17	1236 ± 115***	286 ± 12*	135 ± 10 (0.6)	210 ± 20 (1.1)	247 ± 20 (1.2)	266 ± 23 (1.3)
TA 1535	14 ± 1	3473 ± 385***	12 ± 1	20 ± 2 (1.4)	27 ± 3* (1.9)	26 ± 3** (1.9)	33 ± 4*** (2.1)

The values were presented as mean ± SE (n ⩾ 6). *$p < 0.05$ vs. ethanol, **$p < 0.01$ vs. ethanol, ***$p < 0.001$ vs. ethanol.
[a] 2 μl Ethanol/plate was used as negative control.
[b] Fold increased relative to negative control.
[c] Positive control in +S9 plate was 2-AA: 5 μg/plate, H_2O_2: 200 mM.
[d] Positive control in −S9 plate: TA 97, 9-Aminoacridine 50 μg/plate; TA 98, 4-NQO 2 μg/plate; TA 100 and TA 1535, Sodium azide 5 μg/plate; TA 102, MMC 0.5 μg/plate, H_2O_2:200 mM.

folds in TA98 and TA1535, and 1.5 folds in TA102 over the negative control. At 200 μg/plate, the TA98 clone formation of 7-Keto and α-Epox showed no significantly different relative to control with the value of 20.2 ± 2 and 24.1 ± 3 respectively in the without S9 mix. The same results were seen in other strains (TA97, TA100, TA102 and TA1535) in with or without S9 mix, indicated 7-keto and α-Epoxy did not show the mutagenic effect.

Surprisingly, in the presence of S9, the number of revertants in α-Triol treated plates was attenuated in all tester strains (Table 1B), suggesting α-Triol induced genotoxicity can be detoxified by S9. Pretreatment with catalase (1000 U/ml) and superoxide dismutase (SOD 200 U/ml) significantly inhibited α-Triol (200 μg/ml) induced increase of revertants (Fig. 1A). These data indicating that reactive oxygen species might be involved in the mutagenic effect induced by α-Triol. Similar data was seen in H_2O_2 treated group. H_2O_2 significantly increased reverstants in all 5 tester strains especially TA102 in without S9 group, and this effect can be inhibited in the presence of S9 (Table 1B), or catalase and SOD (Fig. 1B).

3.2. Induction of chromosome aberration by cholesterol-3β,5α,6β-triol in CHO cells

The in vitro effect of α-Triol on chromosome was further investigated with CHO-K1 cells (Table 2). The incidence of CHO-K1 cells with structural chromosome aberrations significantly increased in BaP and mitomycin C treated cells, and was used as positive control in the presence (Table 2B) and absence (Table 2A) of S9 respectively. In the absence of S9, α-Triol (1, 5 and 10 μg/ml) dose-dependently increased structure chromosome aberrations at 3 h treatment as compared with solvent control (0.1% ethanol) (Table 2A). However, when cotreated with S9, the number of aberrant cells was decreased in α-Triol (5 and 10 μg/ml) treated CHO-K1 cells (Table 2B). A similar inhibitory effect was also seen in SOD and catalase treated groups (Fig. 2).

3.3. Cholesterol-3β,5α,6β-triol induced reactive oxygen species formation in CHO-K1 cells

Previous results indicated that ROS might be important in inducing genotoxic effect by α-Triol. ROS generation induced by α-Triol was further examined using a DCFH-DA fluorescence probe in CHO-K1 cells. We found that α-Triol (5, 10, 20 μg/ml) increased the fluorescent intensity, an indication of an increase of ROS level in a concentration-dependent manner (Fig. 3). α-Triol (20 μg/ml)-induced fluorescent intensity was potentiated up to 88.6 ± 0.63% relative to control. Pretreatment with catalase (1000 U/ml) and superoxide dismutase (SOD, 200 U/ml) inhibited α-Triol (10 μg/ml) induced ROS generation.

4. Discussion

In this study, we found that α-Triol is a direct mutagen in bacteria and causes chromosome aberration in CHO cells. Results showed that α-Triol induced the increased of reverstants to all five Salmonella strains TA 97, TA 98, TA100, TA102 and TA1535. In the presence of S9, which contains several metabolic enzymes, the

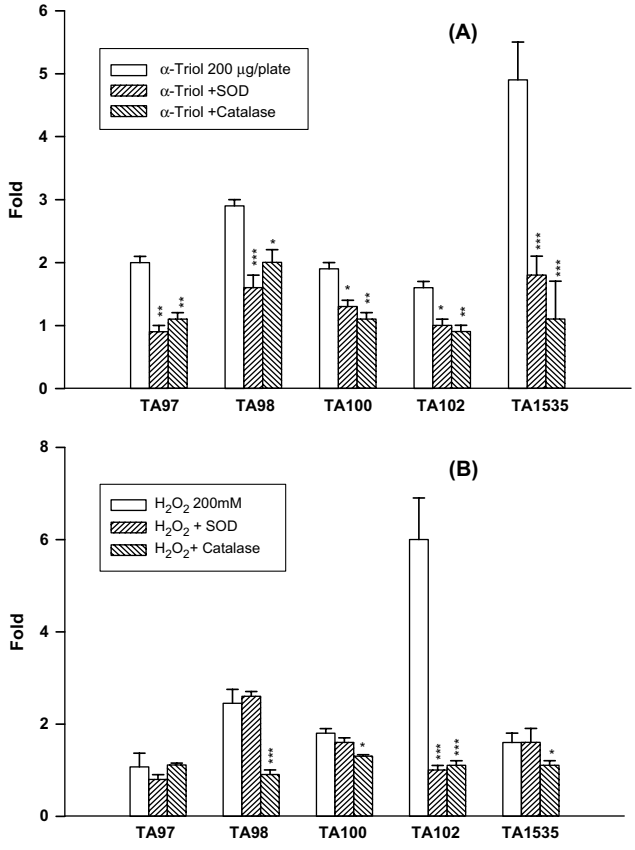

Fig. 1. Effects of antioxidants on α-Triol and H_2O_2 induced revertants on Ames test. SOD (200 U/ml) and catalase (1000 U/ml) significantly inhibited α-Triol (A), and H_2O_2 (B), induced folds revertants on TA97, TA98, TA100, TA102 and TA1535 relative to control. The results were expressed as fold relative to the control of revertants on bacteria. Data with error bars are the mean ± SE from 6 independent experiments. Ethanol was used as the solvent control. $^{*}p < 0.05$ vs. the positive control, α-Triol or H_2O_2.

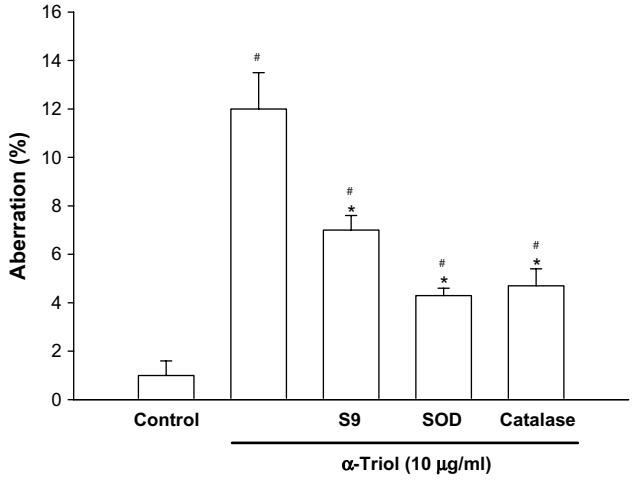

Fig. 2. Effects of antioxidants on α-Triol and H_2O_2 induced chromosome aberration. SOD (200 U/ml) or catalase (1000 U/ml) significantly inhibited α-Triol (10 μg/ml) induced abnormal chromosomes on CHO-cells. The results of chromosome aberration were expressed as percentage relative to control. Data with error bars are the mean ± SE from six independent experiments. Ethanol was used as the solvent control. $^{\#}p < 0.05$ vs. the control. $^{*}p < 0.05$ vs. the α-Triol (10 μg/ml).

mutagenic effect was attenuated. These results indicate that the mutagenic effect induced by α-Triol could be reduced by metabolic enzymes. Some liver enzymes has been shown to detoxify the carcinogenic compound, such as glutathione-S-transferase, SOD and catalase (Turesky, 2004).

DNA brakes and the formation of clastogen can be detected by in vitro CHO cells chromosome aberration test. α-Triol dose-dependently increased the number of abnormal structure chromosomes in the absence of metabolic activation in CHO cells. However, the addition of

Table 2
Chromosome aberrations of CHO-K1 cells treated with α-Triol with and without metabolic activation (S9)

Treatment	Aberration cells (%)	Number of aberrations/100 cells[a]						
		G	B	D	R	g	b	e
(A) without S9								
Solvent[b] (ethanol)	1.3 ± 0.3	0	0	0	0	1.3 ± 0.3	0	0
MMC[b] (1 μg/ml)	13.0 ± 1.2***	0.7 ± 0.3	0	0.3 ± 0.3	0	12.3 ± 0.9	0	0
α-Triol (μg/ml)								
1	3.4 ± 0.3*	0.7 ± 0.3	0	0	0	2.7 ± 0.3	0	0
5	6.7 ± 0.9**	1.0 ± 0.1	0	0.7 ± 0.3	0.3 ± 0.3	4.7 ± 0.3	0	0
10	11.0 ± 0.9***	0.7 ± 0.3	0	1.3 ± 0.9	0	8.7 ± 0.3	0	0
(B) With S9								
Solvent[b] (ethanol)	2.0 ± 0.1	0	0	0	0	2 ± 0.1	0	0
BaP[b] (5 μg/ml)	8.0 ± 0.6*	1.3 ± 0.7	0	0	0	6 ± 1.0	0	0.7 ± 0.7
α-Triol (μg/ml)								
1	3.0 ± 0.1	0	0	0	0	3 ± 0.1	0	0
5	5.3 ± 0.3*	0	0	0	0	5 ± 0.2	0	0.3 ± 0.3
10	7.0 ± 0.6$^{*,\#}$	0.7 ± 0.7	2.0 ± 0.1	1.3 ± 0.7	0	6.7 ± 0.3	0	1.0 ± 0.6

The values were presented as mean ± SE (n = 3). $^{*}p < 0.05$ vs. ethanol, $^{\#}p < 0.05$ vs. Triol 10 μg/ml (without S9).

[a] G = chromosome gap; B = chromosome break; D = dicentric; R = ring; g = chromatid gap; b = chromatid break; e = exchange.

[b] α-Triol was dissolved in ethanol and the solvent control (ethanol) did not exceed 0.1%. BaP was positive control in with S9 medium.

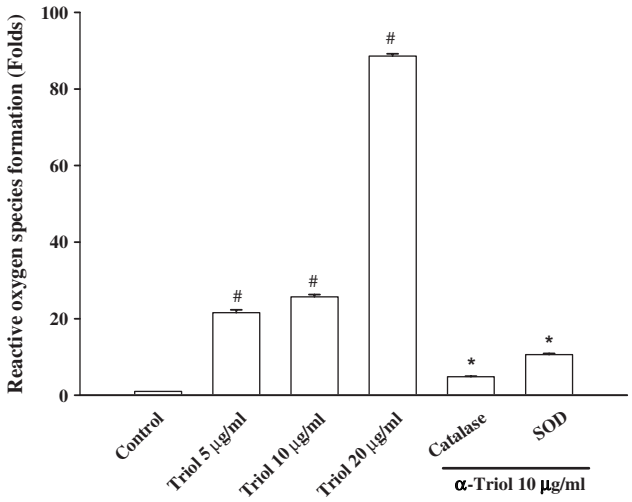

Fig. 3. α-Triol induced ROS generation in CHO-K1 cells and the effect of antioxidants. The cells (2×10^6) were incubated with various concentrations of α-Triol (5, 10 and 20 μg/ml) for 3 h then trypsinized and stained with 20 μM DCFH-DA at 37 °C for 30 min. SOD (200 U/ml) and catalase (1000 U/ml) were pretreated 5 min before the addition of α-Triol. Then cells were collected after PBS washing for fluorescence measurement. The results were expressed as folds to the control of fluorescent intensity. Data with error bars are the mean ± SE from six independent experiments. Ethanol was used as the solvent control. $^{\#}p < 0.05$ vs. the control. $^{*}p < 0.05$ vs. the α-Triol (10 μg/ml).

S9 attenuated the α-Triol induced aberrations. These data suggested that α-Triol can induce chromosome aberration without metabolic activation in CHO cells, and the addition of S9 can diminish the genotoxicity induced by α-Triol.

Mixtures of oxysterols have been shown the mutagenic effects on *S. typhimurium* TA1537, TA1535 and TA98 (Ansari et al., 1982; Smith et al., 1986). Pure oxysterols like 7α-hydroperoxide cholesterol and 5α-hydroperoxide cholesterol have a mutagenic effect on *S. typhimurium* TA1537 (Chien et al., 1998) and the mutagenic effects were attenuated by catalase and superoxide dismutase (SOD) (Smith et al., 1986). Both catalase and SOD can inhibit the mutagenic effect in bacterial and chromosome aberrant effects in CHO cells, suggesting that ROS might also play important role in the α-Triol induced genotoxicity observed in this study. This is further supported by the fact that the level of ROS was increased in α-Triol treated CHO cells. Both α-Triol and H_2O_2 induced genotoxic effect can be inhibited by S9, suggesting that the antioxidative enzymes present in S9 mix, such as catalase, SOD and glutathione (Jurczuk et al., 2004), might be responsible for the detoxifying effect observed. However, both addition of S9 or the antioxidative enzymes, SOD and catalase, could not completely inhibit the genotoxic effects induced by α-Triol, suggesting that α-Triol induced genotoxic effects might be through multiple pathways. Further investigation is needed to determine the detail mechanism.

In this study, we have examined three oxysterols (7-Keto, α-Epox and α-Triol), and showed that only α-Triol has genotoxic effect. α-Triol, which, although not a major dietary oxysterol, may arise from hydrolysis of α-Epox in the acidic environment of the stomach (Maerkar et al., 1988). These findings raise the possibility that α-Triol plays an important role in mutagenicity. In conclusion, results in this study provide evidence indicating the potential genotoxic effects of oxysterol, α-Triol in vitro.

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