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In Vitro Effects of 5α-Cholestane-3β,5,6β-triol on Cultured Rat Cardiomyocytes†

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One of the most toxic among the cholesterol oxidation products generated in food following heat, radiation, or storage procedures is 5α-cholestane-3β,5,6β-triol. When the medium of cultured rat cardiomyocytes was supplemented with scalar concentrations (0.1−5 μM) of triol, a close correlation was found between the concentration of triol in the medium and the amount of triol incorporated into cell lipids. Identification of triol and other compounds was by capillary gas chromatography and gas chromatography−mass spectrometry. Furthermore, triol supplementation caused a significant reduction in cell protein content, without affecting cell viability. This study is one of the first reports on triol toxicity to cardiomyocytes and indicates that triol could exert its effect by affecting cell proliferation at a concentration significantly lower than concentrations used in experiments on other cell lines.

Keywords: *Cholestanetriol; oxysterols; cultured cardiomyocytes; lipids; gas chromatography; cytotoxicity*

INTRODUCTION

Cholesterol, ubiquitously present in mammalian tissues, is essential for formation and function of cellular membranes and is the obligate precursor of the bile acids and steroid hormones. Cholesterol is subject to oxidation by diverse active oxygen species, yielding the biologically active oxysterols. Oxysterols are uniformely toxic in a wide variety of *in vivo* tests and *in vitro* bioassays (Smith and Johnson, 1989).

Occurrence of oxidized lipids in food has long been a concern from the quality standpoint to the technologist and from the safety standpoint to the toxicologist. A spectrum of cholesterol oxidation products (COPs) is generated in food products following heat, radiation or storage procedures in the presence of oxygen (Pie et al., 1990). The presence of COPs in food is a growing concern due to their relation to potential deleterious health problems which include atherogenicity, cytotoxicity, mutagenicity, and possible carcinogenicity (Addis and Warner, 1991). In the light of the capacity of humans to absorb COPs from food sources and of the potential deleterious impact on human health of COPs, there is an urgent need to investigate the biological effects of COPs themselves.

In previous works (Caboni et al., 1993; Hrelia et al., 1994) we evaluated the effects of one of the main COPs, cholesterol 5α,6α-epoxide, when supplemented to the medium of cultured cardiomyocytes; supplementation in the range 1−100 μM caused a reduction in cellular protein level concomitant to a dose-related epoxide incorporation into cardiomyocyte lipids, without affecting cell viability. Furthermore, the hydrolytic metabolite of epoxide, 5α-cholestane-3β,5,6β-triol (triol), was

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identified in the lipid fraction of all the epoxide supplemented cells.

Since triol is considered one of the most potent of angiotoxic COPs (Addis and Warner, 1991), we have investigated the consequences of the supplementation to cultured cardiomyocytes of scalar concentrations of triol, evaluating cellular protein content and incorporation of triol itself into cellular lipids. Identification of individual compounds was made by gas chromatography–mass spectrometry (GC–MS). This paper confirms the successful application to cell lipid analysis of the previously reported methodology (Caboni et al., 1993) for COPs identification and quantification.

MATERIALS AND METHODS

Reagents. Lup-20(30)-ene-3β,28-diol (betulinol) was purchased from Roth (Karlsrhue, Germany); 5α-cholestane-3β,5,6β-triol, pure 5-cholesten-3β-ol (cholesterol), and trypan blue were from Sigma Chemical Co. (St. Louis, MO). Horse serum, fetal calf serum, trypsin, and Ham F10 medium were obtained from Boehringer (Mannheim, Germany). All chemicals and solvents were of the highest analytical grade.

Cell Cultures. Primary heart cell cultures were obtained by isolation of cardiomyocytes from the ventricles of 2–4-day-old Wistar rats, as previously reported (Bordoni et al., 1991). Cardiomyocytes were grown in nutrient mixture Ham F10 supplemented with 10% v/v fetal calf serum and 10% v/v horse serum (control medium). Before the final seeding in Petri dishes, cells were divided into different groups: (a) control cells, grown in the control medium; (b) cells grown in the same control medium but supplemented with 0.1, 0.5, 1, or 5 μM triol in ethanol vehicle (T 0.1, T 0.5, T 1, and T 5 cells, respectively).

In control cells, the same ethanol concentration (0.04% v/v) was added to the culture medium. Cells were incubated at 37 °C, 95% humidity, and 5% CO_2, and the culture media were replaced with the corresponding fresh media every 48 h.

Cardiomyocytes were grown in the above-mentioned conditions for 96 h; after this period, control cells appeared completely confluent in a monolayer.

The culture medium was collected from each dish to evaluate the release of cellular lactate dehydrogenase (LDH), as described by Korzeniewski and Callewaert (1983). Cells were washed three times with medium supplemented with 10% horse serum and 10% fetal calf serum and two times with phosphate-buffered saline (PBS). Preliminary experiments demonstrated that triol is completely removed by these washings and medium is not carried over into the excised cells.

Some dishes of each group were scraped off in 0.9% NaCl, and protein concentration was evaluated in each dish according to the method of Bradford (1976).

Cells from other dishes were detached with 0.25% trypsin and tested for viability; they were immediately incubated with 0.1% trypan blue and counted in a Burker chamber.

Lipid Extraction and Cold Saponification. Some dishes of each group were scraped off in ice-cold methanol, and total lipids were extracted according to the procedure of Folch et al. (1957). Twenty microliters of betulinol in *n*-hexane (1 mg/mL) was added to each sample as an internal standard. The organic extract obtained from each dish was dried, 5 mL of 2 N KOH in methanol was added, and the mixture was shaken vigorously until it became free of dispersed fat particles. Saponification was conducted at room temperature overnight according to the procedure described by Sander et al. (1989). A 10 mL portion of distilled water was added to the saponified mixture, and the unsaponifiables were extracted three times, each time with 10 mL of diethyl ether (peroxide free). Water was removed from the pooled diethyl ether extracts with anhydrous sodium sulfate. The extract was filtered and concentrated in a rotary vacuum evaporator and freed of solvent on a nitrogen flush evaporator. The dried unsaponifiable components were redissolved in 100 μL of pyridine, to which 100 μL of hexamethyldisilazane/trimethylchlorosilane (2:1 v/v) was added to obtain Sweeley's silylating mixture (Sweeley et al., 1963). The silanized extracts were dried, resuspended in 20 μL of *n*-hexane, and analyzed by capillary gas chromatography (CGC).

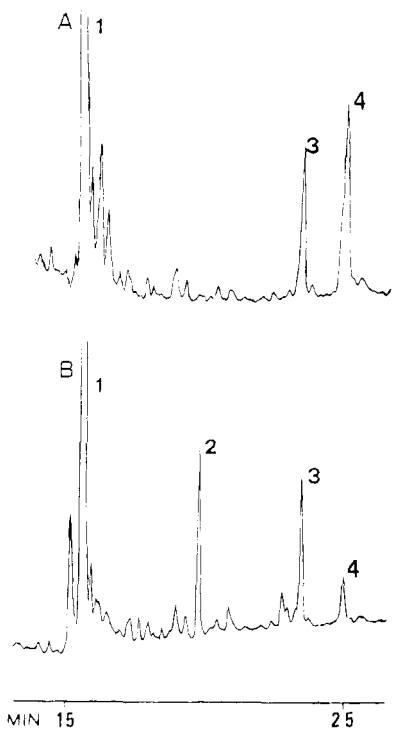

Figure 1. Typical CGC traces of the unsaponifiable fraction derived from control and triol-supplemented cardiomyocytes. The gas chromatohraphic traces of cell lipids of two representative groups of cardiomyocytes (control and T 5) were obtained in the conditions reported under Materials and Methods: (A) control cells; (B) T 5 cells. Peak identification: 1, cholesterol; 2, triol; 3, internal standard (betulinol); 4, unidentified compound.

Capillary Gas Chromatographic Analysis. Capillary gas chromatographic analysis was performed on a Carlo Erba Mega series gas chromatograph (Rodano, Milano, Italy) equipped with a flame ionization detector (FID) and a 25 m × 0.32 mm i.d. fused silica capillary column coated with SE52 (0.1 μm film thickness). The injection was by the on column system–Grob's cold injector (Galli et al., 1979) when the oven temperature was 60 °C. Temperature was programmed from 60 to 260 °C with a gradient of 25 °C/min and from 260 to 310 °C with a gradient of 2 °C/min. Detector temperature was 350 °C, and gas carrier (helium) flow rate was 2 mL/min. CGC traces and quantitative evaluations were obtained by using a Spectra-Physics 4290 computing integrator (San José, CA). The FID response factors were calculated on the corresponding standards and applied to normalize CGC areas.

Mass Spectral Analysis. The GC–MS analyses were performed with a Carlo Erba QMD 1000 apparatus, equipped with the same capillary column used for CGC analyses and in the corresponding conditions of temperature reported for CGC analyses. Temperatures were 250 °C for the transfer line and 200 °C for the source; ionization energy was 70 eV. Mass spectra were recorded for trimethylsilyl ether sterols in the mass range (m/z) 40–650; scan speed was 0.9 scan/s.

Triplicate dishes were used for each experimental condition, and data represent results from three to five independent experiments. Results are expressed as mean ± SD for each condition presented.

RESULTS

In Figure 1 the CGC traces of the unsaponifiable lipids derived from control and triol-supplemented cells are visualized. The use of cold saponification protected

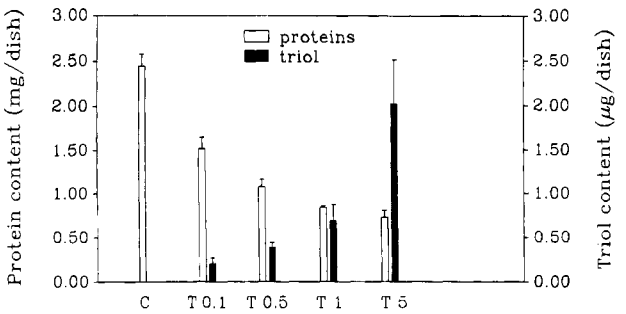

Figure 2. Protein and triol contents in control and triol-supplemented cardiomyocytes. Protein and triol contents were determined as reported under Materials and Methods. Values are means ± SD of triplicate determinations performed on three to five different cell cultures. C, control cardiomyocytes; T 0.1, T 0.5, T 1, and T 5, cardiomyocytes supplemented with 0.1, 0.5, 1, and 5 μM triol, respectively. Triol content in control cells was not detectable in the analytical conditions reported under Materials and Methods. When expressed as micrograms per milligram of protein, the amount of triol incorporated into cardiomyocytes was as follows: control cells, not detectable; T 0.1 cells, 0.135 ± 0.024; T 0.5 cells, 0.358 ± 0.037; T 1 cells, 0.820 ± 0.036; and T 5 cells, 2.767 ± 0.081.

against loss of triol, and frequent N_2 flushes during workup procedures helped to protect against artifactual oxidation. Preliminary experiments performed on pure cholesterol standard, subjected to the procedure reported herein, demonstrated the absence of cholesterol oxidation products after the complete procedure. None of the standards of pure cholesterol oxidation product subjected to this procedure formed any other cholesterol oxidation product.

All peaks observed in CGC analysis were confirmed by MS analysis and by utilizing the single ion monitoring system (SIM). The m/z values of trimethylsilyl ether sterols were as follows: cholesterol, 458, 443, and 368; triol, 546, 456, 403, 367, and 321. These m/z values were identical to those obtained on authentic standards and to those reported previously (Park and Addis, 1985).

Figure 2 presents data on triol content in lipids of control and triol-treated cardiomyocytes. GC–MS analysis of control cells did not reveal the presence of triol. Results on triol-supplemented cardiomyocytes showed that the level of triol incorporated into cell lipids ranged from 0.205 to 2.02 μg/dish; a linear correlation was found between the concentration of triol incorporated into cardiomyocyte lipids and triol supplemented to the culture medium ($y = 0.38x + 0.16$; $r^2 = 0.990$, $p < 0.001$). An even closer linear correlation between these two parameters ($y = 0.54x + 0.10$; $r^2 = 0.996$, $p < 0.001$) was found when triol incorporation into cell lipids was expressed as micrograms per milligram of protein (control cells, not detectable; T 0.1 cells, 0.135 ± 0.024; T 0.5 cells, 0.358 ± 0.037; T 1 cells, 0.820 ± 0.036; and T 5 cells; 2.767 ± 0.081).

Protein total content, evaluated at the time of complete confluence of control cells, is visualized in Figure 2. Protein content was significantly lower in triol-supplemented than in control cardiomyocytes. Protein total content was about 40% lower in T 0.1 than in control cells ($p < 0.001$); in cardiomyocytes supplemented with a 10-fold higher triol concentration (T 1), protein total content was about 70% lower than in control cells ($p < 0.001$), while in cardiomyocytes supplemented with a 50-fold higher triol concentration (T 5) protein content was not significantly different from that in T 1 cells. Protein content was proportional to triol incorporation into cellular lipids only for triol supplementation to the culture medium ≤1 μM; therefore, an equation can be constructed which could be useful in predicting the level of protein content from triol incorporation into cell lipids in the range of triol supplementation 0–1 μM ($y = -2.24x + 2.19$; $r^2 = 0.930$, $p < 0.001$).

Cell counting correlated well with data of protein total content, and cell viability was more than 90% in each group. No alterations in cell size were detected by phase contrast light microscopy.

The permeability of cytoplasmic membrane to LDH was not affected by the exposure of cardiomyocytes to triol [control cells, 0.020 ± 0.005 unit mL^{-1} (mg of protein)$^{-1}$; triol-treated cells, 0.022 ± 0.004 unit mL^{-1} (mg of protein)$^{-1}$].

DISCUSSION

Heat, air, and radiation result in the formation of numerous COPs in food. Recently, evidence has been reported (Emanuel et al., 1991) that human subjects have the capacity to absorb COPs from food sources into the bloodstream, where they may produce toxic effects. COPs may also be generated endogenously via free radical reactions (Smith et al., 1981; Bjorkhem et al., 1987); thus, it is important that the mode of toxicity of these compounds be understood.

Oxidation of the double bond between carbon atoms 5 and 6 produces the two isomers cholesterol 5α,6α- and 5β,6β-epoxide, which are converted in 5α-cholestane-3β,5,6β-triol (Smith, 1981). Triol has been demonstrated to be one of the most toxic COPs in a wide range of cellular systems: rabbit vascular smooth cells (MacDougall et al., 1965); white New Zealand rabbit liver (Imai et al., 1978); and mouse fibroblasts, macrophages, and vascular muscle cells from pigs (Baranowski et al., 1982). Furthermore, in cell cultures of smooth aortic muscle cells from rabbit, triol supplementation resulted in necrosis in up to 25% of cells, using a minimal concentration of 10 μg/mL culture medium, while no cytotoxic concentration was found for pure cholesterol up to 100 μg/mL (Peng et al., 1979).

On the other hand, in cases where several related cell lines have been examined under different culture medium conditions, different sensitivities of cells to triol have been observed, so extrapolation of specific *in vitro* results obtained in the above-mentioned cell systems to other situations is uncertain. Since Mersel et al. (1984) reported inhibition of cellular proliferation and impairment of synchronous beating in rat myocardial cells exposed to different COPs, we have evaluated the effect of triol supplementation on rat cultured cardiomyocytes, using different triol concentrations.

Triol was incorporated into cardiomyocyte lipids in a dose-related manner; a close correlation was found between the concentration of triol supplemented to the medium and the amount of triol incorporated into cell lipids. GC–MS determination revealed the absence of a detectable amount of triol in control cells, while triol was unambiguously identified in treated cardiomyocytes. The possibility of misidentification of triol as of other COPs was discussed in several previous publications (Park and Addis, 1985, 1986; Addis, 1986) and was dealt with by conducting mass spectral studies on the gas chromatographic peaks obtained from cardiomyocyte lipids.

It is documented that COPs inhibit cholesterol synthesis (Kandutsch and Chen, 1978) by inhibiting the

enzyme 3-hydroxy-3-methylglutaryl-CoA-reductase. However, the correlation between the ability of COPs to inhibit cholesterol biosynthesis and their cytotoxicity is inconsistent (Zander et al., 1977; Peng et al., 1979). More recently, Herian et al. (1985) indicated that the cause of the toxicity of triol and other COPs could lie in their direct incorporation in the cell membrane. COPs have been shown to insert into plasma membranes, altering membrane fluidity and organization. As such it could have significant effects on cell function and morphology (Kucuk et al., 1992). Insertion of COPs into cell membranes may also compromise their ability to maintain various functions necessary for continued cell viability, such as transmembrane ion gradients (Holmes and Yoss, 1984; Boissonneault and Heiniger, 1985).

Our data clearly indicate that, in cardiomyocytes, triol was incorporated into cell lipids in a dose-related manner.

Triol supplementation caused a significant reduction in cell protein content; the protein content was used as a parameter in the toxicity test because it correlates very well with cell number. This reduction was inversely correlated to the content of triol incorporated into cell lipids and, therefore, to the concentration of triol supplemented to the medium in the range of concentration 0−1 μM. No significant differences in protein content were found between T 1 and T 5 cardiomyocytes. Even though the supplementation of the culture medium with a triol concentration as high as 5 μM determined a proportional incorporation of triol into cell lipids, no additional decrement in protein content were detected.

The observed reduction in cardiomyocyte protein content cannot be accounted for by increased protein degradation. In fact, neither an increase in protein content of the culture medium (which could be due to the release of proteolytic fragments) nor modification of LDH release (which is a common indicator of cell damage) was ever detected in all of the experimental conditions. Furthermore, at the concentration of triol in culture medium used in our experiment, cell viability was always more than 90% and no alterations in cell size were detected. Therefore, the decrease in cardiomyocyte protein content cannot be attributed to an increased protein degradation or to modifications in cell size or viability. The reduction in cell number and in cell protein content observed in triol-supplemented cardiomyocytes compared to control cardiomyocytes indicates that triol could exert its effects by affecting cell proliferation. A depression in protein synthesis by triol supplementation was already observed by Henning et al. (1988) in vascular endothelial cells; Sevanian et al. (1991), in a similar cell system, concluded that the major effect of triol may be inhibition of cell division. Our data indicate that triol has a similar effect even on cardiomyocytes.

Our study represents one of the first reports on triol toxicity performed on cardiomyocytes. We have demonstrated that triol is incorporated into cardiomyocyte lipids in a dose-dependent manner, and it is able to cause adverse effects on cellular protein content even at concentrations significantly lower than concentrations used in experiments on other cell lines (Peng et al., 1979). Cardiomyocytes appear to be very sensitive to triol supplementation. It is very important considering that COPs are thought to compromise transmembrane barrier function, causing an elevation of membrane permeability to extracellular ions, particularly calcium (Boissonneault et al., 1991). The consequent modification of intracellular calcium concentration could be deleterious in cardiac cells, thus inducing us to consider COPs to increase not only the risk for development of atherosclerosis but also the risk for cardiac disrythmias.

ABBREVIATIONS USED

COPs, cholesterol oxidation products; triol, 5α-cholestane-3β,5,6β-triol; GC−MS, gas chromatography−mass spectrometry; PBS, phosphate-buffered saline; CGC, capillary gas chromatography; FID, flame ionization detector; SIM, single ion monitoring.

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