

## The effect of tocopheryl phosphates on atherosclerosis progression in rabbits fed with a high cholesterol diet

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### Abstract

The effect of tocopheryl phosphate on atherosclerosis progression has been studied in rabbits, fed with a 2% cholesterol diet and compared with an equivalent amount of  $\alpha$ -tocopheryl acetate. The results show that the atherosclerotic-preventing effect of the phosphate derivative was more pronounced than that of the acetate derivative.  $\alpha$ -Tocopheryl phosphate was also more potent in diminishing the expression of CD36 than the acetate derivative.

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$\alpha$ -Tocopherol has been shown to regulate a number of cell properties, including signal transduction, cell proliferation, and gene expression [2,8,12]. It has also been shown, in animal models, to be able to protect against the progression of atherosclerosis caused by a high cholesterol diet [9–11]. Tocopheryl phosphate has been shown to be more effective than tocopherol in the regulation of cell properties [1,5,6]. The possibility that the active form of tocopherol may be tocopheryl phosphate has been considered [6]. In fact, tocopheryl phosphate has been found in tissues and cells and it has also been shown to be synthesized by cells in vitro [4]. The scope of this study was to investigate the effects of tocopheryl phosphate on an animal model of atherosclerosis and to compare them with the effect produced by  $\alpha$ -tocopheryl acetate.

### Methods and materials

#### Animal experiments

#### Experimental design

Twenty-five New Zealand albino rabbits (1–2 months old) were divided into five groups, which were treated with different diets for 4 weeks according to the following procedure. Group I, the control rabbits, were only fed a vitamin E deficient diet, without additions and treatments. Group II rabbits were fed with a vitamin E deficient diet containing 2% cholesterol. Group III rabbits were fed with a vitamin E deficient diet containing 2% cholesterol and tocopherol acetate (1 g/kg chow). Rabbits in groups IV and V were fed with a vitamin E deficient diet containing 2% cholesterol and increasing amounts of tocopheryl phosphate, respectively (0.33 and 1.33 g/kg chow).

The indicated amounts of tocopheryl phosphate and tocopheryl acetate were mixed into the diet. All rabbits were kept in separate cages and were fed 100 g of their respected diets per rabbit per day. Cholesterol was added to

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Table 1  
Serum concentrations of cholesterol,  $\alpha$ -tocopherol, and tocopheryl phosphate (TP)

Group	Cholesterol (mg/dL)		$\alpha$ -Tocopherol ( $\mu$ g/mL)	TP ( $\mu$ g/mL)
	0. week	4th Week	4th Week	4th Week
Control	97.6 $\pm$ 25.8	44.8 $\pm$ 18.6	0.66 $\pm$ 0.27	0.10 $\pm$ 0.07
Cholesterol	74.8 $\pm$ 16.2	2568 $\pm$ 1172.2 <sup>†</sup>	3.96 $\pm$ 2.27	8.08 $\pm$ 6.44
Cholesterol + TPm (0.33 g/kg)	99.8 $\pm$ 31.7	2727.2 $\pm$ 883.1 <sup>†</sup>	11.95 $\pm$ 7.14 <sup>*</sup>	15.02 $\pm$ 1.29 <sup>*</sup>
Cholesterol + TPm (1.33 g/kg)	101.4 $\pm$ 17.7	2072.6 $\pm$ 863.5 <sup>†</sup>	9.02 $\pm$ 11.89	7.14 $\pm$ 6.20
Cholesterol + TA (1 g/kg)	96.6 $\pm$ 25.9	2752.6 $\pm$ 1128.8 <sup>†</sup>	32 $\pm$ 11.79 <sup>**</sup>	4.80 $\pm$ 6.39

Compared with 0. week, <sup>†</sup> $p < 0.001$ , <sup>\*</sup> $p \leq 0.5$  significant, <sup>\*\*</sup> $p \leq$  highly significant, via Student's  $t$  test.

the diet as a diethyl ether solution. All diets were dried of the solvent before use. The amount of tocopherols added to the chow was analyzed and found to correspond to the added amounts (data not shown).

After 4 weeks, following withdrawal of food overnight, rabbits were anesthetized, by using 50 mg/kg ketamine hydrochloride. Blood was taken; serum was separated and kept at  $-20^{\circ}\text{C}$  until they were analyzed for  $\alpha$ -tocopherol, tocopheryl phosphate, and cholesterol. Their thoracic aortas were removed, adventitia layer was cleaned and immediately taken into RNA stabilization reagent (Qiagen) and were frozen at  $-20^{\circ}\text{C}$  until they were analyzed for CD36 mRNA expression. Aortas obtained from the rabbits from each group were removed up to the celiac bifurcation and stained with Sudan IV to permit plaque size en face evaluation.

#### Determination of serum cholesterol, $\alpha$ -tocopherol, and tocopheryl phosphate

Serum cholesterol levels were determined using an automated (Hitachi Modular P800) enzymatic technique (Roche, Boehringer Ingelheim).

The assay of  $\alpha$ -tocopherol was performed by extraction of serum in subdued lighting to produce an  $\alpha$ -tocopherol-rich fraction. Briefly, 1 ml serum was shaken vigorously in 12 mL of 9% w/v KOH in 90% ethanol, plus 1 mL ascorbic acid solution (10% w/v), and incubated at  $80^{\circ}\text{C}$  for 30 min. After cooling, 12 mL NaCl (10% w/v) was added, plus 12 mL petroleum ether (BP 60– $80^{\circ}\text{C}$ ), the samples vortexed and centrifuged. The upper organic extract was collected into a new tube and the aqueous phase was re-extracted with petroleum ether twice more. The organic extracts were pooled, washed three times with water, then dried under nitrogen in glass vials and stored at  $-80^{\circ}\text{C}$  until they were assayed for  $\alpha$ -tocopherol by HPLC. Samples were dissolved with 2 mL methanol and RP-HPLC was undertaken on a C18 Nova-Pak column (Alltech). The mobile phase was 100% methanol and detection by fluorescence was with excitation at 292 nm and emission at 350 nm.

$\alpha$ -Tocopheryl phosphate levels were measured by HPLC. To 1 ml of serum, 1 ml of 0.1% ascorbic acid in 90% ethanol (prepared freshly), 120  $\mu$ l of acetonitrile, and 800  $\mu$ l of 2 M HCl were added and shaken vigorously for 1 min. Four milliliters of hexane was added, shaken vigorously for 2 min, and centrifuged at 2500g for 3 min. The upper phase was removed and placed into a new tube. The extraction was repeated with hexane twice more, the hexane phases

pooled and dried under nitrogen gas. Acetic acid (16  $\mu$ L) and isopropanol (144  $\mu$ L) were added to the sample vial and sonicated for 10 min. The dissolved sample was injected into a 150  $\times$  4.6 mm Phenomenex Luna C8 5  $\mu$ m column (Lane Cove, Australia) using a Waters HPLC system (600 controller, 717 autosampler, 486 detector, 2475 fluorescence detector, Empower Pro software) (Sydney, Australia) with the column heated to  $40^{\circ}\text{C}$ . Mobile phase A was 0.2% phosphoric acid in isopropanol and B was water. The flow rate was 0.4 ml/min with the gradient beginning with 60% A to 100% A over 20 min, 100% A maintained for 10 min, then to 60% A over 4 min, and maintained at 60% A for 15 min before the next injection. Detection was by UV at 286 nm, and by fluorescence with excitation at 297 nm and emission at 319 nm.

#### Macroscopic analyses of the aortas

The aorta obtained from each rabbit was cleaned of excess adventitial tissue, opened longitudinally and fixed onto a plain ground. After fixation in 10% formalin overnight, the aortas were rinsed in 70% ethanol for 5 min and stained with 2% Sudan IV (Sudan IV, S-8756; Sigma) prepared in 70% ethanol. After staining, the aortas were differentiated in 80% alcohol for 5 min, and washed in running tap water for 1 h to remove excess dye [3]. The stained aortas were kept in PBS at  $4^{\circ}\text{C}$  until they were photographed. Images of Sudan IV-stained aortas were taken with a standard Nikon digital camera. Images were stored and analyzed with Adobe Photoshop 7.0. Atherosclerotic index was calculated by dividing the pixels of atherosclerotic lesions to the pixel of the whole aorta multiplied by 100. Results were expressed as a percentage.

#### CD36 mRNA expression

To analyze the expression of CD36 mRNA expression, homogenates were prepared from the thoracic aorta and total RNA was isolated from the media of each rabbit thoracic aorta with an RNA extraction kit (RNeasy Midi kits Qiagen). Reverse transcription-polymerase chain reaction (RT-PCR)<sup>1</sup> was applied to total RNA isolated from rabbit smooth muscle cells with some modifications. Semi-quantitative assays for CD36 mRNA expression were performed

<sup>1</sup> Abbreviations used: RT-PCR, reverse transcription-polymerase chain reaction; TP, tocopheryl phosphate; TPm, tocopheryl phosphate and bis-tocopheryl phosphate (2/1).

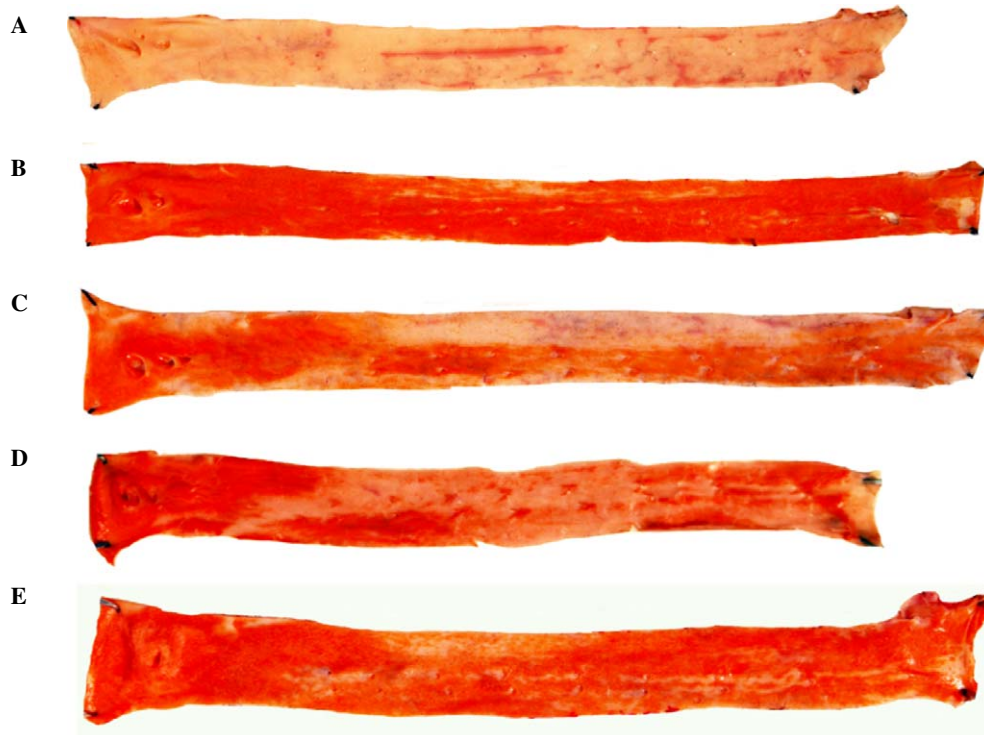


Fig. 1. En-face pictures of rabbit aorta stained with Sudan IV. Cholesterol fed rabbits developed a lesion equivalent to 78% of the surface. Treatment of the rabbits with tocopheryl acetate results in very low protection, equivalent to approximately 17%. Treatments with different amounts of tocopheryl phosphate resulted in a protection of about 60%. (A) Control (B) Cholesterol (C) Cholesterol + TPm (0.33 g/kg chow) (D) Cholesterol + TPm (1.33 g/kg chow) (E) Cholesterol + TA. A → Control diet (no cholesterol, no treatments), B → 2% cholesterol diet alone, C → 2% cholesterol + TPm 0.33 g/kg in the diet (62% diminution of plaque formation), D → 2% cholesterol + TPm 1.33 g/kg in the diet (60% diminution of plaque formation), E → 2% cholesterol + TA (17% diminution of plaque formation).

with a RT-PCR kit (GeneAmpR RNA PCR Kit, Perkin Elmer) with HotStarTaq™ DNA Polymerase (Qiagen) and primers specific for rabbit CD36; CD36PCR: 5'-TTG GTGTGTTTTATCCTTAC-3' and CD36 PCRR: 5'-GG TTCCAGTCTCATTAAGCC-3' for 40 cycles at 95°C, 30s, 56.1°C, 30s, and 72°C, 30s. Control reactions were performed with primers specific for rabbit glyceraldehyde-3-phosphate dehydrogenase; GAPDHR: 5'-TGCCGAAG TGGTCGTGGATGACCT-3' and GAPDHF: 5'-GCG CCTGGTCACCAGGGCTGCTTT-3' for 30 cycles at 95°C, 30s, 64°C, 30s, and 72°C, 30s. The PCR products were loaded on a 2.5% agarose gel and were quantified with a Lumi-Imager (Roche). CD36 mRNA expression was normalized to GAPDH. Quantification of different groups was made by considering CD36 mRNA expression for control samples as 100%.

## Results and discussion

### Analyses of serum cholesterol, TP, and T in the different animal groups

Table 1 indicates the concentrations of total cholesterol present in the rabbits for each treatment group. The effect of the cholesterol rich diet alone indicates a very large increase in the amount of cholesterol in serum (approximately a 34-fold increase). This value however, is not sig-

nificantly affected with any treatment that was tested (i.e., the tocopheryl phosphate or tocopheryl acetate treatments).

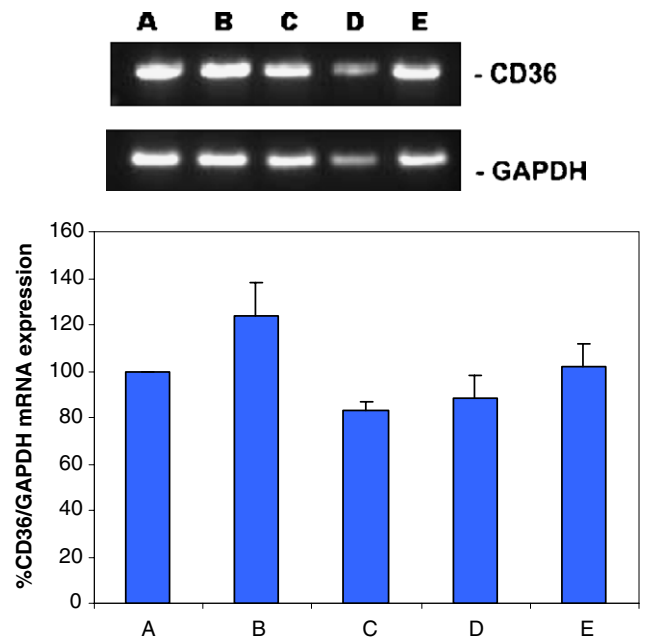


Fig. 2. Expression CD36 mRNA in rabbit aorta (A) Control (B) Cholesterol (C) Cholesterol + TPm (0.33 g/kg chow) (D) Cholesterol + TPm (1.33 g/kg chow) (E) Cholesterol + TA (four rabbits for each value).

The analysis of the concentrations of  $\alpha$ -tocopherol and  $\alpha$ -tocopheryl phosphate in the animal sera (Table 1) can be commented upon as follows. Both tocopherol and tocopheryl phosphate in the serum were, as expected, very low in the absence of tocopherol added to the diet. The reason why cholesterol in the diet increased the serum concentrations of T and TP is probably due to the facilitated transport of the hydrophobic tocopherols associated with cholesterol uptake. The presence of TA in the diet resulted in a 48-fold increase of total tocopherol (free + tocopherol liberated from TA during extraction) in the serum. This was not the case when TP (both at low and high concentrations) was present in the diet. The amounts measured were not significantly different. However, when serum TP was measured, a significant increase of this ester was found in animals treated with the low dose of TPm (0.33 g/kg).

The low uptake of TP in the supplemented animals is interesting in view of the fact that animals treated with TPm are more protected than those treated with TA (see macroscopic pictures of the aortas), indicating that TP (in the absence of increase in plasma tocopherol) may be acting as such and perhaps not by producing tocopherol. The low concentrations may also indicate that, rather than staying in the circulation, TP is rapidly transferred to the target tissues. Studies on bioavailability and plasma clearance are being carried out.

Altogether the data are indicative of a primary effect of tocopheryl phosphate in protecting against diet induced atherosclerosis in rabbits.

#### Macroscopic analyses of the aortas

In Fig. 1 the en face pictures are shown of Sudan IV stained aortas, from control, cholesterol-treated and tocopherol-treated rabbits. The results indicate that, in this typical series of analyses, the cholesterol fed rabbits developed a lesion equivalent to 78% of the surface. Rabbits treated with tocopheryl acetate resulted in very low protection, equivalent to approximately 17%, while rabbits treated with tocopheryl phosphate resulted in a protection of up to 60%. The data clearly shows that tocopheryl phosphate protects much more effectively than tocopheryl acetate against cholesterol induced atherosclerosis in this rabbit model.

#### Analysis of CD 36

The analyses of CD36 are shown in Fig. 2. The data indicates that cholesterol is producing a 24% increase in the expression of CD36 in this region of aorta. This is referred to in the figure as relative to GAPDH, a house keeping gene used as internal standard.

When compared to cholesterol group, TPm (0.33 and 1.33 g/kg diet) shows a CD36 expression diminution of %30 whereas with TA treatment the diminution is 17%. This is much greater compared to the effect of TA).

The data can be compared with those obtained by injecting tocopherol (50 mg/kg/day). In this case the values of

CD36 were 100, 174, and 75% for control, cholesterol treated and cholesterol plus vitamin E i.m., respectively, with an effect of protection of 57% [7]. Altogether the data on CD36 confirms in vivo, what had been seen at a cellular level, that efficacy of  $\alpha$ -tocopheryl phosphate in regulating gene expression is more pronounced than that of the non-esterified form.

#### Conclusions

The model chosen (rabbits fed a 2% cholesterol in the presence of TA or TPm in the diet) has given positive results with respect to the effect of TPm at 0.33 g/kg diet and 1.33 g/kg feed) in almost all the analyses made (macroscopic images and CD36 expression). The protection by TP is higher in the CD36 expression experiments than that of TA. The effect of TPm (0.33 and 1.33 g/kg) administered orally compares positively with the protection by tocopherol administered i.m. It is to be underlined that in the latter case the bioavailability is most probably much higher (data of TP and TA bioavailability are being carried out), due to the non-physiological parenteral administration of tocopherol.

The very large cholesterol load imposed on these animals (34-fold increase), was shown to be hardly counteracted by TA. On the other hand, the detrimental effects of the cholesterol load were effectively neutralized by equivalent or smaller TPm concentrations in the diet. The form by which TP is absorbed in this model will be revealed by the bioavailability analyses of TP, TA, and T in plasma and tissues.

The macroscopic protection by TP against plaque formation, positive in all aortas but not proportional to the TP concentration in the diet, indicates that the protection against this phenomenon may saturate already at low TP concentrations.

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