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Comparison of different vehicles to study the effect of tocopherols on gene expression in intestinal cells

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Abstract
Recent studies have focused on the ability of tocopherols to regulate gene expression. For such experiments, the methodology used to deliver molecules to the cells is crucial and could lead to different results depending on the vehicle used. The objective of the present study was to compare commonly used tocopherol vehicles (ethanol, BSA and mixed micelles) in terms of toxicity, stabilization of tocopherols, uptake efficiency of tocopherols by cells and effect on gene expression. Lactate dehydrogenase measurements did not reveal cytotoxicity of any of the tested vehicles. Tocopherol recovery measurements showed that $\sim$80% of the tocopherol was lost in ethanolic solutions, while only $\sim$30% and 10% were lost in BSA and mixed micelles, respectively. After 24 h incubation, Caco-2 cell monolayers treated with mixed micelles exhibited the highest α-tocopherol intracellular concentrations (5-times those measured with the two other vehicles). Similar results were obtained with γ-tocopherol. Vehicles, except mixed micelles that activate the FXR/bile acids signalling pathway, did not affect expression of nuclear receptors involved in lipid metabolism or their target genes. This study establishes mixed micelles as the best vehicle to deliver tocopherols to intestinal cell monolayers in culture.

Keywords: *Gene regulation, tocopherol, Caco-2, intestine, vitamin E, mixed micelles*

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Introduction

Vitamin E is a major fat-soluble antioxidant. This compound is naturally present in eight different forms ((R,R,R)-α, -β, -γ, -δ-tocopherols and tocotrienols), but (R,R,R)-α-tocopherol and (R,R,R)-γ-tocopherol are the two forms mainly found in our diet [1]. Besides its well established antioxidant properties, vitamin E displays other activities related to gene expression [2]. Indeed, α-tocopherol is an inhibitor of smooth muscle cell proliferation via the modulation of PKC phosphorylation level [3]. Furthermore, several vitamin E vitamers were presented as potential regulators of xenobiotic metabolism via their Pregnane X Receptor (PXR) ligand properties [4]. Now it is assumed that several signalling pathways are modulated by vitamin E. For example, the activities of NF-κB and AP-1, two major players of the response to oxidative stress, were linked to tocopherols [5] and protein kinase B also seemed to be involved in tocopherol mediated gene regulation [6].

The intestinal absorption of vitamin E has not been completely elucidated, but recent studies reported the involvement of two membrane lipid transporters: scavenger receptor class B type I (SR-BI [7]) and an ATP binding cassette (ABC [8]). Furthermore,

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microsomal triglyceride transfer protein (MTP), which is well known to participate in the incorporation of triglycerides into chylomicrons, is also involved in the incorporation of tocopherol in these intestinal lipoparticles [8,9]. In this context, it is reasonable to hypothesize that other proteins involved in lipid and sterol metabolisms could also be implicated in vitamin E metabolism. Because most of these proteins are known to be regulated by molecules they are dealing with and because tocopherol is able to regulate gene expression, it is interesting to evaluate the ability of tocopherol to regulate the expression of genes coding for proteins involved in intestinal lipid metabolism. Such an approach would be relevant to evaluate the consequence of vitamin E treatment on its own intestinal metabolism, as well as on lipid and sterol metabolisms.

As with many hydrophobic bioactive compounds, tocopherols need to be solubilized in an aqueous phase using an appropriate vehicle. Different vehicles have been previously used: ethanol [10–12], bovine serum albumin (BSA [13–15]) and bile salts or bile salt-rich micelles [7,16]. However, the ability of these vehicles to efficiently provide tocopherols to cells and their effects on gene expression have not been addressed. The objective of the present work was thus to compare these vehicles with regard to different key parameters: (1) toxicity towards cells, (2) ability to protect tocopherol from degradation/oxidation, (3) efficiency of tocopherol delivery to cells and (4) effects on gene expression.

Materials and methods

Chemicals

(RRR)-α-tocopherol ($\geq$99% pure) and (RRR)-γ-tocopherol ($\geq$97% pure) were purchased from Fluka (Vaulx-en-Velin, France). Tocol, used as an internal standard for HPLC analysis, was purchased from Lara Spiral (Couternon, France). 2-Oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine (phosphatidylcholine), 1-palmitoyl-sn-glycero-3-phosphocholine (lysophosphatidylcholine), monoolein, free cholesterol, oleic acid, sodium taurocholate, fatty acid free bovine serum albumin and pyrogallol were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose and trypsin-EDTA (500 mg/L and 200 mg/L, respectively) was purchased from BioWhittaker (Fontenay-sous-Bois, France). Foetal bovine serum (FBS) came from Biomedia (Issy-les-Moulineaux, France). Non-essential amino acids, penicillin/streptomycin and PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS-CM) were purchased from Invitrogen (Cergy Pontoise, France).

Preparation of ethanolic or BSA solutions of tocopherols

DMEM medium was supplemented with 1% non-essential amino acids, 1% antibiotics and tocopherols as ethanolic solutions in the range of 5–60 μM. In the case of BSA solutions, BSA (5 mg/ml in water) was incubated with ethanolic solutions of tocopherol for 1 h and then DMEM medium supplemented with 1% non-essential amino acids and 1% antibiotics was added. The final concentration of ethanol in all samples did not exceed 0.1%. Controls were media with the corresponding percentage of ethanol and/or BSA.

Preparation of tocopherol-rich mixed micelles

Mixed micelles, with similar lipid compositions to in vivo micelles [17], were prepared as previously described [7] to obtain the following final concentrations: 0.04 mM phosphatidylcholine, 0.16 mM lysophosphatidylcholine, 0.3 mM monoolein, 0.1 mM free cholesterol, 0.5 mM oleic acid, 5–90 μM tocopherol [18] and 5 mM taurocholate. The concentration of tocopherol in the micellar solutions was checked by HPLC before each experiment.

Cell culture

Caco-2 clone TC-7 cells [19,20] were a gift from Dr M. Rousset (U178 INSERM, Villejuif, France). Cells were cultured in the presence of DMEM supplemented with 20% heat-inactivated FBS, 1% non-essential amino acids and 1% antibiotics (complete medium).

For each experiment, cells were seeded and grown on 6-well plates to obtain confluent differentiated cell monolayers. Twelve hours prior to each experiment, the medium was replaced by a serum-free complete medium.

Cytotoxicity of vehicles and tocopherol on Caco-2 cells

Differentiated cell monolayers were incubated with media containing ethanol, BSA or mixed micelles with or without tocopherols for 24 h. In recovered media, lactate dehydrogenase, a classical marker of toxicity, was measured by the Cytotoxicity Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany), using the Roche MODULAR automated system (Roche Diagnostics GmbH).

Measurement of tocopherol uptake by Caco-2 cell monolayers

At the beginning of each experiment, cell monolayers were washed twice with 0.5 mL PBS. The cell monolayers were exposed to tocopherols (from 0–60 μM) solubilized in different vehicles and incubated for various times (1–24 h) at 37°C. Cells were then rinsed with PBS containing taurocholate (1 mM) before being scrapped in PBS. Media was also

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recovered at the end of the incubation periods. All of the samples were stored at $-80°C$ under nitrogen with 0.5% pyrogallol as an antioxidant before tocopherol extraction and HPLC analysis.

Tocopherol extraction

Distillated water was added to sample volumes (125 µl) to reach a final volume of 500 µL. An equal volume (500 µL) of tocol in ethanol was added to the samples as an internal standard. The mixture was extracted once with one volume of hexane. The hexane phase obtained after centrifugation (500 *g*, 10 min, 4°C) was evaporated to dryness under nitrogen and the dried extract was dissolved in 200 µL methanol. A volume of 5–20 µL was used for HPLC analysis.

Tocopherol analysis

α-, γ-Tocopherol and tocol were separated using a 250 × 4.6 nm RP C_{18}, 5 µm Zorbax column (Interchim, Montluçon, France) equipped with a guard column. The mobile phase was 100% methanol, the flow rate was 1.5 mL/min and the column was kept at a constant temperature (30°C). The HPLC system was a Dionex separation module (P680 HPLC Pump and ASI-100 Automated Sample Injector, Dionex, Aix-en-Provence, France) connected to a Jasco fluorimetric detector (Jasco, Nantes, France). Tocopherols were detected at 325 nm after light excitation at 292 nm and characterized by retention time compared with pure ($>95\%$) standards. Quantification was performed using the Chromeleon software (version 6.50 SP4 Build 1000) by comparing peak areas with standard reference curves. All solvents used were HPLC grade from SDS (Peypin, France).

Effect of tocopherol-free vehicles on Caco-2 cell nuclear receptors and target gene mRNA levels

Differentiated cell monolayers were incubated with either ethanol or BSA solutions or mixed micelles for 24 h. Total RNA (~ 50 µg) was isolated from the cells using Trizol (Invitrogen). cDNA was prepared by reverse transcription of 1 µg total RNA using random hexamers as primers with M-MLV reverse transcriptase (Invitrogen). Five microlitres of cDNA were used for quantification by real-time quantitative PCR, as previously reported [21]. The sequences of the primers used are reported in Table I.

Reactions were performed in duplicate with a Stratagene MX3005P apparatus (Stratagene, Amsterdam, The Netherlands) using SYBR green kits (Eurogentec, Angers, France), according to the manufacturer instructions. The relative levels of mRNA were calculated using the comparative $\Delta\Delta C_t$ method [22].

Table I. Sequences of oligonucleotides used for real-time PCR.

Oligonucleotide	Sequence (5′–3′)
PPARα for	cgtgcttcctgcttcataga
PPARα rev	cacagacaggcaatctcagc
PPARβ for	AAAAAGAAGGCCCGCAGC
PPARβ rev	CACAAAGGGCGCCGTG
PPARγ for	GGCGAGGGCGATCTTGA
PPARγ rev	CCCATCATTAAGGAATTCATGTCA
LXRα for	TGTAACCGGCGCTCCTTTT
LXRα rev	TGGTGCCATGGGCCA
LXRβ for	CCTGCAGCACAGACTGGGT
LXRβ rev	GGCCCTTCTTTCGCTTGC
FXR for	gaggaagactcagtccagaatcc
FXR rev	ccttctacgatgtcttctacctcct
SHP for	agggaccatcctcttcaacc
SHP rev	ttcacacagcacccagtgag
L-FABP for	CCAGGAAAACTTTGAAGCCTTC
L-FABP rev	TTCCCCTTCTGGATGAGCTCT
SREBP1c for	CCATGGATTGCACTTTCGAA
SREBP1c rev	CCAGCATAGGGTGGGTCAAA
18S for	cgccgctagaggtgaaattct
18S rev	cattcttggcaaatgctttcg

Statistical analysis

Results are expressed as means ± SD. For mRNA expression levels, differences were tested using the Student's *t*-test. For other experiments, differences were tested by two-way ANOVA. The Tukey-Kramer test was used as a post-hoc test when ANOVA showed significant differences between groups. Values of $p < 0.05$ were considered significant. All statistical analyses were performed using the Statview software 5.0 (SAS Institute, Cary, NC).

Results and discussion

In order to determine the most appropriate vehicle to deliver tocopherols to intestinal cell monolayers, we evaluated toxicity of different vehicles, their effects on the stability of tocopherols and on the efficiency of tocopherol uptake by the cell monolayer and the effects of vehicles alone on gene expression. In this study, we used the Caco-2 cell line as a recognized model of intestinal function (absorption, gene regulation, etc.).

Cytotoxicity of the different vehicles

In order to assess the cytotoxicity of the vehicles as well as that of the tocopherols, LDH released into the culture medium was measured. As a positive control, Caco-2 cells were incubated for 10 min with 1% Triton X-100. As expected, this treatment induced a significant toxic effect. In contrast, no toxic effect was observed with the vehicles alone or with the vehicles enriched with tocopherols (Table II). These results are in agreement with Gysin et al. [23], who reported no apparent cell death in Caco-2 cells under tocopherol treatment when evaluated by the trypan

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Table II. Dosage of lactate dehydrogenase (LDH).

Culture conditions	LDH (UI/L)	*t*-test
Positive control (1% Triton X-100)	3946 ± 403	***
Standard culture medium	65 ± 18	
Ethanol solution		
Control	83 ± 30	ns
α-tocopherol	71 ± 27	ns
γ-tocopherol	65 ± 13	ns
BSA solution		
Control	47 ± 4	ns
α-tocopherol	52 ± 18	ns
γ-tocopherol	64 ± 10	ns
Mixed micelles		
Control	78 ± 10	ns
α-tocopherol	63 ± 8	ns
γ-tocopherol	47 ± 8	ns

Cells were incubated with different solution containing tocopherol for 24 h. LDH was quantified in the medium as described in Materiel and methods. Significant differences between standard culture medium and other conditions were tested by Student t-test. Means $\pm$SD; *** $p < 0.01$.

blue assay. It is noteworthy that long-term incubation (24 h) of Caco-2 cells with similar concentrations of mixed micelles was not cytotoxic and did not affect the monolayer or cell morphology [24], even if these structures contained bile acids (taurocholate) and lysophosphatidylcholine that are known for their detergent properties [25]. Nevertheless, our data are in good agreement with previous studies using these vehicles to study of the absorption of lipophilic molecules [7,26,27].

Stability of tocopherols in different vehicles

Another quality of a vehicle is its ability to protect tocopherols against degradation/oxidation during the time of an experiment. Whereas mixed micelles allowed a very good recovery of tocopherols (~ 90% of the tocopherol was recovered after 24 h), only 70% was recovered when tocopherols were incorporated into BSA and less than 20% when dissolved in ethanol (Figure 1). Note that a significant difference in stability between α- and γ-tocopherol was observed in the case of ethanolic solutions. We hypothesize that tocopherol in ethanol was exposed to oxidative damage, due to the presence of Fe^{2+} and Cu^{2+} in the culture medium. Indeed, it has been reported that these ions are involved in the oxidation of tocopherol [28]. Moreover, it is known that phenolic compounds in culture medium could generate H_2O_2 [29]. The mechanism involved is not fully understood, but the consequence could be massive oxidative degradation of phenolic compounds incubated in the medium. Due to the phenolic structure, such a phenomenon is highly likely to occur with tocopherols. The stability discrepancy between α- and γ-tocopherol in ethanolic solution could be due to the fact that α-tocopherol is consumed by more side reactions than γ-tocopherol in an oxidative environment [30]. When associated with BSA, which displays well known antioxidant properties [31], tocopherols were partly protected from oxidative damage. In the case of mixed micelles, the amphiphilic nature of these structures could allow efficient protection against oxidative damage of the internalized tocopherols.

Effect of the vehicle on tocopherol uptake efficiency

In order to choose the best vehicle, we tested the efficiency of uptake of tocopherol incorporated in the different vehicles (Figure 2). Note that the tocopherol concentrations tested varied from 5–60 μM,

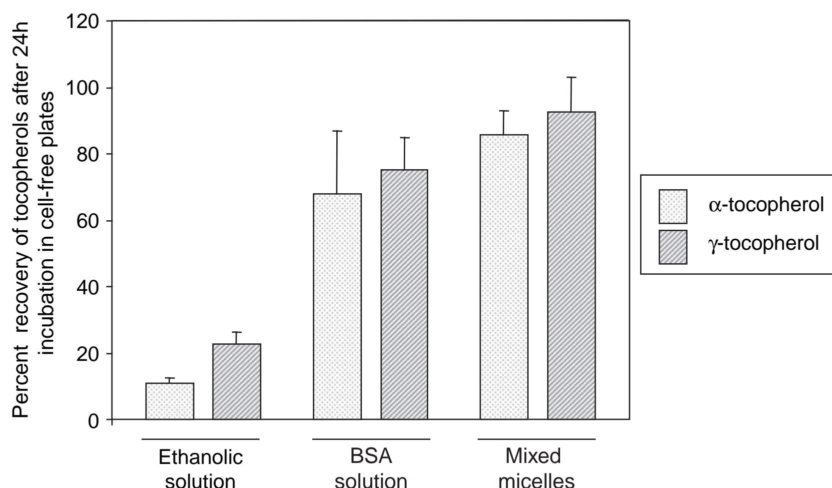

Figure 1. Percentage recovery of α- and γ-tocopherol as a function of vehicle. Ethanolic and BSA solutions of tocopherols or tocopherol-rich mixed micelles were incubated for 24 h in a cell-free tissue culture environment. Tocopherols were extracted at the end of the incubation and quantified by HPLC as described in Materials and methods. Data are means $\pm$SD. Two-way ANOVA, with tocopherol vitamer and vehicle as factors, showed an effect of both the molecules (α-tocopherol vs γ-tocopherol in ethanol) and vehicles on recovery. The post-hoc Tukey-Kramer test showed significant ($p < 0.001$) differences between the three vehicles.

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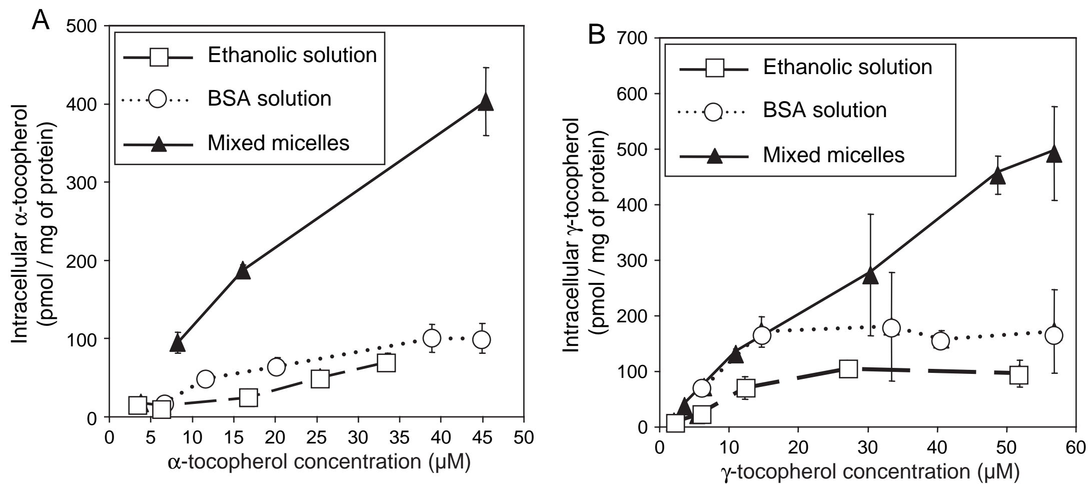

Figure 2. Effect of vehicle type and tocopherol concentration on tocopherol uptake by Caco-2 cells. Caco-2 cells were incubated for 24 h with medium containing ethanol, BSA or mixed micelles as delivery vehicles for α-tocopherol (A) or γ-tocopherol (B). Tocopherols incorporated into Caco-2 cells were extracted and quantified by HPLC as described in Materials and methods. Data are means $\pm$SD. Two-way ANOVA, with concentration and vehicle as factors, showed an effect of both factors on uptake ($p<0.001$). The post-hoc Tukey-Kramer test showed significant ($p<0.001$) differences between the three vehicles and between concentrations.

corresponding to physiological concentrations in human intestinal lumen [18]. We observed a strong discrepancy in α-tocopherol uptake when it was provided in mixed micelles (426 ± 99 pmol/mg of protein) vs ethanol (70 ± 10 pmol/mg of protein) or BSA solutions (100 ± 19 pmol/mg of protein) (Figure 2A), the two latter being $\sim$5-times less efficient in terms of uptake after a 24 h incubation ($p<0.001$). For this incubation time, tocopherol uptake for ethanol or BSA solutions was not significantly different. In the case of mixed micelles, a clear relationship appeared between the amount of intracellular tocopherol and the amount of tocopherol added in the medium. Such a relationship did not occur for ethanol and BSA. The same pattern of response was also observed for γ-tocopherol (Figure 2B). The amount of intracellular α- or γ-tocopherol did not differ significantly ($p=0.19$) when mixed micelles were used as a vehicle. Similar absorption patterns for α-tocopherol and γ-tocopherol were previously observed [7]. This is also in agreement with previous data that reported no discrimination between the two vitamers for intestinal uptake [32].

Kinetic experiments were performed only with γ-tocopherol because no major discrepancy between the vitamers was observed in dose effects. As shown

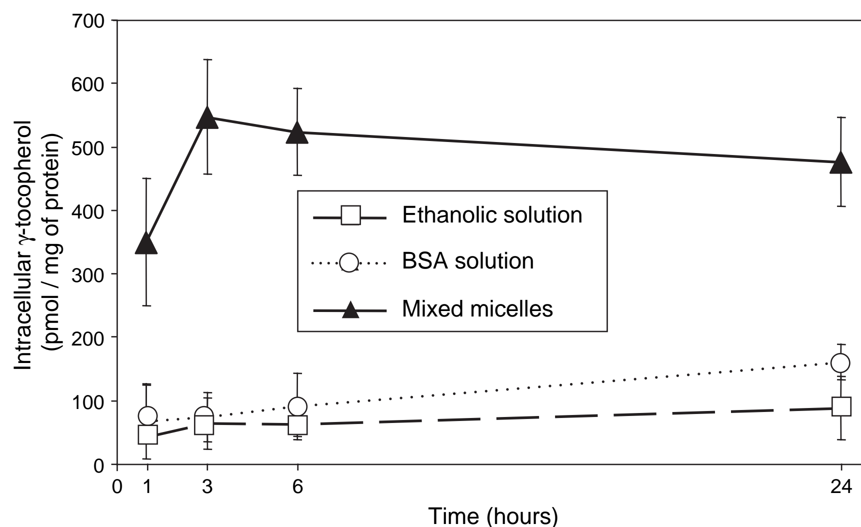

Figure 3. Effect of vehicle type on kinetics of γ-tocopherol uptake by Caco-2 cells. Caco-2 cells were incubated for 24 h with medium containing 60 μm γ-tocopherol solubilized in the aqueous medium with either ethanol, BSA or mixed micelles. γ-tocopherol was extracted at various times of incubation and quantified by HPLC as described in Materials and methods. Data are means $\pm$SD. Two-way ANOVA, with time and vehicle as factors, showed an effect of both factors on uptake ($p<0.001$). The post-hoc Tukey-Kramer test showed significant ($p<0.001$) differences between values observed for mixed micelles and those observed for the other vehicles and for values observed at 1 h and those observed at 24 h of incubation.

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in Figure 3, when γ-tocopherol was supplied in mixed micelles, the amount of γ-tocopherol was significantly greater (× 5) as compared to ethanol or BSA solution, which did not differ significantly. Under these conditions, the maximum incorporation was achieved for short incubation times and remained constant over a period of 24 h for mixed micelles. Ethanol or BSA led to quick incorporation, which also remained constant for 24 h.

The very low absorption efficiency of tocopherol with ethanol can be explained by the lower recovery rate of this vitamin in ethanol that we have previously shown. However, this phenomenon cannot explain the huge difference between mixed micelles and BSA. One hypothesis is that the uptake of tocopherol requires the involvement of transporters that interact with mixed micelles to transfer tocopherol into cells, whereas this phenomenon does not occur in the presence of BSA. As a matter of fact, recent data have demonstrated the involvement of the scavenger receptor type BI (SR-BI) in tocopherol transport across the enterocyte membrane [7] and we suggest that such an interaction requires the presentation of tocopherol in a physiological manner that is achieved only when it is incorporated into mixed micelles.

Effect of the vehicles on mRNA levels of transcription factors involved in lipid metabolism

We evaluated the impact of the different vehicles on several genes because the presence of vehicle molecules, especially in mixed micelles, may affect gene expression independently of tocopherols. Thus, we evaluated the mRNA levels of the nuclear receptors Peroxisome proliferators activated receptors (PPARs) [33], Liver X receptors (LXRs) and Farnesoid X receptor (FXR) [34]. Indeed, the PPAR isoforms are activated by fatty acids and/or metabolites of these fatty acids [35] and present an auto regulation loop. LXRs are sensors for oxysterols that come from cholesterol [36]. FXR is the nuclear receptor activated by bile acids [37–39]. We also measured prototypical target genes of PPARs, LXRs and FXR, i.e. Liver fatty acid bindind protein (L-FABP) [40], Sterol response element binding protein 1c (SREBP1C) [41] and Small heterodimer partner (SHP) [42,43]. As shown in Figure 4, we did not observe major effects of the vehicles in terms of mRNA level modulations. The only significantly induced gene was SHP, which was induced by mixed micelles and is a classical FXR target gene. This

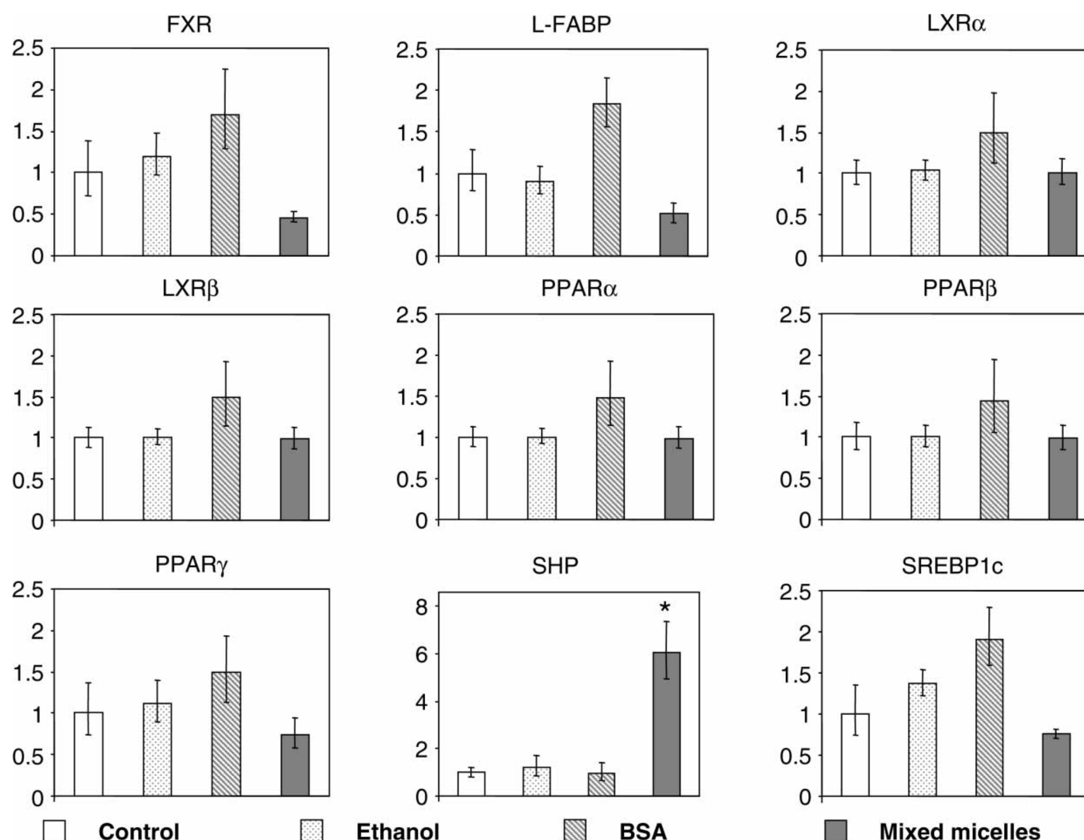

Figure 4. Modulation of mRNA levels of genes involved in lipid metabolism by delivery vehicles. Caco-2 cell monolayers were incubated for 24 h with medium containing either ethanol, BSA or mixed micelles as delivery vehicles for tocopherols. Relative mRNA levels (studied mRNA/18S rRNA) were quantified by real-time quantitative PCR. Data are means ± SD. * $p < 0.005$ as assessed by Student's *t*-test. FXR, Farnesoid X receptor, L-FABP, Liver fatty acid binding protein, LXR: Liver X receptor, PPAR, Peroxisome proliferators activated receptor, SHP: Small heterodimer partner, SREBP1c: Sterol response element binding protein 1C.

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result suggests that FXR target genes are induced by mixed micelles. Consequently, we suggest that the entire intestinal bile acid signalling pathway was regulated in the presence of mixed micelles. This observation was confirmed by microarray experiments, where several FXR target genes were upregulated in the presence of mixed micelles (data not shown). Because tocopherols have not yet been reported to act as FXR ligands, such an induction of the FXR signalling pathway by mixed micelles is unlikely to interfere with the effect of tocopherol on Caco-2 cells. Indeed, no activation of nuclear receptors has been reported with tocopherol except for Pregnane X receptor (PXR), a nuclear receptor involved in xenobiotic metabolism, which is activated by tocopherols and tocotrienols [4]. Furthermore, in intestinal lumen, tocopherols are incorporated into mixed micelles, thus it is much more relevant, in a physiological point of view, to take into account the effect of this vehicle when evaluating the effect of tocopherols.

Concluding remarks

Mixed micelles appeared to be the best vehicle to deliver tocopherols to Caco-2 cells, since this method displayed no toxicity at the concentrations assayed, promoted the stability of tocopherols and led to the best absorption efficiency. Although mixed micelles can affect expression of some genes, the induction of the bile acid target genes is not an issue since it is a physiological response that happens *in vivo*.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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