Biochemical and Biophysical Research Communications 446 (2014) 798–804

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Biological activities of Schottenol and Spinasterol, two natural phytosterols present in argan oil and in cactus pear seed oil, on murine miroglial BV2 cells

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ARTICLE INFO

Article history:
Available online 25 February 2014

Keywords:
ABCA1
ABCG1
Argan oil
BV2 cells
Cactus oil
Ficus indica
LXR
Phytosterols
Schottenol
Spinasterol

ABSTRACT

The objective of this study was to evaluate the biological activities of the major phytosterols present in argan oil (AO) and in cactus seed oil (CSO) in BV2 microglial cells. Accordingly, we first determined the sterol composition of AO and CSO, showing the presence of Schottenol and Spinasterol as major sterols in AO. While in CSO, in addition to these two sterols, we found mainly another sterol, the Sitosterol. The chemical synthesis of Schottenol and Spinasterol was performed. Our results showed that these two phytosterols, as well as sterol extracts from AO or CSO, are not toxic to microglial BV2 cells. However, treatments by these phytosterols impact the mitochondrial membrane potential. Furthermore, both Schottenol and Spinasterol can modulate the gene expression of two nuclear receptors, liver X receptor (LXR)-α and LXRβ, their target genes ABCA1 and ABCG1. Nonetheless, only Schottenol exhibited a differential activation *vis-à-vis* the nuclear receptor LXRβ. Thus Schottenol and Spinasterol can be considered as new LXR agonists, which may play protective roles by the modulation of cholesterol metabolism.

1. Introduction

It's widely accepted that among plant natural food ingredients, several micro-nutriments contribute to the basis of their health benefits. In this regard, it is relevant that supplementation of diet with these plant micro-nutriments could help preventing and fighting the threat of metabolic dysregulation, mainly in cardiovascular and neurodegenerative diseases. Among these micro-nutriments, plant sterols (also called phytosterols), which are structural analogues of cholesterol found mainly in plant fruits and seeds, have been recognized to efficiently reduce serum concentrations of cholesterol and LDL-cholesterol (for extensive review read Genser et al. [1]). Here, we focused our work on two phytosterols, Schottenol and Spinasterol, found in argan oil (*Argania spinosa [L.] Skeels*) and seed oil from cactus pear (*Opuntia ficus indica*) [2,3] [and the present work].

Argan oil is used as a traditional food ingredient in the 'Amazigh diet', bringing almost 25% of total diet fat intake to indigenous consumers [2]. Health benefits of this virgin oil have been documented by several studies highlighting its capacity in lowering of plasma LDL-cholesterol and lipoperoxides concomitant to increasing plasmatic tochopherol concentration [4], resulting in hypocholesterolemic and hypotriglyceridemic effects in consumer populations [4–7]. However, mediator molecules of these health benefits still remain to be clarified.

Abbreviations: AO, argan oil; CSO, cactus seed oil; AOSE, argan oil sterol extract; COSE, cactus oil sterol extract; LXR, liver X receptor; PS, phytosterols.

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http://dx.doi.org/10.1016/j.bbrc.2014.02.074
0006-291X/

Recently, some interesting researches were developed around *O. ficus indica*, which is a cactus specie well adapted to diverse regions around the world, including the Mediterranean basin and South Africa among others, and is cultivated for its edible fruit (prickly pear) [8]. Data from few clinical trails show that consumption of cactus dehydrated leaves significantly increases HDL-cholesterol [9]. Short-term supplementation in healthy humans with cactus pear fruit or dried prickle-pear cactus decreases lipid oxidative damage, improves antioxidant status, and lowers of cholesterol and triglycerides [10,11]. Nevertheless, regarding seed oil of *O. ficus indica*, to our knowledge, only the study of Ennouri et al. [12] reported the effects of seed oil administration in rat, showing decrease in serum glucose concentration concomitant to a decrease in circulating cholesterol and LDL-cholesterol [12].

Phytosterols (PS) can be considered as good candidates for the beneficial effects of both AO and *O. ficus indica* on cholesterol metabolism. Daily intake of 0.8–4 g of PS leads to 10–15% decrease in plasma cholesterol [13]. Analysis of numerous trails revealed that intake of 2 g/day of phytostanols (saturated PS with no double bonds in the sterol ring) can lower plasma LDL-cholesterol up to 9–14% [14]. Several phytosterols are revealed as ligands for liver X receptor (LXR) α and LXRβ [14–16]. These nuclear receptors are considered as integrators of metabolic and inflammatory signaling, particularly in the brain [17]. The role played by LXRs in neurodegenerative diseases, particularly in Alzheimer's disease and in amyotrophic lateral sclerosis-Parkinson's dementia, is still under investigation [18,19]. LXRs regulate brain cholesterol homeostatic genes and sensitivity of motor neuron and glial cells to phytosterols [18,19].

In attempt to precise the biological characteristics of phytosterols on the nervous system, we focused our investigations on two phytosterols, Schottenol and Spinasterol, present in AO and CSO, studying their effects on the murine microglia cell line BV2. We reported the composition of AO, CSO and their sterol extracts. We analyzed several parameters related to the cytotoxicity of these compounds especially at the mitochondrial level with the MTT test and after staining with DiOC6(3) allowing the measurement of the transmembrane mitochondrial potential. The gene expression and

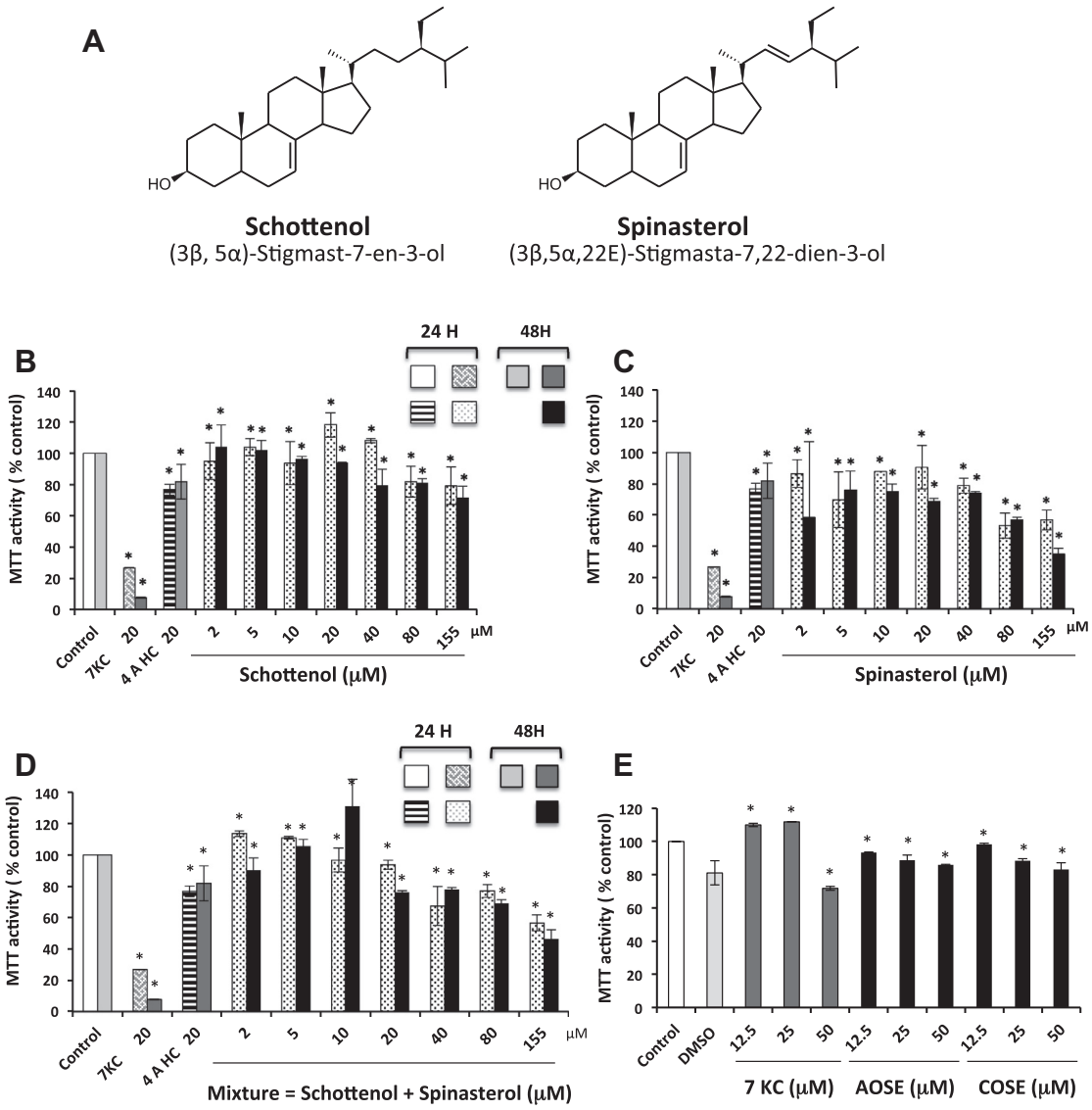

Fig. 1. (A) Planar structure of Schottenol and Spinasterol. (B–E) Effects of sterols on cell proliferation and viability measured with the MTT test on BV2 microglial cells treated for 24 and 48 h. 7-Ketocholesterol (7KC) was use as positive control and 4 alpha-hydroxycholesterol (4A HC) as negative control for cell death induction in a concentration range from 2 to 155 μM for Schottenol (B), Spinasterol (C) or their mixture (D), and from 12 to 50 μM for sterol extracts AOSE (argan oil) or COSE (cactus oil) (E). Values were normalized to the control and were considered statistically significance (Student-*t* test) at $P < 0.05$ (*).

activation of the nuclear receptors LXRα and LXRβ were analyzed by qPCR and gene reporter activation essay respectively. Our data showed that phytosterols from AO and CSO are not toxic to BV2 cells and they can modulate the mitochondrial membrane potential. Among these phytosterols, both Schottenol and Spinasterol can modulate the gene expression of liver X receptor (LXR)-α or LXRβ their target genes ABCA1 and ABCG1 in BV2 cells.

2. Materials and methods

2.1. Materials

N-(2,2,2-trifluoroethyl)-N-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]-benzenesulfonamide (T0901317), a potent synthetic LXRα and LXRβ agonist [20], and 7-ketocholesterol (7KC) are from Sigma–Aldrich. 4α-hydroxycholesterol (4αOHC) and 4β-hydroxycholesterol (4βOHC) were prepared as previously described [21].

2.2. Synthesis of Schottenol and Spinasterol

Schottenol [(3-beta,5-alpha)-Stigmast-7-en-3-ol] and Spinasterol [(3-beta,5-alpha,22E)-Stigmasta-7,22-dien-3-ol] (Fig. 1) were prepared according to Kircher and Rosenstein [22].

2.3. Cell culture and treatments

Murine microglia BV2 cell line (BV2) were seeded at 1.2×10^5 -cells per well in 12-well microplates containing 1 mL of Dulbecco's modified Eagle medium (Lonza) supplemented with 5% (v/v) heat-inactivated fetal bovine serum (Pan Biotech) and 1% antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin) (Pan Biotech). The treatments of BV2 cells with sterols (4β-OHC, 7KC, Schottenol and Spinasterol) were previously defined [21].

2.4. Gene reporter activation assays

Investigation of the potential activation of LXRα and/or LXRβ by Schottenol and Spinasterol was performed using the luciferase gene reporter essay as described previously [21]. The synthetic ligand T0901317 was used as positive LXR agonist. HEK293T cells were seeded in triplicate in 96 well plates using the Lipofectamine 2000 transfection protocol (Invitrogen/Life Technologies).

2.5. Quantitative PCR analysis

BV2 cells total RNA was extracted using the RNeasy Mini kit (Qiagen) following the manufacturer's instructions. Real-Time PCR System was performed as described previously [23]. The primer sequences were chosen using the Beacon Designer Software (Bio-Rad). Sequences are available from M.C-M on request. Gene expression was quantified using cycle to threshold (Ct) values and normalized by the reference gene, RPL27 encoding the ribosomal protein. To this end, the quantitative gene expression was determined according to $2^{-\Delta\Delta Ct}$ with ΔCt = (Ct of the gene studied) − (Ct of the RPL27 gene).

2.6. Colorimetric MTT assay

MTT assay was carried out on BV2 cells as previously described [21]. Cells plated in 48-wells were treated for 24 or 46 h with the different compounds in order to evaluate cell proliferation and/or viability. The plates were read at 570 nm with a microplate reader.

2.7. Measurement of the mitochondrial membrane potential ($\Delta\Psi m$) variations with DiOC6(3) by flow cytometry analysis

3,3′-Dihexyloxacarbocyanine iodide (DiOC6(3)) (Invitrogen) allows estimation of the percentage of cells with low $\Delta\Psi m$. Mitochondrial depolarization is indicated by a decrease in green fluorescence collected through a 520/10-nm band pass filter. DiOC6(3) was used at a 40 nM [21]. Flow cytometry analyses were performed on a Galaxy flow cytometer (Partec). Ten thousand cells were acquired for each sample. Data were analyzed with Flomax software (Partec) or FlowJo software (Tree Star Inc.) [21].

3. Results and discussion

3.1. Sterol composition of argan, cactus seed and olive oils and their sterol extracts

Analysis of the sterol content (Table 1) has been performed by GC–MS, revealing that cactus seed oil contains 5 times more sterols than AO. When compared to sterol extracts, we still have higher yield of sterols in COSE extract, more than twice of olive oil extract and almost 3 times than AO (Table 1). Interestingly, the composition of AO sterol fraction exhibited mainly 2 PS, Schottenol (56.97%) and Spinasterol (43.03%). This is in accordance with the previously published work of Khallouki et al. [24]. However, the AO origin and the extraction method can influence the physico-chemical characteristics of component analysis [25]. This may explain the lower content of total sterols in our AO sample. By contrast, both olive oil and cactus oil sterol extracts (OOSE and COSE) showed more than 80% of Sitosterol as the major PS (Table 1). In addition, Campesterol was found at low content in olive and cactus oils. Campestanol and Sitostanol, the two saturated forms of Campesterol and Sitosterol respectively, were detected to a lesser extents in OOSE and in COSE (Table 1).

3.2. Chemical synthesis of Schottenol and Spinasterol

Khallouki et al. [24] reported that the two major sterol found in AO are Schottenol and Spinasterol, which are also present in CSO. As these compounds are not commercially available, we synthesized them using the hydrogenation of 7-dehydrostigmasteryl acetate for Spinasterol and complete hydrogenation in Ni-catalyzed reduction of 7-dehydrostigmasterol [22]. Planar structures of Schottenol and Spinasterol are shown in Fig. 1A.

Table 1
GC–MS sterol analysis of argan, cactus seed and olive oils and oil-extracts.

Sterol	Argan oil	Cactus seed oil	Sterol extract		
			AOSE	COSE	OOSE
Schottenol (%)	91.24[a] (56.97)[c]	11.61[a] (1.29)[c]	7.65[b] (58.53)[c]	2.90[b] (7.1)[c]	0.01[b] (0.08)[c]
Spinasterol (%)	68.92[a] (43.03)[c]	14.44[a] (1.60)[c]	5.42[b] (41.47)[c]	1.69[b] (4.14)[c]	0.07[b] (0.36)[c]
Campesterol (%)	ND	57.94[a] (6.43)[c]	ND	0.96[b] (2.34)[c]	1.28[b] (6.97)[c]
Sitosterol (%)	ND	737.85[a] (81.92)[c]	ND	33.31[b] (81.50)[c]	15.22[b] (82.80)[c]
Campestanol (%)	ND	7.37[a] (0.82)[c]	ND	ND	0.10[b] (0.55)[c]
Sitostanol (%)	ND	71.47[a] (7.94)[c]	ND	2.01[b] (4.94)[c]	1.70[b] (9.25)[c]
Total sterol	160.16[a]	900.68[a]	13.08[b]	40.87[b]	18.38[b]

Values represent a: mg/100 g oil; b: mg/100 ml extract; c: % of total sterols.
AOSE: argan oil sterol extract. COSE: cactus oil sterol extract. OOSE: olive oil sterol extract.

3.3. Effects of PS on mitochondrial status microglial BV2 cells

MTT test was performed to evaluate the effect of natural PS on mitochondrial function and BV2 cells viability. Production of formazan by mitochondrial dehydrogenases is proportional to the number of viable cells and reflects the metabolic activity of mitochondria.

7-α Ketocholesterol (7KC) and 4-α hydroxycholesterol (4<alpha>OHC) respectively were used as control molecules for which MTT test has been reported elsewhere [21]. Fig. 1B shows that MTT reduction indicated a decreased number of viable cells when compared to the control. 7KC has a strong effect on cell viability and exerts its known cytotoxicity at 20 μM after 24 or 48 h of treatment respectively, while at the same concentration 4<alpha>OHC showed a very slight effect (Fig. 1B). Treatment with Schottenol had a slight effect and even at 155 μM we still have 80% and 70% of viable cells at 24 and 48 h respectively (Fig. 1B). On the other hand, high concentration of Spinasterol seems to be more cytotoxic than Schottenol, as reported in Fig. 1C, showing 60% and less than 40% of viable cells at 24 and at 48 h respectively for 155 μM (Fig. 1C). The treatment with a mixture containing both Schottenol and Spinasterol at a final concentration of 155 μM showed an intermediary effect (Fig. 1D). Sterol extracts were prepared from argan oil (AOSE) and cactus seed oil (COSE), and sterols content was reported in Table 1. Parallel treatments with these extracts revealed a slight dose dependent diminution of cell viability, which stands at 80% after 48 h (Fig. 1E). Compared to microglial BV2 cells, the human prostate cell lines LNCaP seems to be more sensitive to sterol extracts, showing 50% inhibition of cell proliferation after 48 h at only 25 μM [24]. This could be explained by the accumulation of sterols in LNCaP cells, for which an absence of cholesterol feedback mechanism was shown [26].

3.4. Effects of PS on mitochondrial membrane potential in microglial BV2 cells

The mitochondrial membrane potential (ΔΨm) was measured using the fluorescent probe DiOC6(3). Treatment with vehicle (i.e. DMSO) showed 20% of negative cells with depolarized mitochondria. For more accuracy, results are standardized in Fig. 2 as percent of the control. After 24 h treatment, the percentage of BV2 cells with depolarized mitochondria observed under treatment with Schottenol or Spinasterol was 40% of the control, revealing an increase of ΔΨm and hyperpolarization of mitochondria in BV2 cells, except in the treatment with Spinasterol at 50 μM (Fig. 2A). By contrast, longer incubation (48 h) with Schottenol leads to a decrease of ΔΨm and depolarization of mitochondria
revealed by a higher percentage of DiOC6 negative cells (Fig. 2A). In a similar way, 48 h treatment with Spinasterol reverses the mitochondria membrane polarity, when compared to the 24 h point, and the same percentage of negative cells as in the control was obtained (Fig. 2A). Treatments with sterol extracts reveal no significant effect on ΔΨm of AOSE, while COSE treatment leads to a hyperpolarization of mitochondria at 24 h and to a depolarization at longer incubation (48 h, Fig. 2B). As Sitosterol represents more than 80% of COSE (Table 1) the effect of COSE could probably be attributed to this compound, which can be hydroxylated in mitochondria [27]. In addition, Sitosterol can be incorporated into the membrane of mitochondria promoting the inner membrane fluidity and therefore leading to the increase of mitochondrial membrane potential [28].

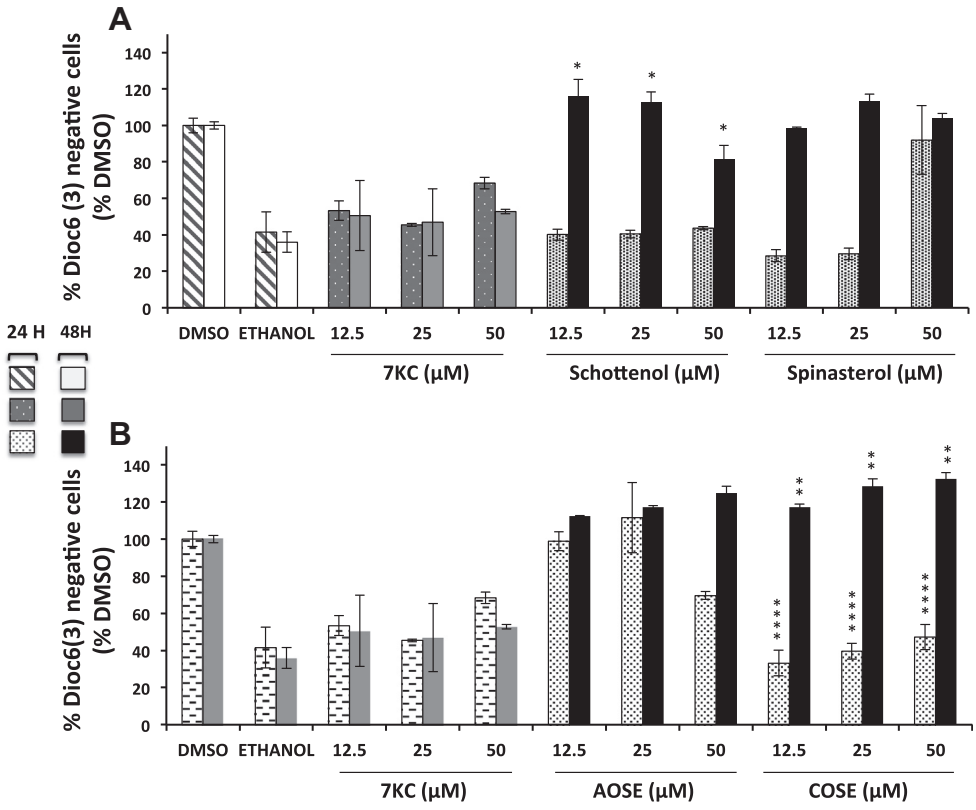

Fig. 2. Effect of Sterols (A: Schottenol and Spinasterol) or sterol extracts (B: argan oil (AOSE) and cactus seed oil (COSE)) on mitochondrial transmembrane potential DIOC6(3) negative cells by flow cytometry, on BV2 microglial cell treated for 24 and 48 h. 7-Ketocholesterol (7KC) was use as positive control. Cells were treated with vehicle (DMSO or Ethanol) or different sterols in a concentration range from 12.5 to 50 μM. Values were normalized to the control and were considered statistically significance (Student *t*-test) at $P < 0.05$ (∗). The percentage of negative cells in the control is 20%.

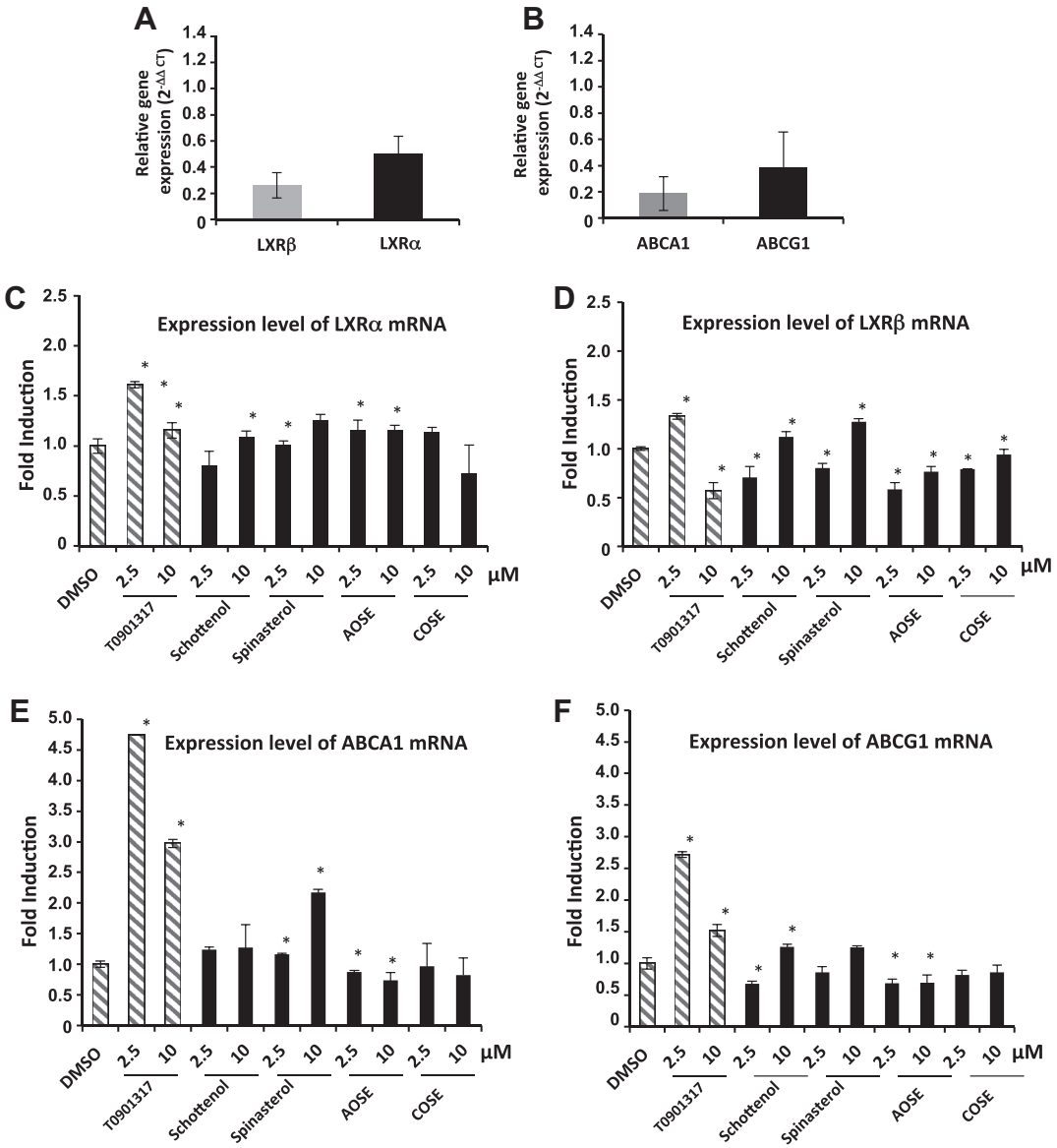

Fig. 3. Gene expression of LXRα and LXRβ and their target genes ABCA1 and ABCG1 in BV2 microglial cells: untreated (A and B) or treated 24 h with vehicle (DMSO), sterols or sterol extracts (C–F). Values of Ct and $2^{-\Delta\Delta Ct}$ in untreated cells were respectively as following: LXRα (35.18, 0.52); LXRβ (29.64, 0.45); ABCA1 (32.7, 0.18) and ABCG1 (31.08, 0.39). Values were normalized to the control and were considered statistically significance (Student *t*-test) at $P < 0.05$ (*).

3.5. Effect of PS on the mRNA expression of LXRα and LXRβ and their target genes

The BV2 cells mRNA levels of genes of interest were measured by quantitative PCR. First the basal expression of LXRα and LXRβ and their target genes ABCA1 and ABCG1 were evaluated. QPCR analysis revealed that in BV2 cells, LXRα expression is very low, while LXRβ is 10-fold higher and both ABCA1 and ABCG1 transcripts are expressed (Fig. 3A and B). The LXR synthetic benzenesulfonamide ligand T0901317 induces both LXRα and LXRβ and their target genes ABCA1 and ABCG1 in BV2 cells (Fig. 3). Induction of ABCA1 mRNA by T0901317 has been reported in mouse peritoneal macrophages [20]. However, in our hands, high concentration of the T0901317 LXR ligand exhibited an inhibitory effect mainly on LXRβ mRNA expression (Fig. 3D). This inhibition is also reflected by the lower expression of ABCA1 and ABCG1 (Fig. 3E and F). Spinasterol seems to be more efficient at 10 μM in the induction of LXRα and LXRβ and their target genes ABCA1 and ABCG1, while Schottenol treatment exhibited a lower, but significant effect in the induction of LXRα and LXRβ and their target genes. Among phytosterols compounds, the Ergosterol derivatives have been shown as ligands of LXRα and LXRβ receptors, and induced the expression of ABCA1 transporter [15]. Curiously, sterol extracts from argan oil (AOSE) reduced the LXRβ mRNA expression, which is accompanied by a significant diminution of mRNA levels of ABCA1 and ABCG1 (Fig. 3D–F). This effect of AOSE is mainly due to Schottenol and/or Spinasterol, which are the only sterols identified in AOSE (Table 1).

3.6. Evaluation of the LXRα and LXRβ activation by PS

The luciferase gene reporter under the control of LXR response element (LXRE) was used to test the activation of either LXRα or LXRβ. As reported previously [15,20,21], T0901317, 4α-OHC and 4β-OHC ligands induced the activation of LXRα and to a lesser extent (at least for T0901317 and 4α-OHC) the activation of LXRβ (Fig. 4A and B). Interestingly, Schottenol induced a significant activation of LXRα, while Spinasterol exhibited only a very slight activation. On the other hand, Spinasterol reduced specifically and significantly the activation of LXRβ. This result suggests that

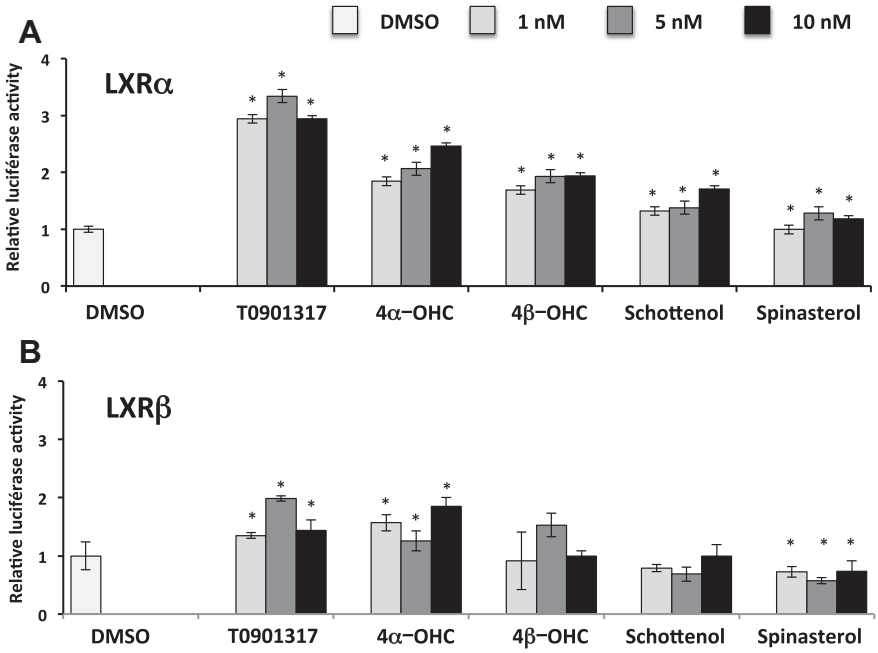

Fig. 4. Evaluation of the effect of sterols (Schottenol and Spinasterol) on the activation of LXRα and LXRβ. HEK293 cells were co-transfected with a luciferase reporter gene, under the control of the LXR response element LXRE, and the expression vectors for LXRα or LXRβ. Cells were then incubated with the vehicle (DMSO), the LXR synthetic ligand T0901317, 4αHC or 4βHC (as controls), Schottenol or Spinasterol at different concentrations (1, 5, or 10 nM). Luciferase activity of the reporter was standardized to β-galactosidase activity and expressed as fold induction relative to the vehicle. Values were normalized to the control and were considered statistically significance (Student *t*-test) at $P < 0.05$ (∗).

Spinasterol is implicated in the observed reduction of mRNA levels of LXRβ-target genes ABCA1 and ABCDG1 (Fig. 3E and F).

Collectively our results show that phytosterols from argan oil or from cactus seed oil are not toxic to microglial BV2 cells and can impact the mitochondrial membrane potential. Both Schottenol and Spinasterol can modulate the gene expression of LXRα or LXRβ and their target genes, ABCA1 and ABCG1. However, only Schottenol exhibited a differential activation *vis-à-vis* of the nuclear receptor LXRβ. The synthetic ligand T0901317 is considered as a potent agonist of LXR and, as other agonists, activates LXR nuclear receptor leading to the modulation of cholesterol metabolism [29,30]. Furthermore, such potent synthetic agonist exhibit several side effects linked to the activation of LXR-responsive genes involved in hepatic lipogenesis. This activation leads to the increase of triglyceride levels in plasma and liver tissue [30]. Accordingly, the need of selective modulators that can modulate cholesterol metabolism without lipogenic side effects is required.

Acknowledgments

This work was supported by the Action Intégrée of the Comité Mixte Inter-universitaire Franco-Marocain (CMIFM, AIMA/10/238, EGIDE) from the PHC Volubulis/Toubkal program, Ministère des Affaires Etrangères, the Conseil Régional de Bourgogne, the Ministère de l'enseignement et de la Recherche and The Projet Sectoriel CNRST.

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