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Decreased OxLDL uptake and cholesterol efflux in THP1 cells elicited by cortisol and by cortisone through 11β-hydroxysteroid dehydrogenase type 1

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ABSTRACT

Background and aims: Data about glucocorticoids role in the development of atherosclerosis are controversial showing different effects in human than in experimental animal models. Atherosclerosis is the result of a chronic inflammatory response to an injured endothelium where an uncontrolled uptake of OxLDL by macrophages triggers the development of foam cells, the main component of fatty streaks in atherosclerotic plaque. There are few data about the direct effect of glucocorticoids in macrophages of atherosclerotic plaque. The aim of the study was to elucidate the role of glucocorticoids in the development of foam cells in atherosclerosis initiation.
Methods: For this purpose we used THP1 cells differentiated to macrophages with phorbol esters and incubated with OxLDL alone or with cortisol or cortisone. THP1 cells were also incubated with cortisone plus an inhibitor of 11β-hydroxysteroid dehydrogenase 1 (11βHSD1) activity to determine the role of this enzyme on glucocorticoid action in this process.
Results: Ours results showed that cortisol and cortisone decreased significantly the inflammation promoted by OxLDL, and also diminished the expression of genes involved in influx and efflux of cholesterol resulting in a reduced lipid accumulation. Likewise cortisol and cortisone decreased 11βHSD1 expression in THP1 cells. The presence of the inhibitor of 11βHSD1 abolished all the effects elicited by cortisone.
Conclusion: Our results indicate a direct effect of glucocorticoids on macrophages braking atherosclerosis initiation, reducing pro-inflammatory markers and OxLDL uptake and cholesterol re-esterification, but also inhibiting cholesterol output. These effects appear to be mediated, at least in part, by 11βHSD1 activity.

1. Introduction

Atherosclerosis is an essential trigger for the development of cardiovascular disease (CVD), which is the main cause of mortality in both developed and developing countries alike [1]. Atherosclerosis occurs as a result of a chronic inflammatory response to an injured vessel wall [2]. One of the main causes of this vessel wall damage is the accumulation of oxidized lipids in low density lipoproteins (OxLDL). As a consequence of lesion, monocytes are recruited to the endothelium, where they polarize to a pro-inflammatory macrophage profile. Then, uncontrolled macrophage uptake of OxLDL leads to the subsequent formation of foam cells, the main components of fatty streaks in atherosclerotic plaque [3].

Abbreviations: EMR1, epidermal growth factor like module-containing mucin-like hormone receptor-like1; CD163, Cluster of Differentiation 163; MMR, macrophage mannose receptor; IL-12b, interleukin-12b; IL-6, interleukin-6; TNFα, tumor necrosis factor α; rIL-10, interleukin-10 receptor; PLA2, phospholipase A2; FAT/CD36, fatty acid translocase; SRA1, Scavenger receptor class A type1; ACAT, acyl-CoA:cholesterol acyltransferase; NCEH1, neutral cholesterol ester hydrolase1; LXRα, liver X receptor α; ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; APO E, apoprotein E; 11βHSD1, 11β-hydroxysteroid dehydrogenase type 1; 11βHSD2, 11β-hydroxysteroid dehydrogenase type 2; G6PT1, glucose-6-phosphate translocase 1; H6PDH, hexose-6-phosphate dehydrogenase; RPL4, ribosomal protein L4.

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Thus, macrophages play a pivotal role in the development and progression of atherosclerosis, and the knowledge of the factors that regulate their metabolism is crucial to control the process. A key step in the formation of foam cell macrophages is the internalization of OxLDL through specific scavenger receptors (mainly SRA1 and FAT/CD36), but the degree of lipid accumulation is also dependent of cholesterol esterification (regulated by ACAT and NCEH) and cholesterol efflux that involves the activity of ATP-binding cassette transporters (ABCA1, ABCG1), scavenger receptor class B (SR-BI) and high density lipoproteins (HDL). Consequently, foam cell formation is the result of a disrupted balance between cholesterol influx, esterification and efflux in macrophages, which occurs when macrophages fail to restore their cellular cholesterol homeostasis via regulation of reverse cholesterol transport [4].

Stress and visceral obesity are main risk factors for CVD [5,6]. Stressful situations are known to activate the hypothalamic-pituitary-adrenal axis (HPA), inducing increased levels of circulating glucocorticoids; however, the role of glucocorticoids in the development of atherosclerosis remains controversial [7,8]. Whereas circulating glucocorticoids correlate positively with CVD in humans [9,10], animal studies (e.g. on rabbits and dogs) suggest an atheroprotective role of both natural and synthetic glucocorticoids [8,11]. An excess of glucocorticoids, as in Cushing's syndrome or with the pharmacological therapy common in autoimmune diseases, is associated with an increase in atherosclerotic and cardiovascular events [12,13]. Normalization of cortisol levels in patients with Cushing's syndrome largely reverses pathophysiological changes in vascular function and structure [14]. However, most atherosclerosis occurs independently of exogenous glucocorticoid administration, and plasma cortisol levels are not normally elevated in atherosclerosis.

Glucocorticoid availability in tissues could differ from circulating levels due to 11β-hydroxysteroid dehydrogenase type 1 and 2 (11βHSD1 and 11βHSD2) activity. In vivo, the 11βHSD1 enzyme predominantly converts inert glucocorticoids (cortisone in humans, 11-dehydrocorticosterone in rodents) into the corresponding active forms (cortisol in humans, corticosterone in rodents). 11βHSD1 is widely expressed, mainly in liver but also at more modest levels in classical glucocorticoid target cells and tissues, e.g. adipose tissue and immune cells [15]. In 11βHSD1 knockout mice, the absence of 11βHSD1 is atheroprotective [16]. Expression of the 11βHSD2 enzyme, which catalyzes the opposite reaction, is restricted to mineralocorticoid target cells or tissues, mainly in kidney but also in skin, lung and adrenal cortex [17]. 11βHSD2 deficient mice show increased atherosclerotic plaque development, probably due to increased activation of mineralocorticoid receptors by glucocorticoids [18].

Few studies have addressed the direct role of glucocorticoids and 11βHSD1 in the control of cholesterol homeostasis on macrophages throughout the atherogenic process. To elucidate it, we studied the changes caused by cortisol and cortisone in THP1 macrophages incubated in the presence of OxLDL (a potent atherogenic stimulus) on internalization of OxLDL, lipid accumulation and cholesterol efflux. In addition, THP1 macrophages were also incubated with an inhibitor of 11βHSD1 activity to unravel its mediating function of glucocorticoid actions in the macrophages involved in the initiation of atherogenic process.

2. Materials and methods

2.1. Isolation of LDL, preparation of oxidized LDL (OxLDL) and acetylated LDL (AcLDL)

The LDL fraction was isolated from plasma obtained from clinically healthy human volunteers after a 12 h fast. The isolation method employed is described elsewhere [19], with some modifications. The density of the isolated plasma was adjusted to 1.21 g/mL with NaBr in the presence of EDTA 1%. Throughout purification, the resulting plasma was kept on ice and protected from light. After ultracentrifugation at 55,000 rpm for 36 h, the lipoproteins were separated by gel permeation on a 1 mL Sephacryl S-300 column. The LDL subfraction was dialyzed against TRIS/HCl buffer (10 mM pH 7.40, containing 1 mM EDTA) overnight, and adjusted to a final protein concentration of 3 mg/mL. The pooled preparations were aliquoted after nitrogen bubbling into cryovials at −70 °C until use (no more than one month) [20].

To obtain OxLDL, 15 mL of human isolated LDL (3 mg protein/mL) were treated in vitro with 0.5 mL copper sulfate at a final concentration of 5 μM at 37 °C under gentle agitation for 8 h in order to produce a medium peroxidation degree of the LDL lipids (about 45 nmol malondialdehyde/mg protein). To stop the peroxidation process, each preparation was treated with a solution of butylhydroxytoluene (BHT; 2,6-di-tbutyl-p-cresol) in PBS at a final concentration of 0.1 mM [21] and immediately subjected to dialysis against PBS (50 mM, pH 7.40, changed every 8 h) for 24 h to eliminate BHT and Cu ions. Cu elimination was tested by atomic absorption spectrometry as described elsewhere [22]. In brief, the samples were diluted with ultrapure water (18 mΩ cm, Carlo Erba) and ultrafiltered using a 0.22 μm Millipore membrane (Milli-Q Purification System, Millipore). Ultrafiltered dissolutions were directly aspirated into the flame 1100 B Spectrophotometer equipped with a cathode lamp (Perkin-Elmer) at a spectral width of 1 nm. Calibrations were performed with a standard solution of $Cu(NO_3)_2$ in HNO_3 0.5 N (Tritrisol from Merck Co.) and 18 Ω cm water ultrafiltered through a Millipore membrane. All measurements were performed in peak height mode (324.7 nm line). The intra- [(SD/ξ).100] and inter- [ΔSD/Δξ).100] assay coefficients of variations were 15.5 and 6.0%, respectively. We routinely obtained a similar equation for the calibration curve (IR = 0.00055 + 0.04788 [Cu, mg/L]) and statistical analyses routinely demonstrated a correlation coefficient of between 0.956 and 0.991.

The LDL were acetylated by the Fraenkel-Conrat method [23]. Firstly, 5 mL of human isolated LDL (3 mg protein/mL) were added to a 5 mL of a saturated solution of sodium acetate with continuous stirring in an ice water bath. Then acetic anhydride was added in multiple small aliquots (2 μL) over 1 h with continuous stirring. After the addition of a total mass of acetic anhydride equal to 1.5 times the mass of protein used, the mixture was stirred for an additional 30 min without further additions. Then the reaction solution was dialyzed for 24 h at 4 °C against a buffer containing 150 mM NaCl and 0.3 mM EDTA, pH 7.4. Preparations of OxLDL and AcLDL were then sterilized by ultrafiltration under vacuum using a 0.22 μm membranes (milli-Q purification System, Millipore), fractioned into small aliquots, stored under nitrogen atmosphere at 4 °C (for no more than 2 weeks), and used for the experimental protocols.

2.2. Cell culture and treatment

About 1×10^6 human monocytes (THP1) were seeded on 6-well plates and incubated for 1 day with 2 mL of RPMI medium supplemented with penicillin/streptomycin (100 units/mL) and 10% FBS at 37 °C in a 5% CO_2 atmosphere. Phorbol esters at 200 nM (PMA) were added to the medium for 24 h to transform monocytes into macrophage type cells.

THP1 macrophages were seeded and grown for 24 h in disposable culture dishes (Falcon) in a humidified atmosphere with PMA. Then, the medium was collected and remaining cells were used for the different experiments. The experiments conducted in presence of OxLDL or AcLDL were carried out at a final concentration in the medium of 100 μg protein/mL in a total volume for well of 2 mL.

Cells were treated for 24 h with OxLDL and increasing concentrations of cortisol, cortisone or cortisone plus 100 μM of BTV.2733 (cat. nº 1756, Axon Medchem) to inhibit 11βHSD1 activity [24]. Cortisol and corticosterone concentrations ranging from physiological to pharmacological were used (from 0.1 to 1000 nM). In addition, a set of cells were treated for 24 h with AcLDL or AcLDL plus 1000 nM of cortisol. The glucocorticoids were dissolved in dimethylsulfoxide (DMSO) and immediately diluted in the culture medium (RPMI). The final concentration of DMSO was 0.1% in the culture medium, and control culture dishes were also supplemented with an equivalent aliquot of DMSO. After the corresponding treatment, the medium was collected and frozen at −80 °C. The cell monolayers were washed three times with cold sterile PBS (5 mL), and Trizol (Invitrogen) reagent was added for RNA extraction.

2.3. Trypan blue dye (TBD) assay test

Cell viability was assessed for each experimental condition. Attached cells were washed with PBS and incubated with 100 μL of a 0.1% solution of trypan blue dye (in PBS, pH 7.4). The cells were examined under optical microscopy to determine viability according to the method previously described [25]. At least four fields of one hundred cells per field were counted and the results were expressed as the percentage of non-viable cells.

2.4. Quantitative real-time PCR

Total RNA (1 μg) was used to generate cDNA using the iScript cDNA synthesis kit (Bio-Rad). Real-time PCR (RT-PCR) amplification was carried out using 10 μL of amplification mixture containing SYBR Green PCR Master Mix (Life Technologies), 10 ng of cDNA and 300 nM of primers. The primers used are shown in Table 2 (supplemental material). Reactions were run on an ABI PRISM 7900 HT detection system (Applied Biosystems) using an annealing temperature of 60 °C. To determine amplification sensitivity and efficiency, PCR linear ranges were previously established for each gene pair of primers and cells cDNA. To evaluate inflammation related to the atherogenic process, we determined the expression of epidermal growth factor like module-containing mucin-like hormone receptor-like 1 (EMR1), Cluster of Differentiation 163 (CD163), interleukin-12b (IL-12b), macrophage mannose receptor (MMR), interleukine-6 (IL-6), tumor necrosis factor α (TNFα), interleukin-10 receptor (rIL-10) and phospholipase A2 (PLA2). For the study of genes involved in cholesterol influx and efflux from macrophages we analyzed fatty acid translocase (FAT/CD36), scavenger receptor class A type 1 (SRA1), liver X receptor α (LXRα), ATP-binding cassette transporters A1 and G1 (ABCA1, ABCG1) and apoprotein E (APO E). To evaluate the cholesterol esterification and hydrolysis of cholesterol esters we determined the expression of acyl-CoA:cholesterol acyltransferase ACAT) and neutral cholesterol ester hydrolase 1 (NCEH1) respectively. To investigate the availability of glucocorticoid in cells, we determined the expression of 11β-hydroxysteroid dehydrogenase type 1 and type 2 (11βHSD1, 11βHSD2), glucose-6-phosphate translocase 1 (G6PT1) and hexose-6-phosphate dehydrogenase (H6PDH). As a housekeeping gene, the expression of ribosomal protein L4 (RPL4) was measured. Ct values were obtained using an automatically calculated threshold and the relative expression of target genes to RPL4 was calculated using the ΔC(t) formula. Data were expressed as the % with respect to OxLDL-THP1 cells without hormone.

2.5. Oil-red staining

Cells were fixed to glass plates by covering them with 10% formaldehyde in PBS overnight at room temperature. Oil red O working solution was prepared from a stock solution (0.5 g Oil Red O in 100 mL isopropanol, Sigma HT50-1-640) by adding 6 mL of stock to 4 mL of bidistilled water. After mixing, it was filtered through Whatman 1 filter paper. Subequently removing fixing buffer carefully, cells were covered with fresh Oil Red O working solution at least one hour at room temperature. Then cells were rinsed several times, carefully with bidistilled water and were allowed to air dry [26]. The macrophages were visualized under optical microscope (Olympus BX-51), and image stacks were captured (Olympus DP-70). Lipid droplets areas were analyzed using the Fiji ImageJ [27].

2.6. TNFα quantification

Determination of TNFα in the cells medium was performed by Human TNF ELISA set (BD Biosciences) following supplier instructions.

2.7. Statistical analysis

The data were analyzed using the GraphPad software program version 5.0 and were expressed as the mean ± SEM. Statistical comparisons for different concentrations of cortisol, cortisone or cortisone plus BT2733 were made by one-way ANOVA and Bonferroni post Test. The Student's *t*-test was used to compare Control with OxLDL and discrete concentration points of cortisol and cortisone or cortisone and cortisone plus BTV.2733. P values <0.05 were considered.

3. Results

The viability of cultured cells treated with OxLDL in all conditions was expressed as percentage of dead cells (Fig. 1) and did not exceed 7–8% of total cells, confirming that our results were not affected by the addition of DMSO, hormones or the inhibitor BTV.2733.

Gene expression of macrophage and inflammation mediator markers as a response to incubation of THP1 cells with OxLDL and cortisol, cortisone and cortisone plus BTV.2733 is given in Table 1. The presence of OxLDL increased the expression of macrophage marker EMR1 and of pro-inflammatory interleukins IL-6, IL-12b

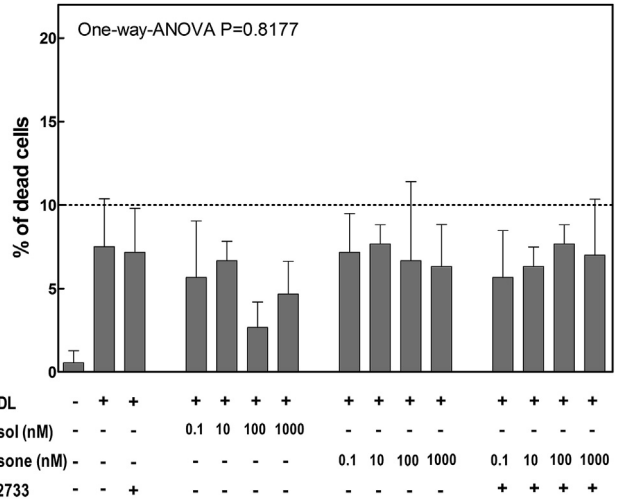

Fig. 1. Viability of cultured THP1 macrophages for all conditions tested expressed as dead cells versus total cells (%). The One way ANOVA significance is shown in the graphic. Bonferroni post Test analysis was not significant in any case.

Table 1
Expression of inflammatory markers in THP1 macrophages treated with OxLDL and glucocorticoids during 24 h.

	Control	With OxLDL	With OxLDL										One way ANOVA		
			Cortisol (%OxLDL)			Cortisone (%OxLDL)			Cortisone plus BVT.2733 (%OxLDL)						
			0.1 nM	10 nM	100 nM	1000 nM	0.1 nM	10 nM	100 nM	1000 nM	0.1 nM	10 nM	100 nM	1000 nM	Cortisol	Cortisone	Cortisone plus BVT.2733
EMR1	2.3 ± 0.7*	100 ± 15	100 ± 8	45.5 ± 6.7^{+}	44.5 ± 5.9^{+}	18.4 ± 0.7	94.3 ± 7.6	98.9 ± 11.7	88.9 ± 11.9●	30.3 ± 7.5●	95.2 ± 16.2	99.8 ± 15.2	137 ± 15	124 ± 15	<0.0001	0.0004	0.3885
TNFα	5.9 ± 1.6*	100 ± 12	112 ± 9	47.2 ± 5.8^{+}	25.8 ± 5.8^{+}	5.9 ± 0.9	104 ± 13	108 ± 10	72.2 ± 16.8	7.6 ± 1.3●	104 ± 11	54.3 ± 16.5	71.1 ± 1.3	63.9 ± 9.3	<0.0001	<0.0001	0.2751
IL-12b	34.6 ± 3.0*	100 ± 10	97.8 ± 10.2	110 ± 17	62.4 ± 12.4	30.1 ± 6.7^{+}	129 ± 13	132 ± 14	133 ± 4	76.7 ± 4.7	139 ± 8	111 ± 6	134 ± 55	77.0 ± 7.3	0.0011	0.6331	0.5973
IL-6	0.9 ± 0.0*	100 ± 2	67.1 ± 8.3	20.2 ± 2.8^{+}	23.8 ± 5.7^{+}	7.0 ± 0.7^{+}	65.6 ± 5.9	89.7 ± 8.3	60.2 ± 10.7	19.3 ± 3.6●	47.2 ± 10.4	86.1 ± 12.8	91.1 ± 13.5	61.1 ± 15.9	<0.0001	<0.0001	0.0525
MMR	4080 ± 88*	100 ± 20	74.0 ± 27.9	96.8 ± 18.6	490 ± 65^{+}	1605 ± 138^{+}	206 ± 97	191 ± 65	155 ± 59	88.6 ± 46.6	203 ± 95	65.7 ± 21.4	105 ± 44	108 ± 22	<0.0001	0.7556	0.3760
CD163	39.8 ± 4.2*	100 ± 12	78.1 ± 1.1	66.7 ± 7.2^{+}	310 ± 2^{+}	269 ± 17	78.3 ± 9.4●	125 ± 6.8●	159 ± 6●	323 ± 84●	52.0 ± 2.5	80.4 ± 3.5	109 ± 13.1	157 ± 4	<0.0001	<0.0001	<0.0001
rIL-10	10.0 ± 1.8*	100 ± 6	75.4 ± 1.6^{+}	52.4 ± 6.1^{+}	76.9 ± 10.3	32.8 ± 1.3	96.7 ± 5.6●	90.6 ± 6.8	81.5 ± 11.0	22.5 ± 5.9●	142 ± 14	95.7 ± 26.4	112 ± 26	127 ± 18	<0.0001	<0.0001	0.5277

The data are the mean ± sem. Statistical significance performed by One Way Anova is shown in the last columns of the table. Student's *t*-test is denoted by symbols: *P < 0.05 control vs OxLDL; +P < 0.05 cortisol vs cortisone; ● P < 0.05 cortisone vs cortisone plus BVT.2733.

and TNFα. In addition, OxLDL strongly decreased the expression of anti-inflammatory marker MMR but increased the expression of rIL-10 and CD163 also related to M2 macrophage profile. Cortisol markedly decreased the expression of EMR1 and pro-inflammatory cytokines such as IL-6, IL12b and TNFα when added together with OxLDL. Consistently with this, cortisol promoted an increase in the expression of MMR and CD163, markers for the alternative pathway (M2) of macrophage differentiation. We observed a significant dose-response effect for cortisol in all cases. Cortisol also diminished the expression rIL-10 but to a lesser extent than for the pro-inflammatory markers mentioned above. At the highest concentration tested, cortisone completely mimicked the effects of cortisol on the expression of EMR1, TNFα, CD163 and rIL-10, but only partially for IL-6. In the case of MMR, and IL12b cortisone had no effect on its expression. The presence of BTV.2733 blocked the effect of cortisone in all cases. In addition, levels of TNFα were measured in culture medium of cells treated with OxLDL or AcLDL and a similar increase was found for both modified LDL, of about 100 fold compared to the control without modified LDL. Cortisol decreased TNFα production in the medium in a 42% and 66% respectively.

To check if PLA2 was involved in the anti-inflammatory effect of cortisol we determined its expression. The OxLDL increased PLA2 expression 7 fold that found in unstimulated THP1 macrophages (14.2 ± 1.3% in control and 100.0 ± 7.6% in OxLDL treated). Cortisol strongly decreased PLA2 expression only at 1000 nM to a 12.5 ± 0.8% and cortisone showed no effect.

Fig. 2 shows the expression of genes coding for proteins involved in macrophage modified LDL uptake (FAT/CD36 and SRA1), esterification of cholesterol (ACAT) and hydrolysis of cholesterol esters (NCEH1). The expression of FAT/CD36, SRA1 and ACAT, was strongly increased by OxLDL. The expression of NCEH1 was also significantly increased by OxLDL to a lesser extent. Cortisol reduced the expression of FAT/CD36, SRA1 and ACAT in a dose-dependent manner, but had no effect on NCEH1. Cortisone at the highest dose exerted the same effect that cortisol, blunted by the addition of the 11βHSD1 inhibitor. The expression of genes that encode for the key transporters involved on efflux of cholesterol (ABCA, ABCG1), as well as LXRα, that up regulates their expression, and ApoE is shown in Fig. 3. ABCA1, ABCG1, LXRα and ApoE expression were increased by OxLDL effect. Cortisol reduced the expression of all genes studied in a dose-dependent manner. In all cases, the effect of cortisol at the highest dose tested was to reduce the expression of these genes to below 50% compared to cells incubated with OxLDL. The weakest effect exerted by cortisol was on ApoE, and the strongest effect was on LXR, with a decrease in expression to 57% and 81%, respectively. Cortisone only mimicked the effect of cortisol at the highest dose, and the presence of BVT2733 abolished this effect. In summary, it is observed a decreased expression of genes involved in OxLDL uptake and cholesterol esterification, but also of those involved in cholesterol efflux without effect in the expression of the main enzyme involved in hydrolysis of cholesterol esters.

Intracellular lipids droplets, measured by oil-red staining (Fig. 4A and B), were increased 3.3 fold by effect of OxLDL, whereas when cortisol and cortisone were also present the increase was lower, about 1.7 and 2.4 fold respectively. Thus, the presence of glucocorticoids caused a significant decrease of cell cholesterol accumulation induced by OxLDL. The presence of BTV.2733 blocked the effect of cortisone. The effect of AcLDL on the charge of lipids was also evaluated (Fig. 4C). In our hands, AcLDL promoted an increase of 3.1 fold compared to that found in control macrophages without AcLDL. Cortisol reduce the lipids charge but to a lesser extents that found under OxLDL incubation.

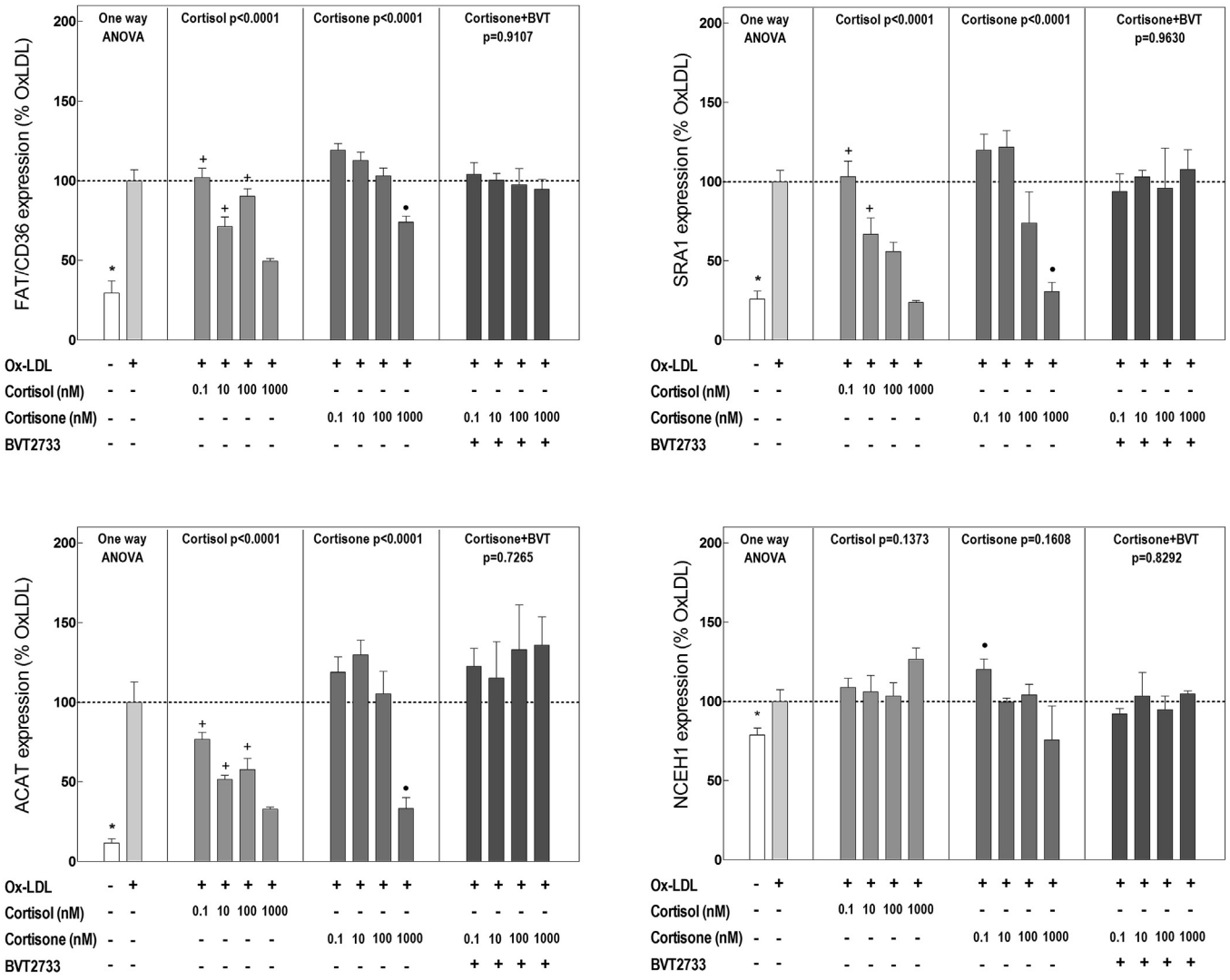

Fig. 2. Expression of genes involved on influx of OxLDL, esterification of cholesterol and hydrolysis of cholesterol esters in THP1 macrophages incubated with OxLDL or with OxLDL plus cortisol or cortisone or cortisone with BVT.2733. Data are expressed as percentage of the expression found in cells incubated only with OxLDL. Fatty acid translocase (FAT/CD36), Scavenger receptor class A type 1 (SRA1), acyl-CoA:cholesterol acyltransferase (ACAT) and neutral cholesterol ester hydrolase 1 (NCEH1). One way Anova significancy for each group of hormone treatment is shown in the graph. Student's *t*-test for discrete points was used. Symbols denote significativity between: * without OxLDL vs with OxLDL; + Cortisol vs Cortisone; ● Cortisone vs Cortisone with BVT.2733. P values <0.05 were considered significant.

The expression of 11βHSD1 and 11βHSD2 is shown in Fig. 5. Interestingly, the expression of both was increased by OxLDL. Cortisol blocked the increased expression of 11βHSD1 elicited by OxLDL in a dose-dependent manner. The expression of 11βHSD2 was unaffected by cortisol at the lowest dose, but was inhibited at a concentration of 10 nM; in contrast, 100 and 1000 nM of cortisol promoted an increase in this expression proportional to the hormone concentration. In addition, the expression of G6PT1 and H6PDH, which are important to supply reduced substrate (NADH + H) for the 11βHSD1 reaction, was decreased by cortisol. The effect of cortisone on 11βHSD1 expression was similar to that of cortisol but weaker, except at 0.1 nM, when it was the same. Cortisone had no effect on the expression of 11βHSD2 with respect to cells incubated with OxLDL alone. Cortisone increased the expression of G6PT1 at 0.1 nM with respect to the same concentration of cortisol or with OxLDL alone; however, increasing doses of cortisone induced inhibition of G6PT1 expression. Cortisone had the same effect as cortisol on the expression of H6PDH.

4. Discussion

The aim of this study was to elucidate the direct effect of glucocorticoids on the macrophages in the atherogenic process. For this purpose we used THP1 macrophages incubated with OxLDL to promote the accumulation of lipids. Our results highlight that cortisol, and to a lesser extent cortisone, arrested the progression of inflammation in a dose-dependent manner, assessed by the reduction in EMR1, TNFα, IL-12b and IL-6 whereas the anti-inflammatory macrophage profile M2 markers as MMR and CD163 [28–30] were increased by cortisol, but only at high doses. It is well established that glucocorticoids exert an anti-inflammatory effect [31] and activate the alternative pathway of macrophage differentiation. Glucocorticoids and IL-10 activate the alternative differentiation to specific M2 macrophages which express several scavenger receptors such as SRA1 and CD163. The increased expression of SRA1 and CD163 contributes to M2 macrophage functions such as apoptotic cell clearance [32]. These subset of specialized M2 macrophages are the cells involved in foam cell

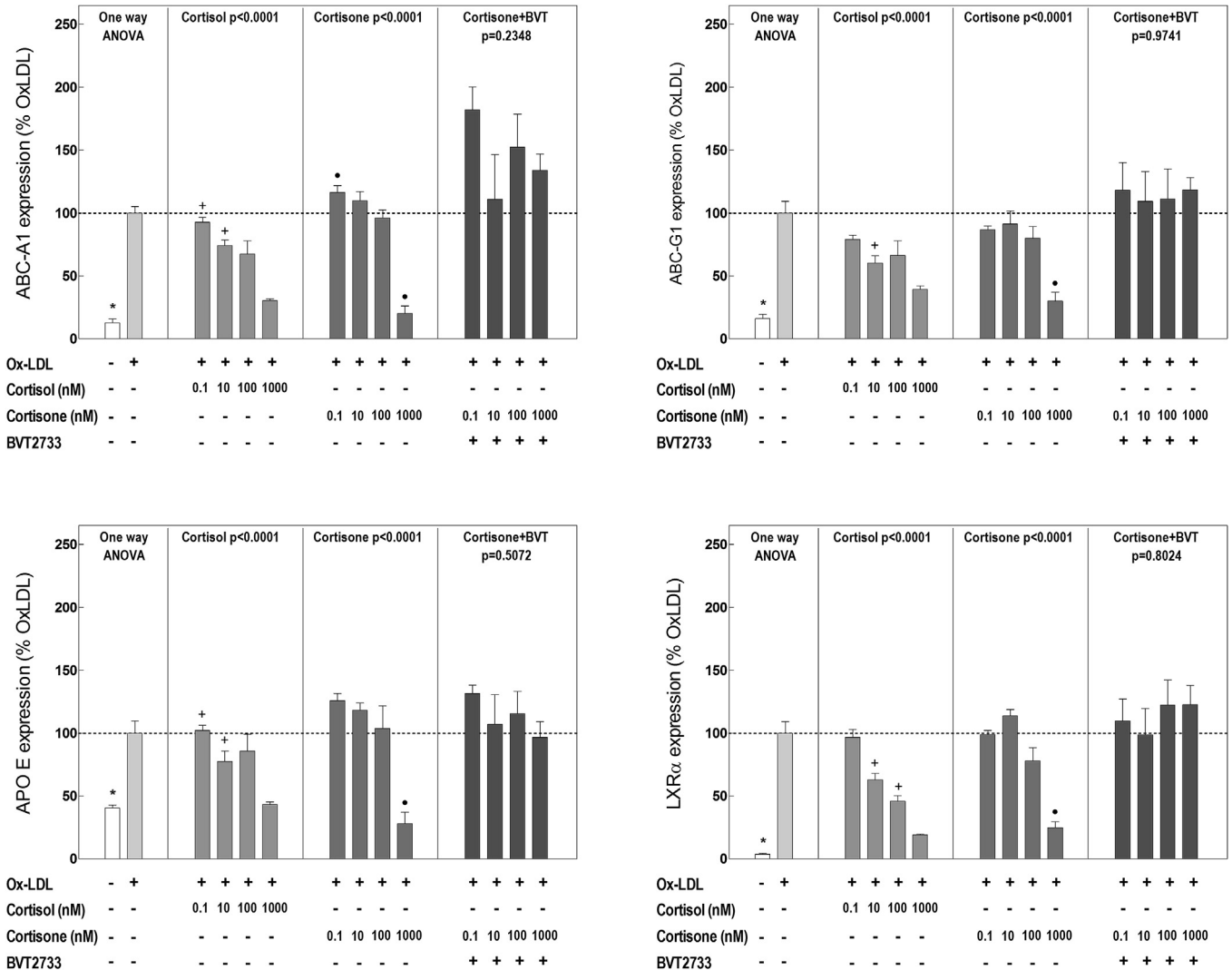

Fig. 3. Expression of genes involved on efflux of cholesterol in THP1 macrophages incubated with OxLDL or with OxLDL plus cortisol or cortisone or cortisone with BVT.2733. Data are expressed as percentage of the expression found in cells incubated only with OxLDL. ATP-binding cassette transporters A1 and G1 (ABC-A1, ABC-G1), apoprotein E (APO E) and liver X receptor a (LXRa). One way Anova significancy for each group of hormone treatment is shown in the graph. Student's *t*-test for discrete points was used. Symbols denote significativity between: * without OxLDL vs with OxLDL; + Cortisol vs Cortisone; ● Cortisone vs Cortisone with BVT.2733. P values <0.05 were considered significant.

formation as a pathological consequence of a normal anti-inflammatory response [32].

In the present study, OxLDL promoted the uptake of cholesterol and its esterification in THP1 macrophages through increased gene expression of CD36 and SRA1, the main pathways of cholesterol uptake in macrophages [33], and of ACAT, which re-esterifies cholesterol inside macrophages [34]. Cortisol inhibited the expression of CD36, SRA1 and ACAT, thus limiting the influx of cholesterol. ATP-binding cassette transporters, specifically ABCA1 and ABCG1, are responsible for most cholesterol efflux from macrophages [35] and to a lesser extent scavenger receptor-BI [36]. In addition, increased apoE secretion could be linked to an augmented cholesterol efflux through ABCA1/ABCG1 in macrophages [37]. Our data show that OxLDL increased gene expression of ABCA1, ABCG1, apoE and LXRα, the transcription factor that regulate its expression [38]. Nevertheless, the presence of the active glucocorticoid cortisol in the culture medium inhibited the expression of genes involved in cholesterol efflux, probably through a reduction in LXRα expression. Cortisone had a more attenuated effect compared with cortisol, which was abolished by the presence of the inhibitor of 11βHSD1 (BVT.2733) due the impossibility of activating cortisone to cortisol. A recent study [39] showed that cortisol decreased cholesterol efflux in THP1 macrophages, in agreement with our results, but the authors also reported a higher influx. Our results indicate that glucocorticoids have the capacity to inhibit the expression of genes related to the influx and the efflux of cholesterol in macrophages, resulting in a reduction of their net lipid content.

The mechanism of action of cortisol is probably multi-factorial, because glucocorticoids can act through multiple systems. First, the reduced efflux could be partially consequence of a minor lipid content inside the cell due to reduced influx, in addition of a direct glucocorticoid action on gene expression supported by the finding of negative glucocorticoid response elements in the promoter region of some of the genes involved, such as ABCA1 that in liver is repressed by glucocorticoid through a direct action [40]. Second, it is known that glucocorticoids mediate their anti-inflammatory action, clearly observed in our data, through transrepression of the NF-kβ transcription factor, which also has a role in foam cell development [41]. It has been described that macrophages from

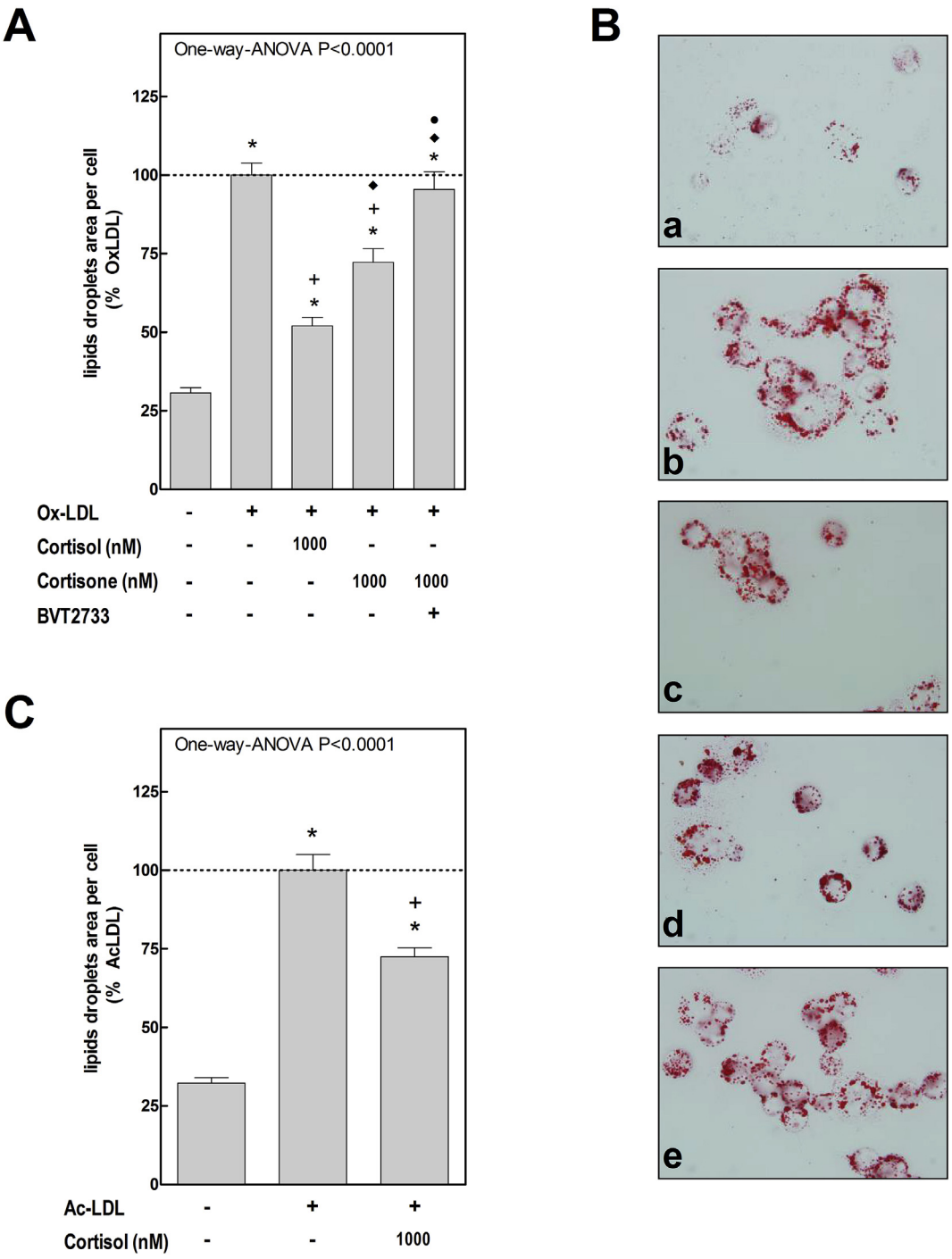

Fig. 4. Intracellular lipids droplets, measured by oil-red staining of THP1 macrophages. A) Lipid droplets deposition in THP1 macrophages incubated without OxLDL, with OxLDL and with OxLDL plus cortisol or cortisone or cortisone and BTV.2733. B) Representative pictures of THP1 macrophages stained with oil-red at 100X. a) THP1 cultured without OxLDL, b) THP1 cultured with OxLDL, c) THP1 cultured with OxLDL plus cortisol, d) THP1 cultured with OxLDL plus cortisone and e) THP1 cultured with OxLDL plus cortisone and BVT.2733. C) Lipid droplets deposition in THP1 macrophages incubated without AcLDL, with AcLDL and with AcLDL plus cortisol. In all cases cortisol and cortisone were used at 1000 nM. One way Anova significancy and Bonferroni post Test analysis are included in the graph: *$p < 0.05$ vs without OxLDL or AcLDL; + $p < 0.05$ vs with OxLDL or AcLDL; ◆ $p < 0.05$ vs OxLDL plus cortisol and ● $p < 0.05$ vs with OxLDL plus cortisona.

p50-KO mice (without transcriptional activity of NF-kβ) show, in agreement with our data, reduced SRA1 expression [42]. In addition, ACAT can be upregulated through activation of NF-kβ [42]. In consequence, NF-kβ repression would result in a reduced ACAT expression. However, data on NF-kβ role on foam cell development is conflicting. Mice overexpressing p65 (with increased transcriptional activity of NF-kβ) show reduced expression of FAT/CD36 and a limited foam cell development [43]. A third option would be that cortisol effects occur through PPRAγ. PPAR inhibition would result in the decrease of FAT/CD36, LXRα, ABCA1 and ABCG1 [44]. Finally, ACAT is upregulated also through MAPK which in turn is inhibited by glucocorticoids [41,44].

The concentration of active glucocorticoids, i.e. cortisol or corticosterone, in tissue depends on 11βHSD1 and 11βHSD2

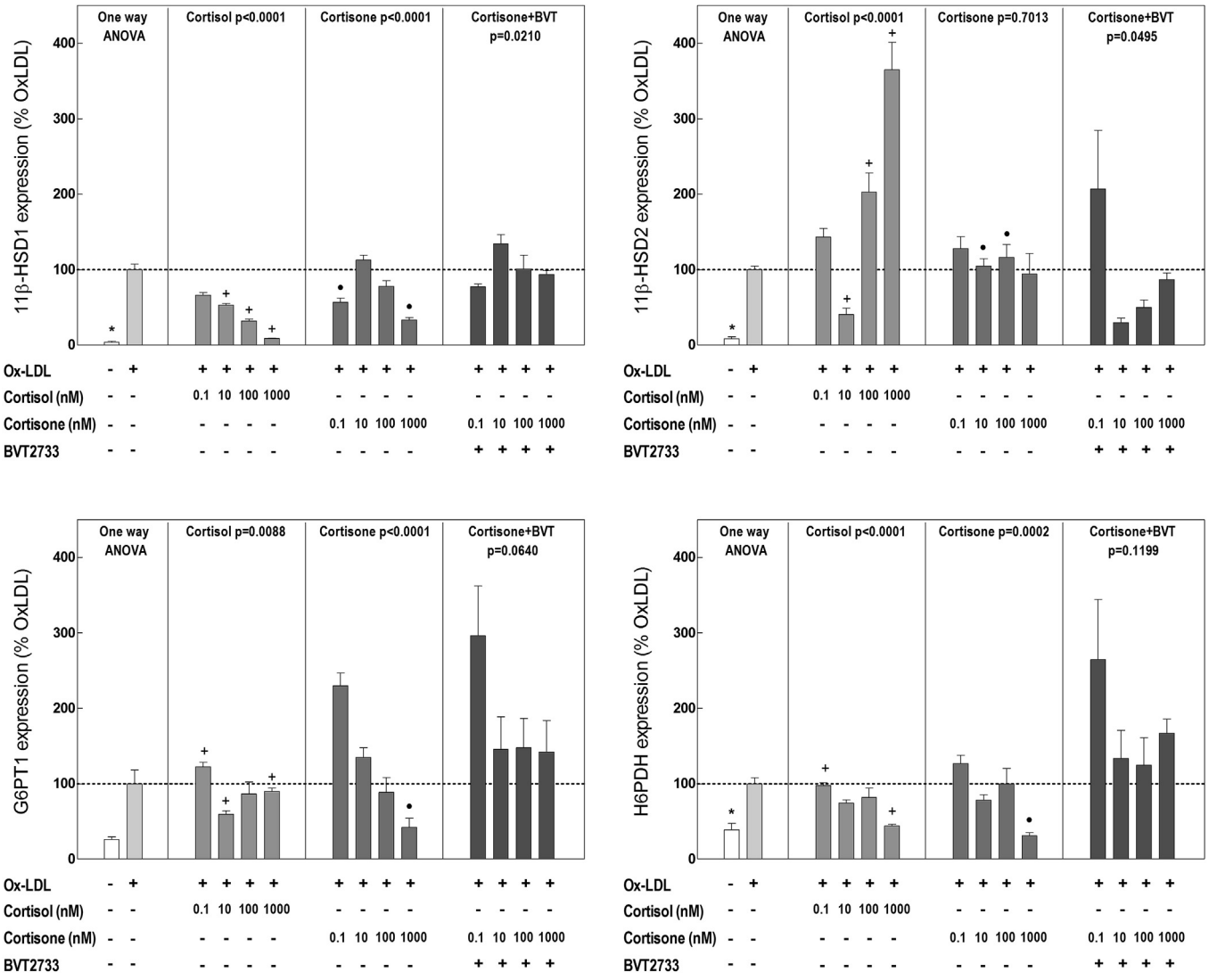

Fig. 5. Expression of genes involved in glucocorticoid availability in THP1 macrophages incubated with OxLDL or with OxLDL plus cortisol or cortisone or cortisone with BVT.2733: 11β-hydroxysteroid dehydrogenase type 1 and type 2 (11βHSD1, 11βHSD2), glucose-6-phosphate translocase 1 (G6T1), hexose-6-phosphate dehydrogenase (H6PDH). Data are expressed as percentage of expression in cells incubated with OxLDL. One way Anova significancy for each group of hormone treatment is shown in the graph. Student's *t*-test for discrete points was used. Symbols denote significativity between: * without OxLDL vs with OxLDL; + Cortisol vs Cortisone; ● Cortisone vs Cortisone with BVT.2733. P values <0.05 were considered significant.

activity, and it has been reported that 11βHSD1 deficiency/inhibition in the organism is atheroprotective whereas 11βHSD2 deficiency/inhibition accelerates atherosclerosis [45]. However, 11βHSD1 and 11βHSD2 expression in macrophages, and their contribution to the development of foam cells and progression of atherosclerosis, remain unclear. The expression of 11βHSD1 in macrophages is dependent on their activation state [46]. Thus, M1 polarization of native macrophages by lipopolysaccharides induces 11βHSD1 expression; in contrast, polarization to M2 by IL4/IL13 has little effect on 11βHSD1 expression. However, if monocytes are differentiated to macrophages in presence of IL4/IL13, the activity of 11βHSD1 is even higher than in M1 macrophages [47]. Here, we found an increase in both isozyme expression elicited by OxLDL but to a greater extent in 11βHSD1. This increase in 11βHSD1 is concordant with the observed elevated expression of TNFα, which is a potent inducer of 11βHSD1 expression [48,49]. On the other hand, the expression of 11βHSD2 in immune cells has only been described in some human diseases, for example synovial macrophage expression [50] and transient expression in peripheral blood mononuclear cells [51] of patients with arthritis rheumatoid. The synovial macrophages in rheumatoid arthritis could reflect an adaptation to enable an altered proliferation and differentiation in a chronically inflamed environment [52] as in the formation of atherosclerotic plaque.

Our results show that cortisol has an inhibitory effect on 11βHSD1 expression and a dual effect on 11βHSD2. Low concentrations did not affect or inhibited 11βHSD2 expression, but the highest cortisol concentration strongly induced its expression. This profile is coherent with a protective response to excessive cortisol levels; thus, when the concentration of cortisol increases, cells tend to reduce its levels through repressing 11βHSD1 and increasing 11βHSD2. Interestingly, cortisone had a different effect. It did not affect 11βHSD2 expression, and the inhibition of 11βHSD1 expression was more attenuated, since cortisone must be activated to cortisol by 11βHSD1 activity. It could be expected cortisone inhibiting 11βHSD2 expression due to an excess of product, but we did not find any effect.

In conclusion, our results indicate a direct effect of

glucocorticoids on macrophages in atherosclerosis progression limiting the accumulation of lipids. Thus, in presence of OxLDL, cortisol reduce the expression of genes involved in OxLDL uptake and cholesterol re-esterification, but also inhibited those involved in cholesterol output perhaps as a consequence of a minor influx (Fig. 6). In addition, cortisone can mimic all the effects of cortisol and the inhibition of 11βHSD1 activity blocked the cortisone action in macrophages. All the facts would indicate a key role of 11βHSD1 modulating the availability and local action of glucocorticoids in atherogenic progression. Our observations point to a direct anti-atherogenic role of glucocorticoids on THP1 macrophages. The molecular mechanisms mediating those effects on cholesterol homeostasis deserve further research.

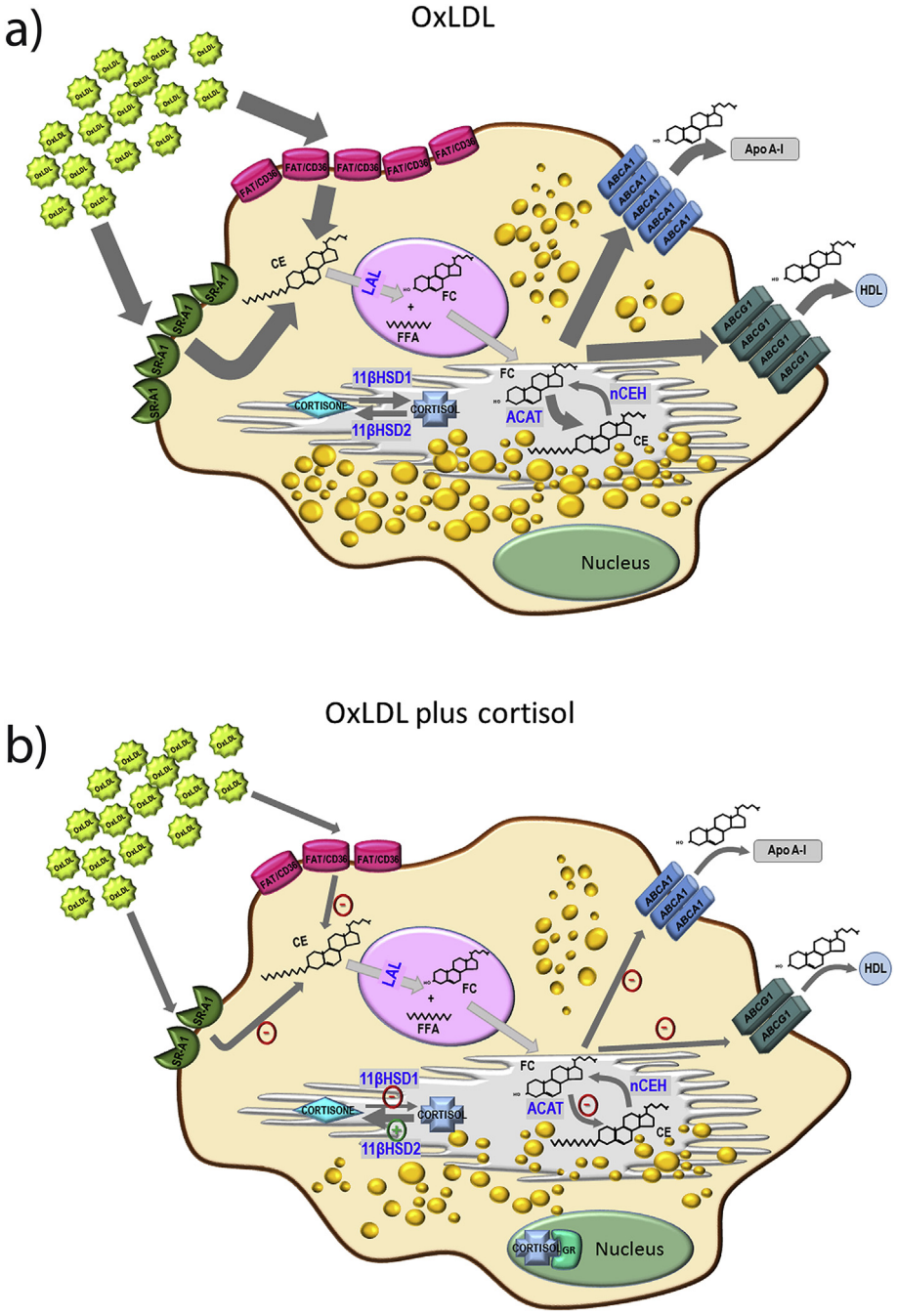

Fig. 6. Schematic representation of the effects caused by OxLDL(a) and OxLDL plus cortisol (b) in THP1 macrophages. The arrows thickness indicates the intensity of the pathway. In b), the pathways are marked in red (−) when cortisol inhibits them and in green (+) when cortisol activates them. Cortisol decreased the influx of OxLDL, reducing SRA1 and FAT/CD36 expression, decreased cholesterol esterification by ACAT and finally diminished the efflux of cholesterol trough ABCA1 and ABCG1 resulting in a minor lipid accumulation inside cells. FAT/CD36 = fatty acid translocase; SRA1 = Scavenger receptor class A type1; ACAT = acyl-CoA:cholesterol acyltransferase; nCEH = neutral cholesterol ester hydrolase 1; ABCA1 = ATP-binding cassette transporter A1; ABCG1 = ATP-binding cassette transporter G; 11βHSD1 = 11β-hydroxysteroid dehydrogenase type 1; 11βHSD2 = 11β-hydroxysteroid dehydrogenase type 2; FC= Free cholesterol; CE= Cholesterol esters; FFA= Free fatty acids; LAL = Lysosomal acid lipase; Apo A-I: Apolipoprotein A1; HDL= High density lipoprotein.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2016.04.020.

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