Atherosclerosis 250 (2016) 84–94

Contents lists available at ScienceDirect

Atherosclerosis

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

# 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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ARTICLE INFO

*Article history:*
Received 8 October 2015
Received in revised form
4 April 2016
Accepted 24 April 2016
Available online 28 April 2016

*Keywords:*
Cortisol
Cortisone
11β-hydroxysteroid dehydrogenase 1
11β-hydroxysteroid dehydrogenase 2
THP1 macrophages
Foam cells

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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http://dx.doi.org/10.1016/j.atherosclerosis.2016.04.020
0021-9150/

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 $1 \times 10^6$ 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.