Pharmacological Reports
2013, 65, 226–234
ISSN 1734-1140

Short communication

Eplerenone promotes alternative activation in human monocyte-derived macrophages

Krzysztof Łabuzek, Sebastian Liber, Łukasz Bułdak, Grzegorz Machnik, Justyna Liber, Bogusław Okopień

Department of Internal Medicine and Clinical Pharmacology, Medical University of Silesia, Medyków 18, PL 40-752 Katowice, Poland

Correspondence: Sebastian Liber, e-mail: sebastian.liber@gmail.com

Abstract

Background: In this study, we have analyzed the response of human monocyte-derived macrophages to mineralocorticoid axis modulators.

Methods: Human monocyte-derived macrophages were incubated with aldosterone alone, eplerenone alone, and the combination of aldosterone and eplerenone. The analyzed variables were nitric oxide and reactive oxygen species production, and the gene and protein expression of inducible nitric oxide synthase, arginase I, and mannose receptor.

Results: We showed that aldosterone promotes a classic inflammatory response in macrophages, whereas its antagonist, eplerenone, attenuates aldosterone-induced activity.

Conclusion: Eplerenone did not quantitatively weaken the response of macrophages to aldosterone but instead qualitatively changed their behavior.

Key words:
human macrophages, eplerenone, aldosterone, mineralocorticoid receptor, alternative activation

Introduction

Eplerenone, along with spironolactone, belongs to a class of mineralocorticoid receptor antagonists, also known as aldosterone antagonists. It is indicated as a treatment for disorders of the renin-angiotensin-aldosterone axis, such as hepatic cirrhosis, heart failure, and hypertension [10].

Aldosterone is a mineralocorticoid hormone that is synthesized by the cortex of the adrenal gland. It is the ligand of a nuclear receptor known as the mineralocorticoid receptor. Mineralocorticoid activity is classically described in relation to its effects on the kidney epithelium, namely salt and water retention. However, in recent years, there has been increased interest in the extrarenal actions of this hormone, especially in the heart, blood vessels and adipose tissue [6].

Most research devoted to the aldosterone axis focuses on the interactions between mineralocorticoid activity and parenchymal cell behavior; frequently, a dysregulation of sodium-potassium ATPase and intracellular oxidative equilibrium is implied. Several recent studies have shown that inflammation, macrophage activity, and macrophage phenotype may be downstream effects of aldosterone [4, 5, 22]. However, at present, it is still unknown whether aldosterone induces significant physiological changes in human macrophages. Alternative activation produces macrophages with an anti-inflammatory

phenotype that opposes the pro-inflammatory and toxic phenotype. Alternative activation is induced by interleukin-4 (IL-4), and it is distinguished by high levels of arginase I and mannose receptor expression and low levels of inducible nitric oxide synthase (iNOS) expression, as well as low levels of nitric oxide (NO) and reactive oxygen species (ROS). Dysregulation of the macrophage phenotype is thought to be involved in the pathogenesis of vascular, granulomatous and parasitic diseases [7]. In this study, we analyzed the influence of aldosterone and its antagonist eplerenone on human monocyte-derived macrophages. We investigated how aldosterone and eplerenone affect the phenotypes of these cells and compared the effects of these compounds to those of IL-4. The phenotypes of these cells and their activity were parameterized with commonly used indices of alternative activation and toxic molecule production.

Materials and Methods

Cell cultures and drug treatments

Twelve healthy volunteers (6 men and 6 women) aged 18–30 years who were taking no drugs participated in the study. Peripheral blood mononuclear cells were separated from blood samples by histopaque density gradient centrifugation using protocols previously described by Flo et al. [3] and Okopień et al. [13]. Monocytes were isolated from the peripheral blood mononuclear cells by negative immunomagnetic separation using Pan-T and Pan-B Dynabeads (Dynal, Oslo, Norway). The isolated cells were labeled with monoclonal antibody (Dako, Glostrup, Denmark) against the monocyte-specific positive antigen CD14. The procedure yielded 92% CD14-positive cells in the isolated fraction. The viability of immunomagnetically isolated monocytes was > 98%, as assessed by the 0.1% trypan blue exclusion test to measure cell membrane integrity. Monocytes were suspended in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 μg/ml Fungizone (Gibco, Grand Island, NY). The cells were counted, and the number of monocytes was adjusted to 1×10^6/ml. Equal numbers of cells (1×10^6 monocytes per well) were placed in each well of a plastic 24-well plate (Becton-Dickinson, NJ, USA) and left intact for 2 h to allow them to adhere to the bottom. Then, the medium was changed, and the cultures were incubated for 24 h. Incubations were performed in triplicate at 37°C in a humidified atmosphere containing 5% CO_2 in air. After a 24-h incubation, the supernatant was carefully removed and replaced with medium supplemented with aldosterone (10^{-7} M), eplerenone (10^{-7} M or 10^{-5} M) and IL-4 (50 ng/ml) (Sigma-Aldrich, USA) for another 24 h. In wells containing both eplerenone and aldosterone, cells were pre-incubated with eplerenone for 1 h before the aldosterone was added. Eplerenone was dissolved in DMSO (Sigma-Aldrich, USA) at an initial concentration of 10 μM. Further dilutions were performed using the appropriate medium. A corresponding amount of DMSO was added to negative controls. The final DMSO concentration in the medium did not exceed 0.05% and, as previously confirmed, did not have any effect on the monocyte cultures. The cells were incubated in the described conditions for an additional 24 h. At the end of the incubation, the supernatant was collected and stored until used for nitrite measurements using colorimetric assay kits according to the manufacturer's recommendation (Cayman Chemicals, Ann Arbor, USA). MTT conversion, ROS measurement and RT-PCR were conducted in the cultures adhered to the bottom of 24-well plates (see below). Cells destined for western blot analysis were seeded onto 35 mm plastic dishes at a density of 2×10^6 per dish. Cell viability was determined using trypan blue and MTT (Sigma-Aldrich, USA).

MTT conversion

The viability of monocytes treated with the studied compounds was evaluated using the MTT conversion method [11]. MTT (2.5 μg/ml) was added to the medium 3 h before the scheduled end of the experiment, and then the cultures were incubated at 37°C in 5% CO_2/95% air. Then, the monocytes were lysed in 100 μL DMSO, which enabled the release of the blue reaction product, formazan (RT, 10 min in the dark). Absorbance at the wavelength of 570 nm was read using a microplate reader (Dynex Technologies, Chantilly, VA, USA,), in three measurements per well, for 12 independent experiments (the number corresponding to the number of the participants).

ROS measurement

Monocytes incubated in 24-well tissue culture plates (1×10^6 cells/well) were treated with the studied compounds (37°C, 5% CO_2/95% air). After 24 h, the cells were resuspended in DMEM containing NBT (1 mg/ml, Sigma-Aldrich). Next, the monocytes were lysed using sonication, and NBT reduction was measured by absorbance at 550 nm in triplicate using a microplate reader.

RT-PCR

The relative gene expression of iNOS, mannose receptor and arginase I was measured in a two-step, reverse transcription-quantitative polymerase chain reaction (RT-QPCR) and normalized against the expression level of the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene. Total RNA was extracted from cells using the TriPure Isolation Reagent (Roche, Switzerland) according to standard protocol. The concentrations and quality of the RNA extracts were estimated by spectrophotometry. Total RNA from each sample (1 μg) was reverse transcribed using MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (Epicentre Technologies, Madison, USA) in a reaction volume of 20 μl.

Quantitative PCR was conducted using Brilliant II SYBR Green QRT-PCR 2X Master Mix (Agilent Technologies Inc., Santa Clara, USA) and 4 μl of cDNA mixture (equivalent to 40 ng of total RNA) in 25 μl of total reaction volume, analyzed in a LightCycler 480 Real-Time PCR System using standard 2-step thermal profile for QPCR (95°C for 2 min., 40 × [95°C 15 s, 60°C 30 s]). The primers used for quantitative analysis (mannose receptor, arginase I and iNOS) were designed using eprimer3 software (EMBOSS platform, [15]). The GAPDH primer pair was published previously [9] and was retrieved from a primer database [14]. All primers were used at a final concentration of 300 nM. After amplification, melting curve was plotted for each sample to confirm the specificity of the reaction, and randomly chosen samples from the QPCR assay were analyzed by agarose gel electrophoresis (Agagel Mini, Biometra, Germany) to confirm the amplification specificity.

Isolation of cytosolic extracts

Cytosolic extracts were prepared using a standard protocol previously described by Towbin et al. [21]. Monocyte cultures were washed from 35 mm dishes with ice-cold PBS, and proteins were extracted with 100 μl of lysis buffer supplemented with protease inhibitors. The cell lysates were incubated on ice, scraped off, and shaken for 20 min on ice, and the nuclear fraction was precipitated by centrifugation at 10,000 × g for 30 min at 4°C.

Protein electrophoresis

The total protein concentrations of each sample were determined spectrophotometrically as described by Bradford [1]. Bovine serum albumin (Fermentas, Lithuania) was used as a concentration standard. Equal amounts of total protein (50 μg) were denatured and resolved on an SDS-polyacrylamide gel [18].

Western blot analysis

Separated proteins were transferred to PVDF membranes (Pall Poland Ltd., Warszawa, Poland) by wet electrotransfer [21]. Membranes were blocked by incubation in blocking buffer (TBST containing 5% non-fat dried milk) for 1 h. Primary antibodies against the mannose receptor, iNOS (C-terminus) (both from Santa Cruz Biotechnology, CA, USA) and arginase I (BD Biosciences, CA, USA) were used at dilutions of 1 : 300, 1 : 500 and 1 : 1000, respectively. Antibodies against β-actin were used at a concentration of 1 : 3000 (Abcam plc, UK). Membranes were incubated with primary antibodies overnight at 4°C and subsequently incubated for 60 min at RT with alkaline phosphatase-conjugated secondary antibodies (diluted 1 : 2000 in blocking buffer) (Bio-Rad Laboratories Inc., Hercules, CA, USA). Finally, membranes were washed, and color precipitates were developed directly on the membrane using AP chromogenic substrates (Bio-Rad Laboratories) [20]. The PageRuler Unstained Protein Ladder (Fermentas, Lithuania) served as molecular weight marker. The integrated optical density (IOD) of the signals was measured semi-quantitatively using ImageJ software (National Institutes of Health, USA) and expressed as the ratio of the IOD of the tested proteins to the IOD of β-actin.

The study was approved by the Bioethics Committee of the Medical University of Silesia. The experiments comply with the current laws of Poland

Statistical analysis

The results are expressed as the mean ± standard deviation (SD). The normality of each distribution was estimated by means of the Shapiro-Wilk test. Statistical analysis of the data was performed using one-way ANOVA followed by the *post-hoc* Tukey honest significant difference test (for normally distributed data) or the Kruskal-Wallis test followed by Dunn's test (for non-normally distributed data). The Bonferroni adjustment was applied for multiple comparisons. Differences were considered significant at $p < 0.05$. Statistical analysis was performed in the SPSS statistical software package (SPSS 19.0 for Windows).

Results

The effects of aldosterone, eplerenone, the combination of aldosterone and eplerenone, and IL-4 on cell viability

The concentrations of mineralocorticoid modulators were selected based on a study conducted in human endothelial vein cells [12], and the concentration of IL-4 was selected based on a study performed on human monocytes [8]. Prior to the evaluation of the MR blockade, we determined whether the compounds used in this study affected the viability of the cells in our experimental settings. Neither the drugs nor their combination affected the cell viability, as determined by MTT and trypan blue exclusion assays (data not shown).

The effects of aldosterone, eplerenone, the combination of aldosterone and eplerenone, and IL-4 on NO production (Fig. 1)

Treatment with 10^{-7} M aldosterone or the combination of 10^{-7} M aldosterone and 10^{-7} M eplerenone significantly increased the production of NO. In contrast, treatment with 10^{-7} M or 10^{-5} M eplerenone, the combination of 10^{-7} M aldosterone and 10^{-5} M eplerenone, and treatment with 50 ng/ml IL-4 had no effect on the production of NO.

The effects of aldosterone, eplerenone, the combination of aldosterone and eplerenone, and IL-4 on ROS production (Fig. 1)

Treatment with 10^{-7} M aldosterone or the combination of 10–7 M aldosterone and 10^{-7} M eplerenone significantly increased ROS production. In contrast, treatment with 10^{-7} M or 10^{-5} M eplerenone, the combination of 10^{-7} M aldosterone and eplerenone at 10^{-5} M, and 50 ng/ml IL-4 had no effect on ROS production.

The effects of aldosterone or eplerenone on the expression of the iNOS, arginase I and mannose receptor genes (Figs. 2–5)

Treatment with 10^{-7} M aldosterone significantly increased the expression of iNOS mRNA but did not affect the expression of arginase I or mannose receptor mRNAs in macrophages. Treatment with 10^{-7} M eplerenone did not influence any of the analyzed variables. However, 10^{-5} M eplerenone up-regulated the expression of both arginase I and mannose receptor mRNAs.

The effects of aldosterone or eplerenone on the expression of iNOS, arginase I and mannose receptor's proteins (Figs. 2–5)

The changes in the expression of iNOS, arginase I and mannose receptor proteins paralleled the changes in the expression of their mRNAs.

The combined effects of eplerenone and aldosterone on the expression of the iNOS, arginase I and mannose receptor genes (Figs. 2–5)

The combination of 10^{-5} M eplerenone and 10^{-7} M aldosterone significantly increased the expression of the iNOS, arginase I and mannose receptor genes. Interestingly, eplerenone attenuated the effects of aldosterone, while aldosterone amplified the effects of eplerenone on iNOS and arginase I mRNAs.

The combined effects of eplerenone and aldosterone on the expression of the iNOS, arginase I and mannose receptor proteins (Figs. 2–5)

The changes in the expression of the iNOS, arginase I and mannose receptor proteins paralleled the alterations in their mRNA levels.

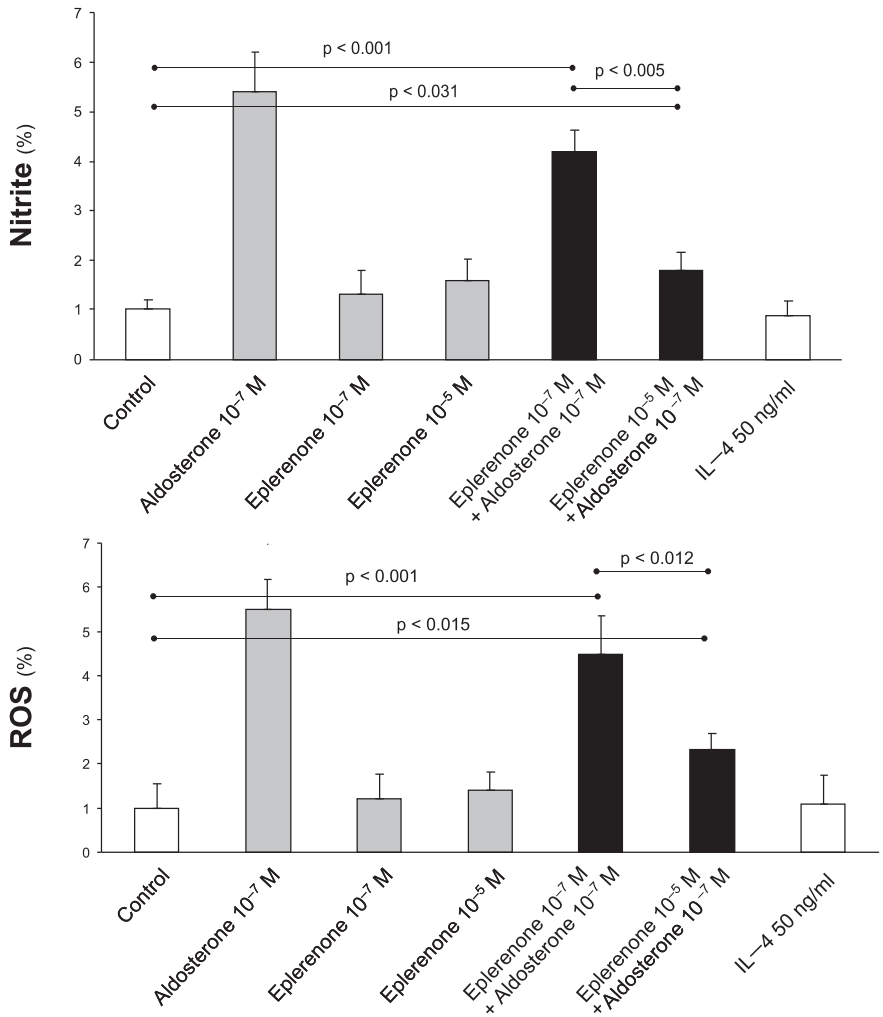

Fig. 1. The effects of aldosterone, eplerenone and interleukin-4 (IL-4) on nitrite and ROS release in cultures of human monocytes. Monocytes were treated with aldosterone (10^{-7} M), eplerenone (10^{-7} M or 10^{-5} M) and IL-4 (50 ng/ml). To inhibit aldosterone receptors, the cultures of macrophages were pre-incubated with eplerenone (10^{-7} M, 10^{-5} M) for 1 h before aldosterone (10^{-7} M) was added, and the cultures were then incubated for 24 h. Nitrite concentrations were evaluated by ELISA. ROS was measured by absorbance at 550 nm using a microplate reader. The results in the control group were set to 1. The results shown are the mean ± SD of three independent experiments

The effects of IL-4 on the expression of the iNOS, arginase I and mannose receptor genes (Figs. 2–5)

Treatment with 50 ng/ml IL-4 significantly increased the expression of the arginase I and mannose receptor genes but did not affect the expression of iNOS mRNA.

The effects of IL-4 on the expression of iNOS, arginase I and mannose receptor's proteins (Fig. 2–5)

The changes in the expression of the iNOS, arginase I and mannose receptor proteins paralleled the changes in their mRNA levels.

Discussion

In this study, we analyzed the response of human monocyte-derived macrophages to modulators of the mineralocorticoid axis. We showed that aldosterone is a pro-inflammatory hormone and that its antagonist, eplerenone, attenuates aldosterone-induced activity. However, eplerenone did not quantitatively weaken the response of macrophages to aldosterone; instead, it qualitatively changed their behavior. Mineralocorticoid receptors are expressed in macrophages [5, 16, 22], and experiments in a mouse model that its agonist, aldosterone, induces macrophages to produce TNF α [22]. In our study, aldosterone induced the re-

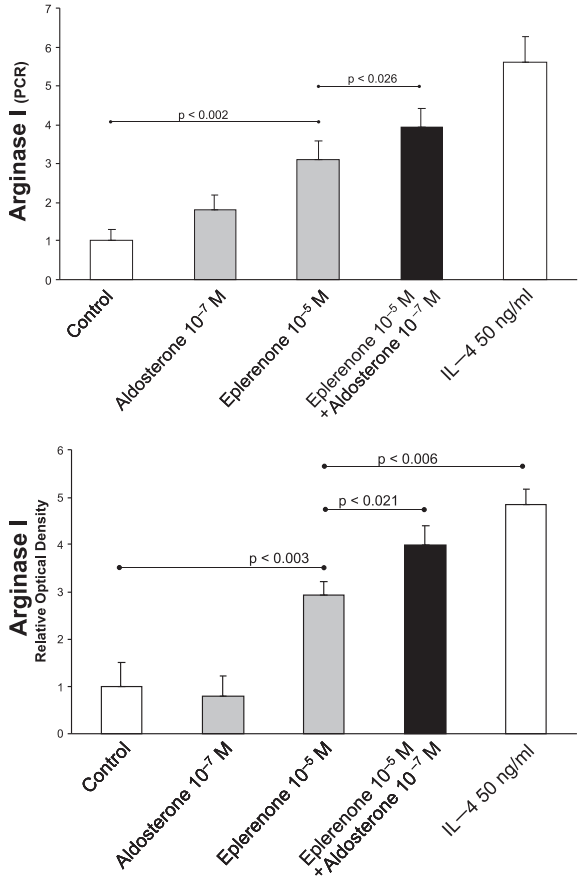

Fig. 2. The effects of aldosterone, eplerenone and interleukin-4 (IL-4) on arginase I transcription (RT-PCR) and protein expression (western blot) in cultures of human monocytes. Monocytes were treated with aldosterone (10^{-7} M), eplerenone (10^{-5} M) or IL-4 (50 ng/ml). To inhibit aldosterone receptors, the macrophage cultures were pre-incubated with eplerenone (10^{-5} M) for 1 h before aldosterone (10^{-7} M) was added, and the cultures were then incubated for 24 h. Transcription was evaluated by RT-PCR and protein expression was evaluated by western blot analysis using antibodies specific for arginase I and β-actin. The results shown are the mean ± SD of three independent experiments

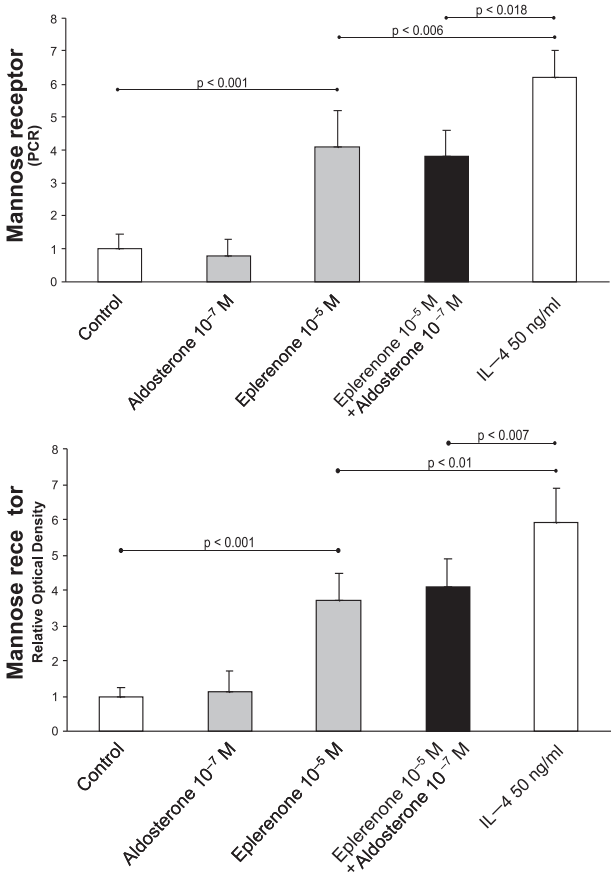

Fig. 3. The effects of aldosterone, eplerenone and interleukin-4 (IL-4) on mannose receptor transcription (RT-PCR) and protein expression (western blot) in cultures of human monocytes. Monocytes were treated with aldosterone (10^{-7} M), eplerenone (10^{-5} M) and IL-4 (50 ng/ml). To inhibit aldosterone receptors, the cultures of macrophages were pre-incubated with eplerenone (10^{-5} M) for 1 h before aldosterone (10^{-7} M) was added, and the cultures were then incubated for 24 h. Transcription was evaluated by RT-PCR, and protein expression was evaluated by western blot analysis using antibodies specific for mannose receptor and β-actin. The results shown are the mean ± SD of three independent experiments

lease of toxic molecules, such as nitric oxide and reactive oxygen species, from macrophages (Fig. 1). Our results are in line with those obtained in healthy rat mesenteric arteries and perivascular fat macrophages [17]. Therefore, aldosterone may exert its deleterious effects by interacting with tissue macrophages, in contrast to the dominant view that aldosterone dysregulates the function of proprietary organ cells. The capacity of macrophages to produce reactive oxygen species and the role of these mediators in the process of fibrosis support this hypothesis.

In our model, the pro-inflammatory action of aldosterone occurred at the level of gene expression, as indicated by the parallel changes in iNOS mRNA and protein level (Figs. 4 and 5). Moreover, the antagonism of mineralocorticoid activity with eplerenone attenuated the above described effects of aldosterone. Therefore, it seems that an aldosterone and mineralocorticoid receptor interaction was the basis for the observed phenomenon.

However, we cannot exclude the possibility that the putative membrane receptor for aldosterone influenced our results [2]. We did not use any general inhibitor of gene transcription or translation, which may have revealed extragenomic effects of aldosterone, and we did not apply any ligand, such as hydrocortisone, that may have distinguished between the effects of aldosterone on membrane and nuclear receptors. Furthermore, we

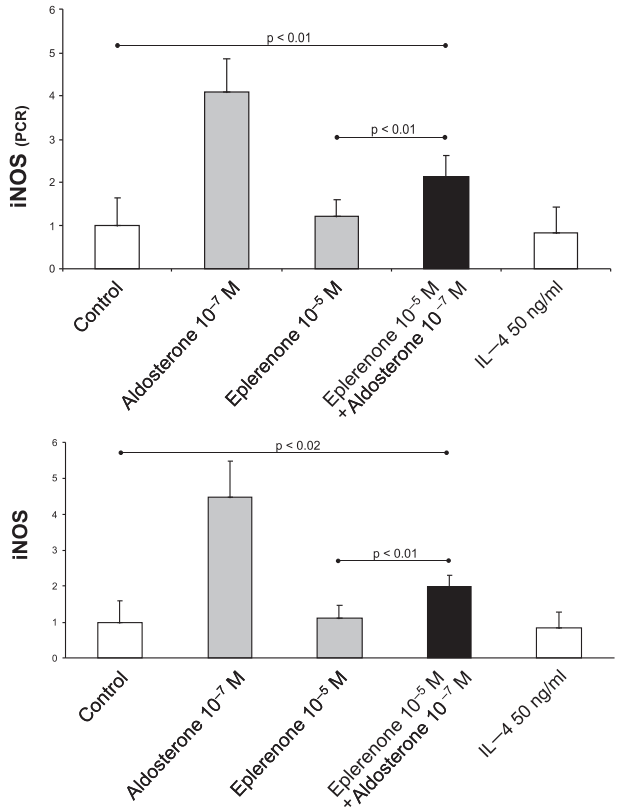

Fig. 4. The effects of aldosterone, eplerenone and interleukin-4 (IL-4) on iNOS transcription (RT-PCR) and protein expression (western blot) in cultures of human monocytes. Monocytes were treated with aldosterone (10^{-7} M), eplerenone (10^{-5} M) and IL-4 (50 ng/ml). To inhibit aldosterone receptors, the cultures of macrophages were pre-incubated with eplerenone (10^{-5} M) for 1 h before aldosterone (10^{-7} M) was added, and the cultures were then incubated for 24 h. Transcription was evaluated by RT-PCR, and expression was evaluated by western blot analysis using antibodies specific for iNOS and β-actin. The results shown are the mean ± SD of three independent experiments

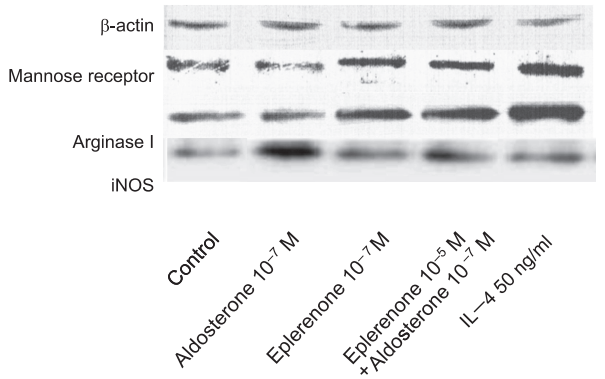

Fig. 5. The representative immunoblots of β-actin, mannose receptor, arginase I and iNOS expression patterns. The cultures of human monocytes were treated with medium containing (a) DMSO alone (control), (b) aldosterone (10^{-7} M), (c) eplerenone (10^{-5} M), (d) eplerenone (10^{-5} M, 1-h preincubation) with aldosterone (10^{-7} M), or (e) interleukin-4 (50 ng/ml). β-Actin was measured as a protein loading control. The integrated optical density (IOD) of the signals was measured semi-quantitatively using Image Pro Plus software and expressed as the ratio of the IOD of the tested proteins to the IOD of β-actin. Each experiment was repeated three times, and the relative density values were subjected to statistical analysis

did not measure our variables on short time scales (i.e., seconds or minutes) after the cells were exposed to aldosterone, which might have revealed some rapid, extragenomic effects for the hormone.

Although eplerenone alone did not change the release of toxic molecules in macrophages, it affected the gene and protein expression in these cells. Notably, it affected the expression of arginase I and the mannose receptor at the mRNA and protein levels (Figs. 2, 3 and 5). Both of these molecules are markers of alternative macrophage activation. This pattern of macrophage activity counteracts inflammation and is detected in healing tissues. In our study, IL-4, which is commonly used to induce alternative activation in macrophages [6, 19], caused the same alterations in gene and protein expression as eplerenone. Fraccarollo et al. [4] have shown that selective MR blockade applied immediately after myocardial infarction augmented macrophage infiltration and transiently increased the expression of healing-promoting cytokines in the injured myocardium, resulting in enhanced infarct neovascularization and reduced early cardiac remodeling and dysfunction. Moreover, the abrogation of macrophages with clodronate, a drug that induces macrophage apoptosis, attenuated the beneficial effects of these cells. It was reported that mineralocorticoid deficiency acts synergistically with IL-4 to enhance alternative activation and protected against cardiac hypertrophy, fibrosis, and vascular damage caused by angiotensin II and L-NAME, a substrate for iNOS, in a mouse model [4]. Our results are in line with those obtained from animal models of vascular and heart disease, suggesting that macrophages and their alternative activation may play prominent role in the effects of mineralocorticoid receptor antagonists on human cardiovascular disorders.

Because aldosterone partly counteracts the effects of eplerenone in our study, it seems that the influence of eplerenone on mineralocorticoid activity likely explain the effects of this drug. In contrast to a mouse model, our results may suggest that eplerenone is an

inverse agonist of the mineralocorticoid receptor and that this receptor is constitutionally active in human macrophages. Therefore, the basal activity of the mineralocorticoid receptor may either suppress alternative activation or promote classic activation in human macrophages.

In summary, the inhibition of mineralocorticoid activity *via* the aldosterone antagonist eplerenone promotes alternative macrophage activation. The resulting phenotype may prevent macrophages from producing toxic molecules in the presence of aldosterone. However, whether eplerenone shows similar activity in aldosterone-primed macrophages and the degree of stability of the phenotype in the presence of other pro-inflammatory molecules remains to be investigated. Furthermore, the cells used in this study were obtained from young, healthy volunteers, and the treatment with aldosterone was short; macrophages harvested from patients affected with chronic disorders of the aldosterone axis may respond differently. The application of eplerenone may modify the course of other diseases in which alternative activation of macrophages has been implicated, such as granulomatous, parasitic, and neoplastic diseases. Nonetheless, our results highlight that human macrophages are an important focus of aldosterone research and that the effects of aldosterone antagonists should be considered in the context of the phenotype of these cells.

Disclosure of conflict of interest:
None of the authors has any conflict of interest.

References:

1. Bradford MM: A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye-binding. Anal Biochem, 1976, 72, 248–251.
2. Dooley R, Harvey BJ, Thomas W.: Non-genomic actions of aldosterone: From receptors and signals to membrane targets. Mol Cell Endocrinol, 2012, 350, 223–234.
3. Flo RW, Naess A, Lund-Johansen F, Maehle BO, Sjursen H, Lehmann V, Solberg CO: Negative selection of human monocytes using magnetic particles covered by anti-lymphocyte antibodies. J Immunol Methods, 1991, 137, 69–94.
4. Fraccarollo D, Galuppo P, Schraut S, Kneitz S, van Rooijen N, Ertl G, Bauersachs J: Immediate mineralocorticoid receptor blockade improves myocardial infarct healing by modulation of the inflammatory response. Hypertension, 2008 51, 905–914.
5. Frieler RA, Meng H, Duan SZ, Berger S, Schütz G, He Y, Xi G et al.: Myeloid-specific deletion of the mineralocorticoid receptor reduces infarct volume and alters inflammation during cerebral ischemia. Stroke, 2011, 42, 179–185.
6. Gilbert KC, Brown NJ: Aldosterone and inflammation. Curr Opin Endocrinol Diabetes Obes, 2010, 17, 199–204.
7. Gordon S, Martinez FO: Alternative activation of macrophages: mechanism and functions. Immunity, 2010, 32, 593–604.
8. Guenova E, Volz T, Sauer K, Kaesler S, Müller MR, Wölbing F, Chen K et al.: IL-4-mediated fine tuning of IL-12p70 production by human DC. Eur J Immunol, 2008, 38, 3138–3149.
9. Kelly GL, Milner AE, Baldwin GS, Bell AI, Rickinson AB: Three restricted forms of Epstein–Barr virus latency counteracting apoptosis in c-myc-expressing Burkitt lymphoma cells. Proc Natl Acad Sci USA, 2006, 103, 14935–14940.
10. Kolkhof P, Borden SA: Molecular pharmacology of the mineralocorticoid receptor: Prospects for novel therapeutics. Mol Cell Endocrinol, 2012, 350, 310–317.
11. Mosmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods, 1983, 65, 55–63.
12. Nagata D, Takahashi M, Sawai K, Tagami T, Usui T, Shimatsu A, Hirata Y et al.: Molecular mechanism of the inhibitory effect of aldosterone on endothelial NO synthase activity. Hypertension, 2006, 48, 165–171.
13. Okopień B, Kowalski J, Krysiak R, Łabuzek K, Stachura-Kułach A, Kułach A, Zieliński M, Herman ZS: Monocyte suppressing action of fenofibrate. Pharmacol Rep, 2005, 57, 367–372.
14. Pattyn F, Speleman F, De Paepe A, Vandesompele J: RTPrimerDB: the Real-Time PCR primer and probe database. Nucleic Acids Res, 2003, 31, 122–123.
15. Rice P, Longden I, Bleasby A. EMBOSS: The European Molecular Biology Open Software Suite. Trends Genet, 2000, 16, 276–277.
16. Rickard AJ, Morgan J, Tesch G, Funder JW, Fuller PJ, Young MJ: Deletion of mineralocorticoid receptors from macrophages protects against deoxycorticosterone/salt-induced cardiac fibrosis and increased blood pressure. Hypertension, 2009, 54, 537–543.
17. Rocha R, Rudolph AE, Frierdich GE, Nachowiak DA, Kekec BK, Blomme EA, McMahon EG et al: Aldosterone induces a vascular inflammatory phenotype in the rat heart. Am J Physiol Heart Circ Physiol, 2002, 283, H1802–1810.
18. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York, 1989, 1.25–1.28.
19. Stein M, Keshav S, Harris N, Gordon S: Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. J Exp Med, 1992, 176, 287–292.
20. Stott DI: Immunoblotting and dot blotting. J Immunol Methods, 1989, 119, 153–187.

21. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA, 1979, 76, 4350–4354.
22. Usher MG, Duan SZ, Ivaschenko CY, Frieler RA, Berger S, Schütz G, Lumeng CN et al.: Myeloid mineralocorticoid receptor controls macrophage polarization and cardiovascular hypertrophy and remodeling in mice. J Clin Invest, 2010, 120, 3350–3364.

Received: March 6, 2012; **in the revised form:** July 18, 2012; **accepted:** August 28, 2012.