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Research Article

# Aldosterone up-regulates MMP-9 and MMP-9/NGAL expression in human neutrophils through p38, ERK1/2 and PI3K pathways

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ABSTRACT

Aldosterone and mineralocorticoid receptors are important regulators of inflammation. During this process, chemokines and extracellular matrix degradation by matrix metalloproteases, such as MMP-9, help leukocytes reaching swiftly and infiltrating the injured tissue, two processes essential for tissue repair. Leukocytes, such as neutrophils, are a rich source of MMP-9 and possess mineralocorticoid receptors (MR). The aim of our study was to investigate whether aldosterone was able to regulate proMMP-9, active MMP-9 and MMP-9/NGAL production in human neutrophils. Here we show that aldosterone increased MMP-9 mRNA in a dose- and time-dependent manner. This hormone up-regulated also dose-dependently proMMP-9 and active MMP-9 protein release as well as the MMP-9/NGAL protein complex. PI3K, p38 and ERK1/2 inhibition diminished these aldosterone-induced neutrophil productions. Furthermore, spironolactone, a MR antagonist, counteracted aldosterone-induced increases of proMMP-9, active MMP-9 and MMP-9/NGAL complex. These findings indicate that aldosterone could participate in tissue repair by modulating neutrophil activity and favoring extracellular matrix degradation.



## Introduction

Aldosterone is now recognized as a hormone implicated not only in homeostasis and blood pressure regulation but also in inflammation under physiopathological conditions. Results obtained from clinical data and animal models have demonstrated direct hypertrophic, thrombotic and fibrotic effects of aldosterone in organs such as heart and kidneys, and mineralocorticoid receptor (MR) antagonists diminish these harmful effects [1–4]. Other data indicate that aldosterone plays an active role in vascular wall inflammation and its remodelling. This hormone promotes notably smooth muscle cell proliferation, NADPH oxidase activity, dysfunction and adhesion molecule increase in endothelial cells, leukocyte adhesion and infiltration [5–9]. Moreover, attenuation

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of atherosclerosis development by MR antagonists or aldosterone synthase inhibitors has been demonstrated [10,11]. Accumulating data show that MR activation of immune cells, like macrophages, is also implicated in these events. Aldosterone increases monocyte/macrophage NADPH oxidase, NO and ROS production [12,13]. Kidneys from rats infused with aldosterone present macrophage infiltration and spironolactone, a MR antagonist, counteracts this effect [14]. Specific deletion of MR from macrophages attenuates the hypertrophic and fibrotic effects, as well as the altered blood pressure normally observed in mice treated with deoxycorticosterone or L-NAME/AngII [15–17]. However, until now, neutrophils have received little attention despite the demonstration that these cells express mineralocorticoid receptors [18]. Moreover, neutrophils are the most abundant blood leukocytes and the first to arrive close to any inflamed tissue and to migrate through it. Activated neutrophils are then able to recruit other leukocytes such as monocytes and lymphocytes by chemokine and leukotriene secretion and help their diapedesis by pro-inflammatory cytokine and matrix metalloprotease (MMP) release [19]. Some chemokines such as CXCL8/IL-8, implicated in neutrophil oxidative bursts and chemotaxis, as well as pro-inflammatory cytokines like proIL-1β are cleaved by MMPs, such as MMP-9, into a biologically active form [20,21]. Degradation of the extracellular matrix by collagenases, like MMP-2 and MMP-9, favors the entry of immune cells in altered tissue, which constitutes a prerequisite for tissue repair [22]. When activated, neutrophils are able to excrete large amounts of pro and active MMP-9 proteins. Cytokines such as IL-8, IL-1β and TNFα or the chemoattractant peptide formyl-Met-Leu-Phe are known to activate neutrophil MMP-9 release, corresponding to inflammatory or/and infectious conditions. A wide variety of other cells like astrocytes, chondrocytes, epithelial cells, fibroblasts, keratinocytes, mesangial cells, smooth muscle cells, and neurons are able also to secrete pro and active MMP-9 in response to cytokines such as IL-1β, TNFα and TGFβ [23,24]. However, only neutrophils synthetize a protein complex termed "MMP-9/NGAL", in which proMMP-9 is linked covalently to neutrophil gelatinase-associated lipocalin (NGAL) [25]. Approximately 20–30% of proMMP-9 is located in this complex where NGAL protects it from protease degradation [26].

Based on the above data, we hypothesized that aldosterone could regulate pro and active MMP-9 neutrophil production as well as that of the MMP-9/NGAL complex.

Herein, we show that aldosterone increases MMP-9 mRNA, proMMP-9, active MMP-9 and MMP-9/NGAL complex expression in granulocyte-differentiated HL-60 cells and in human neutrophils. This up-regulation is mediated by the activation of PI3kinase, p38 and to a lesser extent ERK1/2 pathways. Finally, we show that the MR antagonist, spironolactone, counteracts aldosterone effects on MMP-9 mRNA, proMMP-9, active MMP-9 and MMP-9/NGAL complex.

## Materials and methods

### Cell culture and treatments

The HL-60 human leukemia cell line was cultured at 37 °C under 5% $CO_2$ in RPMI 1640 medium containing 10% heat inactivated fetal calf serum (Eurobio, Courtaboeuf, France), 100 U/mL penicillin, 100 μg/mL streptomycin, 10 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate (all purchased from Sigma-Aldrich) and 1 × non-essential amino-acids (Gibco-BRL, Invitrogen, Baisley, UK). HL-60 cells were differentiated into the neutrophil-lineage by adding 1.3% of DMSO during three days and then 0.65% of DMSO for again three days [27]. Polymorphonuclear leukocytes (PMN) were purified, using the Polymorphprep method (Abcys, Paris, France), from blood collected from five human volunteers. Blood samples were carried out in the "Department of Biological Haematology", Centre Hospitalier Universitaire, Vandoeuvre-les-Nancy, France. Informed consent was obtained from all donors. This center complies with local ethical rules. Flow cytometry experiments revealed a purity >95%. These cells were cultured under the same conditions as for the HL-60 cells except that no DMSO was added to the culture medium. Differentiated HL-60 cells and PMN, at a cellular density of $0.8 \cdot 10^6$ cells/mL, were incubated for different time periods with aldosterone at various concentrations ($10^{-7}$, $10^{-8}$, and $10^{-9}$ M). Then, cell viability was evaluated using Trypan blue and annexin V assays. For all studies, inhibitors were always added 30 min before aldosterone. As these inhibitors were dissolved in DMSO, this molecule was used as solvent control. Aldosterone, spironolactone (MR antagonist), signal transduction inhibitors (Ly294002, PD98059, and SB203580), actinomycin (mRNA synthesis inhibitor) and cycloheximide (protein synthesis inhibitor) were purchased from Sigma-Aldrich (L'Isle d'Abeau Chesnes, France).

### Reverse transcription and quantitative real-time PCR

Total RNA was extracted from cultured cells using Trizol reagent (Euromedex, Mundolsheim, France) and treated with DNase I to remove eventual genomic DNA traces (MBI Fermentas, Euromedex). For cDNA synthesis, 2 μg of total RNA, 40 units of Mu-MLV reverse transcriptase (MBI Fermentas), 2 mM dNTP (MBI Fermentas), 0.25 μM oligo dT15 (Invitrogen), RT 1 × buffer and 10 units of RNAse inhibitor (MBI Fermentas) were incubated 1 h at 37 °C. Sequences of primers used to amplify MMP-9 transcripts (GenBank accession number NM_004994) were sense 5′-cac ttc ccc ttc atc ttc-3′; antisense 5′-cag ggt ttc cca tca gca tt-3′. For RPS-29 (GenBank accession number NM_001032), used as housekeeping gene, oligonucleotide sequences were sense 5′-aag atg ggt cac cag cag ctg tac tg-3′ and antisense 5′-aga cac gac aag agc gag aa-3′. PCR conditions were as follows: activation for 3 min at 95 °C, then 40 cycles of amplification of 10 s at 95 °C and 45 s at 60 °C followed by 1 min at 95 °C. Finally, for each PCR, melting curves were processed from 50 °C to 95 °C to check the quality of the amplification. MMP-9 mRNA levels were normalized to those of RPS-29. Each PCR was performed in triplicate and repeated at least two times.

### Gelatin zymography

10 μL of culture supernatant from $0.8 \cdot 10^6$ cells/mL was mixed with loading buffer (zymography sample buffer, BioRad, Ivry sur Seine, France) and submitted to electrophoresis on a 10% (w/v) SDS-polyacrylamide gel containing 1 mg/mL of gelatin. After electrophoresis, gels were rinsed twice for 15 min with 2.5% Triton X-100 to remove SDS. Then, gels were placed for 15–16 h at 37 °C in an activation buffer (50 mM Tris–HCl at pH 7.4, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 100 mM NaCl, 1% Triton X-100; all provided from Sigma-Aldrich). Finally, gel coloration was carried out with