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Aldosterone increases VEGF-A production in human neutrophils through PI3K, ERK1/2 and p38 pathways

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ABSTRACT

Aldosterone is now recognised as an important actor in inflammation processes. Neoangiogenesis plays a crucial role in this complex process and immune cells, such as neutrophils, appear to be able to secrete different forms of (pro)angiogenic molecules, especially VEGF-A. The present work was undertaken to investigate whether aldosterone was able to regulate VEGF-A production in human neutrophils. The HL-60 (progranulocytic) cell line and human polymorphonuclear leukocytes were incubated for different time periods with aldosterone. Total cellular RNA extraction, submitted to reverse transcription and real time semi-quantitative PCR, was used to study VEGF-A mRNA expression. Cell supernatants were collected and ELISA tests were performed to analyse VEGF-A protein production. Aldosterone increased VEGF-A mRNA and protein expression in a dose- and time-dependent manner in both cell types. Inhibitors of PI3 kinases, ERK1/2, and to a lesser extent of p38 MAPK, decreased this aldosterone-induced immune cell activation. Western-blot performed with HL-60 cells confirmed that ERK1/2 and p38 MAPK pathways were stimulated by aldosterone. Mineralocorticoid receptors are implicated in this VEGF-A up-regulation because HL-60 cells pre-treated with spironolactone, an aldosterone receptor antagonist, diminished the effects of aldosterone. Aldosterone was also able to increase VEGF-A production of phagocytic cells such as neutrophils. These results suggest that this hormone could play an active role in the neovascularisation process by favouring entry of plasma proteins and fluids into the vascular wall, cell proliferation and tissue rebuilding.

1. Introduction

Accumulating evidence suggests that aldosterone is not only implicated in water, potassium and sodium regulation homeostasis but also possesses inflammatory properties when produced in excess. In animal models, direct hypertrophic and fibrotic effects of aldosterone in organs such as the heart and kidney have been reported and its antagonists can counteract these deleterious effects [1–4]. In humans, different studies have demonstrated that mineralocorticoid receptor blockers like spironolactone or eplerenone diminished considerably morbidity and mortality after myocardial failure [5,6]. Over the past decade, numerous data have shown that aldosterone could play an active role in vascular inflammation and remodelling. Attenuation of atherosclerosis development by mineralocorticoid blockers has been described [7,8]. Aldosterone causes endothelial cell dysfunction, increases ICAM-1 [9,10], and promotes leukocyte adhesion to endothelial cells and thence their infiltration [11,12]. In smooth muscle cells, aldosterone increases NADPH oxidase activity and promotes their proliferation [13,14]. In a mice ischemic hindlimb model, Michel et al. have demonstrated that aldosterone increased vessel density in the setting of ischemia and that this phenomenon implicated MR activation, angiotensin and VEGF [15]. In mouse vessels with endothelial damage and in human vessels from patients with atherosclerosis, aldosterone enhances placental growth factor (PGF) expression, a member of the VEGF family known to promote vascular cell proliferation and inflammation [16]. Although an abundant literature exists for aldosterone's actions in non-immune cells, in comparison little is known for leukocytes which are implicated in all steps of inflammation. Aldosterone increases monocyte/macrophage oxidative metabolism and integrin levels [17,18]. In murine models of vascular damage induced by deoxycorticosterone/salt treatment or L-NAME/angiotensin II, selective deletion of mineralocorticoid receptors from macrophages protects the animals against cardiac hypertrophy, fibrosis, remodelling and increased systolic blood pressure [19,20]. Recently, it has been described that the neutrophil, another immune cell, possesses mineralocorticoid receptors [21]. This cell plays a key role in inflammation because they are the first to infiltrate massively and rapidly phagocyte pathogens and/or dead cells in case of tissue injury. Moreover, they recruit monocytes and lymphocytes by chemokines, leukotriene B_4 secretion and promote their diapedesis by pro-inflammatory cytokine release [22]. Optimal leukocyte recruitment and coordination will be crucial for tissue repair and inflammation resolution. Neoangiogenesis

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constitutes an important part of this complex process and neutrophils are a rich source of released (pro)angiogenic molecules. Members of the vascular endothelial growth factor (VEGF) family, notably VEGF-A, stimulate strongly endothelial cell migration and proliferation. Moreover, they induce neutrophil, monocyte/macrophage and smooth muscle cell chemotaxis, mediate increased microvascular permeability, facilitate neutrophil adhesion to endothelial cells and their transmigration [23–26]. Different cells, such as monocytes and neutrophils, are able to secrete large amounts of VEGF-A, one of the most powerful angiogenic molecules [27].

Since angiogenesis and immune cells are intimately implicated in inflammation and tissue repair, we investigated whether neutrophils treated with aldosterone could increase their VEGF-A production and consequently could induce a proangiogenic phenotype. We have shown that aldosterone increases VEGF-A mRNA and protein expression in human neutrophils and granulocyte-differentiated HL-60 cells. This upregulation required activation of PI3kinase, ERK1/2, and, to a lesser extent, p38 MAPK pathways. Spironolactone, an aldosterone antagonist, was able to diminish significantly the effects of aldosterone on increased VEGF-A production.

2. Materials and methods

2.1. Reagents

Aldosterone, spironolactone, DMSO, transduction signal inhibitors (Ly294002, PD98059, SB203580), actinomycin and cycloheximide were purchased from Sigma-Aldrich (L'Isle d'Abeau Chesnes, France).

2.2. Cell culture and treatments

The human progranulocytic cell line, HL-60, was cultured at 37 °C under 5% CO_2 in RPMI 1640 medium containing 10% heat inactivated foetal 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). To differentiate HL-60 cells into the neutrophil-lineage, dimethylsulfoxide (DMSO) 1.3% was added to the cell culture medium over three days, followed by three days with DMSO at 0.65% [28]. Polymorphonuclear leukocytes were collected from blood of five human volunteers and purified with the Polymorphprep method (Abcys, Paris, France). These cells were then cultured under the same conditions as for the HL-60 cells and FACS experiments revealed a purity>96%. The HL-60 cells and neutrophils were incubated for different time periods with aldosterone (Aldo) at 10^{-7}, 10^{-8}, 10^{-9} M. At the beginning of all kinetics, cellular density was $0.8\,10^6$ cells/mL. Cell viability in the presence or absence of inhibitors was measured by cell counting with Trypan blue and by Annexin V assays. For experiments with inhibitors (all solubilised with DMSO), aldosterone at 10^{-7} M served as the control and was compared to "aldosterone at 10^{-7} M + DMSO". Inhibitors were always added 30 min before aldosterone.

2.3. Real time semi-quantitative PCR

Total RNA was extracted from cultured cells using Trizol reagent (Euromedex, Mundolsheim, France). For cDNA synthesis, 2 μg of total

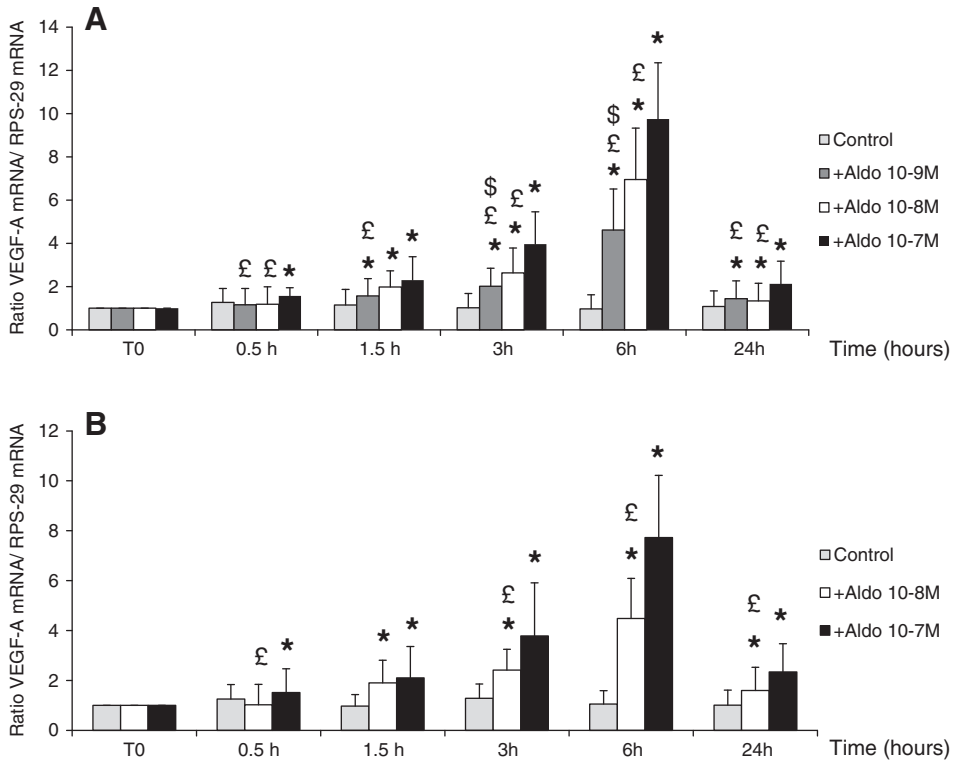

Fig. 1. Aldosterone increases VEGF-A mRNA levels in HL-60 cells and in neutrophils in a dose- and time-dependent manner. Neutrophils provided from blood of healthy volunteers were purified with Polymorphprep solution. For both cell types, after aldosterone (Aldo) incubation for different time periods, RNA extraction, cDNA synthesis and semi-quantitative real time PCR were carried out with specific primers for RPS-29 and VEGF-A. The results are expressed as ratios of VEGF-A mRNA/RPS-29 mRNA and representative of a minimum of three separate experiments. Arbitrary units were used and the mean value of 1 was attributed to the duration 0 min. (A): Ratios of VEGF-A mRNA/RPS-29 mRNA in HL-60 cells. The error bars are S.D. and "*"represents a significant difference ($P<0.05$) between control and aldosterone-treated cells, $^{£}P<0.05$ aldosterone at 10^{-7} M vs aldosterone at 10^{-8} M, 10^{-9} M, and $^{$}P<0.05$ aldosterone at 10^{-8} M vs aldosterone at 10^{-9} M. (B): Ratios of VEGF-A mRNA/RPS-29 mRNA in neutrophils. The error bars are the S.D. and "*"represents a significant difference ($P<0.05$) between control and aldosterone-treated cells, $^{£}P<0.05$ aldosterone at 10^{-7} M vs aldosterone at 10^{-8} M.

RNA was retrotranscribed with 40 units of Mu-MLV (MBI Fermentas, Euromedex), 2 mM dNTP (MBI Fermentas), 0.25 μM oligo dT15 (Invitrogen), RT 1× buffer, 10 units of RNAse inhibitor (MBI Fermentas) during 1 h at 37 °C. Oligonucleotide sequences used to amplify VEGF-A (GenBank accession number *AF022375*) were respectively [29]: sense 5′-ggagggcagaatcatcacgaag-3′ and antisense 5′-cacacaggatggcttgaagatg-3′. For RPS-29 (GenBank accession number *NM001032*) 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, 40 cycles of amplification for 10 s at 95 °C, 45 s at 58 °C (VEGF-A) or 60 °C(RPS-29) followed by 1 min at 95 °C, 1 min at 55 °C and then 80 cycles of 10 s at 55 °C. VEGF-A mRNA levels were normalised to those of RPS-29 mRNA levels.

2.4. Measurement of total VEGF-A by ELISA

After 24 h in culture, HL-60 cell and neutrophil supernatants were centrifuged to remove any dead cells. VEGF-A concentrations were determined by enzyme-linked immunosorbent assay with VEGF-A kits (R&D Systems, Lille, France). Assays were performed as recommended by the manufacturer's protocol and experiments were duplicated and repeated three to five times each.

2.5. Protein analysis and quantification

Cells were lysed with cell lysis buffer (Cell Signaling Technology, Beverly, USA) and extraction was performed in the presence of a complete protease inhibitor mixture (Roche Molecular Biochemicals, Mannheim, Germany). Protein concentrations were determined by a DC protein assay (Bio-Rad).

2.6. Western blotting analysis

Equal amounts of protein were subjected to electrophoresis on a SDS–polyacryamide gel (Bio-Rad) and transferred onto polyvinylidene difluoride membranes (Millipore, Molsheim, France). After

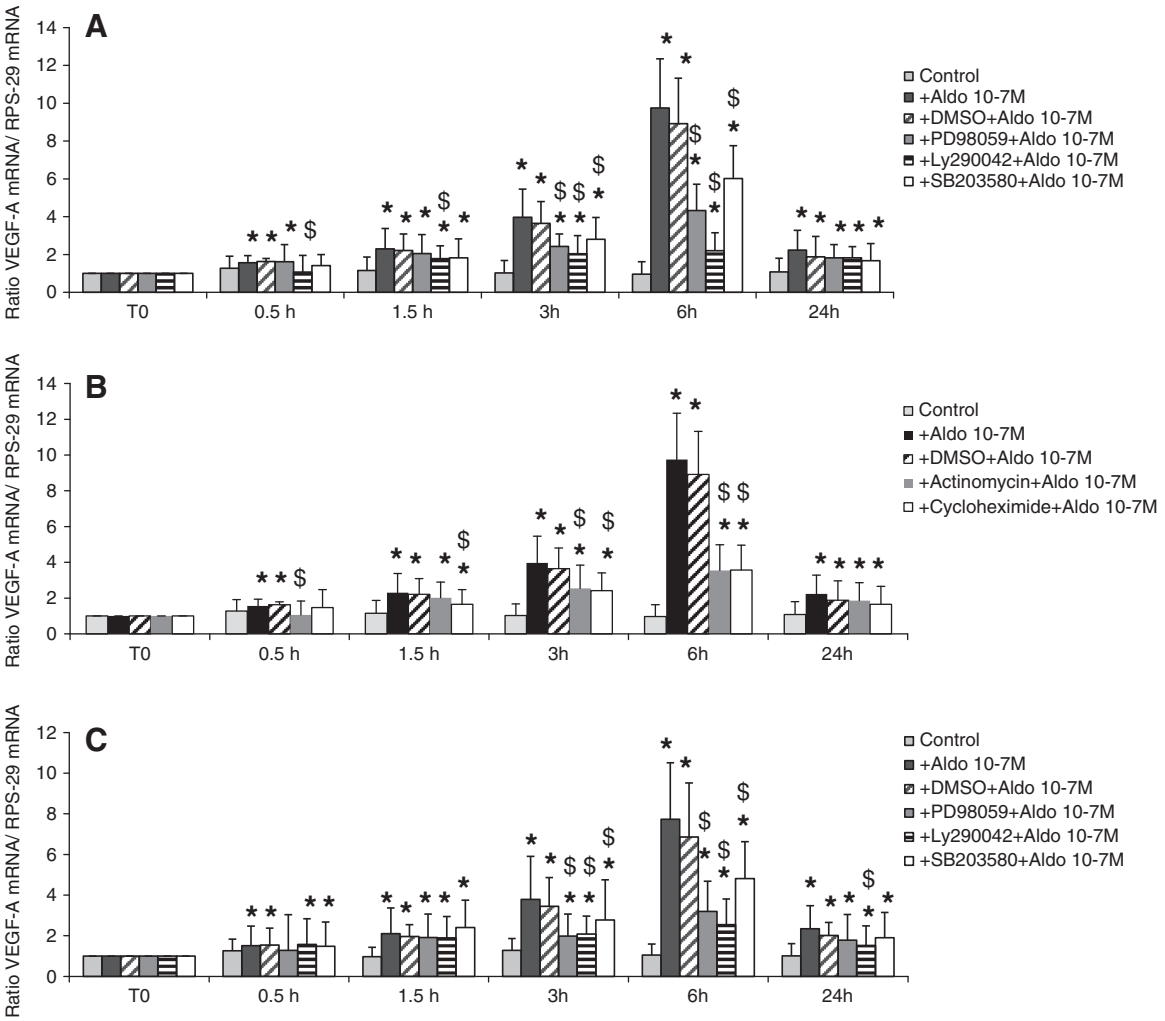

Fig. 2. Aldosterone increases VEGF-A mRNA levels in a PI3K, ERK1/2 and p38 dependent pathway in HL-60 cells and in neutrophils. This up-regulation requires for a part a de novo RNA and protein synthesis. HL-60 cells and neutrophils were preincubated for 30 min at 37 °C with PD98059, a MEK 1 inhibitor, Ly290042, a PI3K inhibitor, SB203580, a p38 MAPK inhibitor, or with DMSO (solvent) and then stimulated with aldosterone at 10^{-7} M for different time periods. Two other inhibitors, cycloheximide (protein synthesis inhibitor) or actinomycin (RNA synthesis inhibitor) were tested on HL-60 cells. All inhibitors were used at the same concentration (10 μM) except actinomycin (0.01 μg/mL). For both cell types, cells were collected for RNA extraction 30 min, 1 h 30 min, 3 h, 6 h, and 24 h after aldosterone addition. cDNA synthesis and semi-quantitative real time PCR with RPS-29 and VEGF-A primers were realised. Results are expressed as ratios of VEGF-A mRNA/RPS-29 mRNA and are representative of a minimum of three independent experiments. Arbitrary units were used and the mean value of 1 was attributed to the duration 0 min. (A, B): Ratios of VEGF-A mRNA/RPS-29 mRNA in HL-60 cells. The error bars are the S.D. and "*"represents a significant difference ($P<0.05$) between control and aldosterone-treated cells, $^{\$}P<0.05$ vs DMSO + aldosterone at 10^{-7} M. (C): Ratios of VEGF-A mRNA/RPS-29 mRNA in polymorphonuclear cells. The error bars are the S.D. and "*"represents a significant difference ($P<0.05$) between control and aldosterone-treated cells, $^{\$}P<0.05$ vs DMSO + aldosterone at 10^{-7} M.

blocking, membranes were incubated with an anti-phospho ERK 1/2 or an anti-phospho p38 MAPK rabbit antibody (Cell Signaling Technology). Membranes were washed and incubated with an anti-rabbit IgG-peroxidase conjugate (Jackson immunolaboratories, West Grove, USA). To determine the amounts of phosphorylated ERK 1/2 or p38 MAPK, blots were stripped and reprobed using respectively an anti-total ERK1/2 or an anti-total p38 MAPK rabbit antibody (Cell Signaling) followed with an anti-rabbit IgG-peroxidase conjugate (Jackson immunolaboratories). Each membrane was subsequently washed and immunocomplexes were visualised by chemiluminescence (LAS 4000 imager, Fuji, Bois d'Arcy, France). The signals were analysed by densitometry (Multi-Gauge, Fuji).

2.7. Statistical and data analysis

All experiments were repeated a minimum of three times. All data are given as mean ± S.D and a Mann–Whitney *U* test was used. A statistically significant difference was set at $P<0.05$.

3. Results

3.1. Effect of aldosterone on VEGF-A mRNA levels in HL-60 and in human polymorphonuclear cells

HL-60 cells were incubated with aldosterone (Aldo) at 10^{-7}, 10^{-8} or 10^{-9} M for different time periods. As shown in Fig. 1A, aldosterone increased VEGF-A mRNA expression in HL-60 cells. This up-regulation appeared as early as 30 min and reached a peak at 6 h whatever aldosterone concentration used. At 6 h, VEGF-A mRNA levels had increased approximately 9.75-fold with aldosterone at 10^{-7} M, 6.9-fold with 10^{-8} M and 4.6-fold with 10^{-9} M compared to unstimulated cells. This increase was also dose-dependent. Thus, aldosterone up-regulates VEGF-A mRNA expression in HL-60 cells in a concentration- and time-dependent manner.

In order to verify that our results were not restricted to the HL-60 cells, human polymorphonuclear leukocytes were used. As shown in Fig. 1B, aldosterone up-regulated VEGF-A mRNA in human neutrophils. This increase was time- and dose-dependent as for the HL-60 cells. Precisely, in the presence of aldosterone at 10^{-7} M, VEGF-A mRNA peaked at approximately 6 h and was of 7.7-fold compared to untreated polymorphonuclear cells. This phenomenon was concentration-dependent since with aldosterone at 10^{-8} M, the increase was only 4.5-fold. Thus, in human neutrophils, VEGF-A mRNA up-regulation mediated by aldosterone was time- and dose-dependent, as observed for the HL-60 cells.

3.2. Effects of different inhibitors on VEGF-A mRNA increase induced by aldosterone

The putative transduction signal pathways used by aldosterone to induce this VEGF-A mRNA up-regulation were further investigated. HL-60 cells were pre-incubated with inhibitors (all at 10 μM) or their solvent (DMSO) and 30 min after, aldosterone at 10^{-7} M was added. Eventual effects of inhibitors and DMSO on cell viability and on VEGF-A mRNA expression were respectively tested by Annexin V assays and real time PCR. At the concentrations used, neither inhibitor nor DMSO had a significant influence on cell death and on VEGF-A mRNA expression (data not shown). Pre-treatment with Ly294002, a PI3K inhibitor, or with PD98059, a MEK1 inhibitor, attenuated significantly aldosterone-increased VEGF-A mRNA ($P<0.05$; Fig. 2A). With SB203580, a p38 inhibitor, this inhibition was also significant but less pronounced compared with ERK1/2 and PI3K inhibition (Fig. 2A). In fact, the increase in VEGF-A mRNA expression, 6 h after aldosterone addition, was only 2.2-fold in the presence of Ly294002, 4.3-fold with PD98059 and 6-fold with SB203580, instead of 9.75-fold observed with aldosterone at 10^{-7} M alone (Fig. 2A).

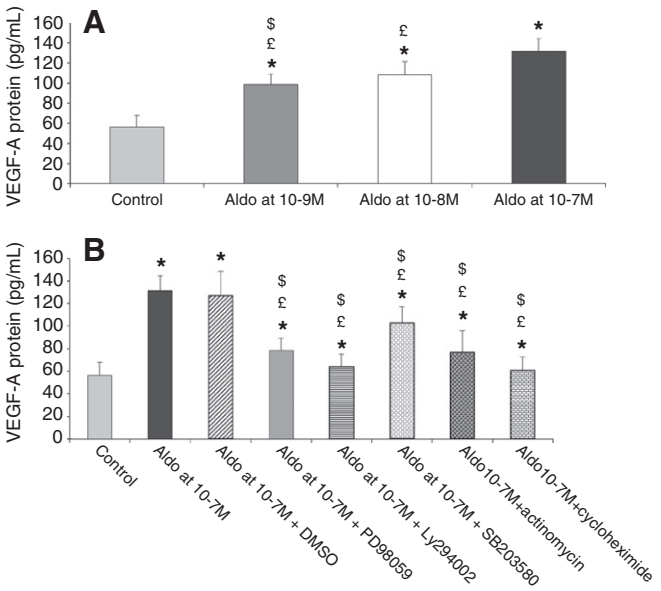

Fig. 3. Aldosterone increases VEGF-A protein release in HL-60 cell culture supernatants in a concentration- dependent manner. The PI3K, MEK1/2 inhibitors and, to a lesser extent p38 inhibitor, block this up-regulation. This phenomenon depends partially of a de novo RNA and protein synthesis. (A): HL-60 cells were stimulated with aldosterone at 10^{-7}, 10^{-8} or 10^{-9} M and culture supernatants were collected 24 h later for ELISA tests. This experiment was repeated in duplicate three to five times. The bars are the S.D. and $P<0.05$ was considered as significant. *$P<0.05$ vs control, $^{£}P<0.05$ vs aldosterone at 10^{-7} M and $^{\$}P<0.05$ vs aldosterone at 10^{-8} M. (B): HL-60 cells were pre-treated with an inhibitor (10 μM each except actinomycin at 0.01 μg/mL) for 30 min at 37 °C and then incubated with aldosterone at 10^{-7} M. Culture supernatants were recovered 24 h later for ELISA tests. Data are presented as the mean ± S.D. of experiments repeated independently three to five times in duplicate. *$P<0.05$ vs control, $^{£}P<0.05$ vs aldosterone at 10^{-7} M, $^{\$}P<0.05$ vs DMSO + aldosterone at 10^{-7} M.

Thereafter, we studied an eventual requirement of de novo RNA and/or protein synthesis for this VEGF-A mRNA up-regulation. Pre-incubation with actinomycin at 0.01 μg/mL, an RNA synthesis inhibitor, or with cycloheximide at 10 μM, a protein synthesis inhibitor decreased significantly VEGF-A mRNA up-regulation orchestrated by aldosterone. Pre-incubated cells increased their VEGF-A mRNA approximately 3.5-fold for both inhibitors compared to 9.75-fold with aldosterone at 10^{-7} M alone (Fig. 2B). Thus, aldosterone increased VEGF-A mRNA levels in HL-60 cells via a PI3K, ERK1/2 and p38 dependent-activation pathway. Moreover, this phenomenon appeared to need de novo RNA and protein synthesis.

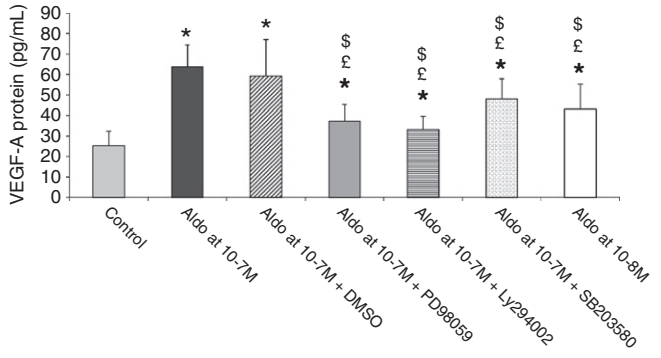

Fig. 4. Aldosterone upregulates VEGF-A protein excretion in neutrophil culture supernatants in a concentration-dependent manner and requires the activation of the PI3K, ERK1/2 and p38 dependent pathways. Neutrophils were incubated with aldosterone and inhibitors in the same conditions as for HL-60 cells. Data are presented as the mean ± S.D. of experiments repeated independently three to five times in duplicate. *$P<0.05$ vs control, $^{£}P<0.05$ vs aldosterone 10^{-7} M, $^{\$}P<0.05$ vs DMSO + aldosterone at 10^{-7} M.

As VEGF-A mRNA up-regulation orchestrated by aldosterone was shared by the HL-60 line and polymorphonuclear cells, we examined if the transduction signal pathways were also preserved between both cell types. Neutrophils were incubated with inhibitors or DMSO and 30 min after, aldosterone was added, as for the HL-60 cells. Pre-treatment of neutrophils with Ly294002, PD98059 or SB203580 diminished significantly the aldosterone-induced VEGF-A mRNA level increase (Fig. 2C). More precisely, 6 h after hormone addition, VEGF-A mRNA increased expression by aldosterone at 10^{-7} M in the presence of Ly294002 was only 2.5-fold, 3.2-fold with PD98059 and 4.8-fold with SB203580, compared with 7.7-fold obtained with aldosterone at 10^{-7} M (Fig. 2C). Thus, in polymorphonuclear cells, aldosterone upregulated VEGF-A mRNA in a PI3K, ERK1/2 and p38 kinase-dependent manner as for HL-60 cells.

3.3. Effect of aldosterone on VEGF-A protein expression in HL-60 cells

HL-60 cells were treated with aldosterone at 10^{-7}, 10^{-8} or 10^{-9} M and cell culture supernatants were collected 24 h later. ELISA tests were performed to analyse total VEGF-A. On average, untreated cells released 56 pg/mL (±11.5) of VEGF-A for 0.8 10^6 cells/mL. In the presence of aldosterone at 10^{-7} M, VEGF-A protein concentration was 131.5 pg/mL (±13; 2.33-fold). Aldosterone used at 10^{-8} or 10^{-9} M increased VEGF-A protein production, respectively 1.9-fold (108.4 pg/mL±13.3) and 1.7-fold (98.6 pg/mL±10), compared to untreated cells (Fig. 3A). Thus, as for VEGF-A mRNA, VEGF-A protein level up-regulation by aldosterone was concentration-dependent.

We focused on the signalling pathways used by aldosterone to increase VEGF-A protein in cell culture supernatants. HL-60 cells were pre-treated with Ly294002, PD98059, or SB203580 transduction signal inhibitors (10 μM each) or DMSO and 30 min after, aldosterone at 10^{-7} M was added as for the VEGF-A mRNA experiments. Cell culture supernatants were taken out 24 h later for VEGF-A ELISA tests. HL-60 cells pre-treatment with these inhibitors decreased aldosterone-induced VEGF-A protein expression. VEGF-A protein production enhancement induced by DMSO + aldosterone at 10^{-7} M (126.9 pg/mL±21) compared to control (56 pg/mL±11.5) was strongly diminished in the presence of these inhibitors: namely 63.9 pg/mL (±11.1) with Ly294002, 78.2 pg/mL (±11) with PD98059. The increase in VEGF-A protein production was only attenuated significantly in the presence of SB203580 (102 pg/mL±19.5), (Fig. 3B). Aldosterone activated PI3K, ERK 1/2 and p38 MAPK pathways to up-regulate VEGF-A protein production in HL-60 cells.

Finally, we studied if this aldosterone-increased VEGF-A protein expression in cell culture supernatants was due to de novo RNA and/or protein synthesis. In the presence of actinomycin at 0.01 μg/mL and in the presence of cycloheximide at 10 μM, the VEGF-A increase mediated by aldosterone was reduced approximately by half (respectively 76.6 pg/mL±19.5 and 60.6 pg/mL±12.1 compared to 126.9 pg/mL±21). Hence, this VEGF-A excretion was partly due to de novo RNA and protein expression (Fig. 3B).

3.4. Effect of aldosterone on VEGF-A protein expression in human polymorphonuclear cells

We further investigated if VEGF-A mRNA up-regulation by aldosterone in neutrophils was followed by an enhanced VEGF-A protein expression. We incubated these cells (0.8 10^6 cells/mL) with aldosterone at 10^{-7} M or at 10^{-8} M during 24 h. Cells' supernatants were used to realise VEGF-A ELISA tests. As shown in Fig. 4, aldosterone was able to up-regulate neutrophil VEGF-A protein release and this effect appeared concentration-dependent: 2.5-fold with aldosterone at 10^{-7} M (63.8 pg/mL±10.6) and 1.7-fold with aldosterone at 10^{-8} M (43 pg/mL±12.2) compared to untreated neutrophils (25.5 pg/mL±6.9). Signal transduction inhibitors (Ly294002, PD98059 and SB203580) added 30 min before aldosterone at 10^{-7} M decreased VEGF-A protein excretion. In detail, with Ly294002, the VEGF-A increase was only 1.3-fold (33 pg/mL±6.5) compared to DMSO + aldosterone at 10^{-7} M (2.35-fold, 59.1 pg/mL±18), with PD98059 a 1.47-fold (37.4 pg/mL±8) and with SB203580 a 1.88-fold (48 pg/mL±9.8) increase. Thus, aldosterone stimulated VEGF-A protein excretion in neutrophils in a PI3K, ERK1/2 and p38 dependent pathway, as observed for HL-60 cells.

3.5. Analysis of the aldosterone stimulation pathway by Western-blot

To corroborate these findings, we analysed by Western blot the ERK 1/2 and p38 MAPK phosphorylation status after aldosterone addition. HL-60 cells were incubated with aldosterone at 10^{-7} M during different time periods. Results presented in Fig. 5 show that

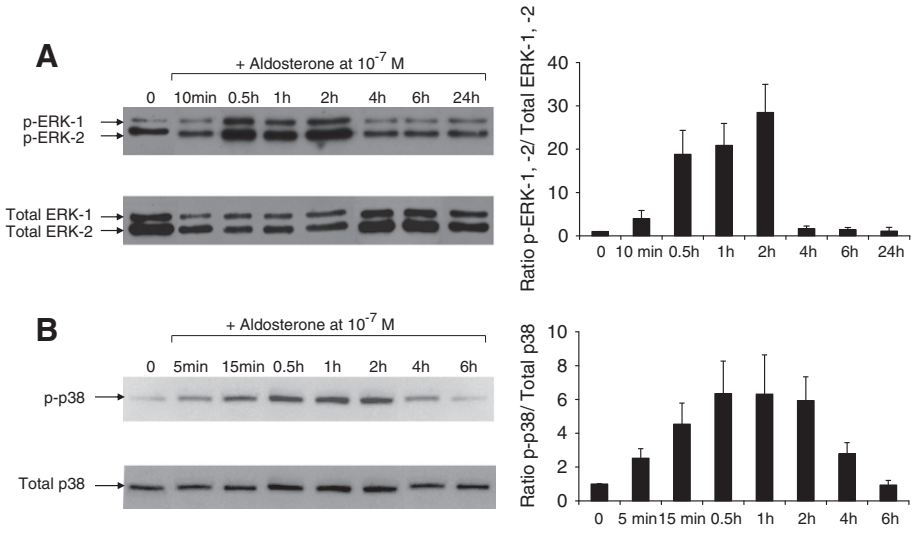

Fig. 5. Effects of aldosterone on ERK1/2 and p38 MAPK phosphorylation status in HL-60 cells. HL-60 cells were incubated with aldosterone at 10^{-7} M for different time periods. After centrifugation, cells were lysed and proteins were quantified. Total cell proteins were separated by SDS–PAGE and analysed by Western blotting. Phosphorylation of ERK-1, ERK-2 and p38 MAPK was studied respectively by using an anti-phospho ERK-1/-2 and an anti-phospho-p38 MAPK rabbit antibody. Densitometric analyses were performed to study the relative changes in their phosphorylation status. To control protein loading, membranes were stripped and reprobed respectively with an anti-total ERK1/2 and an anti-total p38 MAPK rabbit antibody. (A): Phosphorylation of ERK 1/2 (p-ERK-1, -2) and its control "Total ERK-1, -2". (B): Phosphorylation of p38 MAPK (p-p38) and its control "Total p38 MAPK". The experiment shown is representative of four independent experiments.

ERK1/2 phosphorylation had started by 30 min after aldosterone addition and peaked approximately 2 h later. A transient increase in ERK1/2 phosphorylation was seen 2 min after aldosterone addition (data not shown). For p38 MAPK, phosphorylation started approximately 15 min after hormone addition and finished approximately 2 h later. Thus, Western blot analyses were in agreement with RT-PCR and ELISA experiments.

3.6. Effect of spironolactone on VEGF-A mRNA and protein increase induced by aldosterone in HL-60 cells

We used an aldosterone antagonist, spironolactone (Spiro), to investigate if this molecule could counteract aldosterone effects on VEGF-A upregulation. Spironolactone was used at 10^{-7} or 10^{-6} M on HL-60 cells and 30 min later, aldosterone at 10^{-7} M was added. RNA extraction was performed 6 h after aldosterone addition when the VEGF-A mRNA peak occurred (Fig. 1). For VEGF-A protein analysis, HL-60 cell culture supernatants were collected 24 h after. As shown in Fig. 6A, spironolactone inhibited partially and dose-dependently the aldosterone effects on VEGF-A mRNA and protein levels. Precisely, the VEGF-A mRNA peak at 6 h was reduced approximately by half in the presence of spironolactone at 10^{-7} M (5.6-fold vs 10.1-fold), and by three quarters in the presence of spironolactone at 10^{-6} M (2.65-fold vs 10.1-fold). At the VEGF-A protein level, this reduction was respectively 28% (95.6 pg/mL ± 11.2 vs 131.5 pg/mL ± 13) and 42% (77.7 pg/mL ± 10.8 vs 131.5 pg/mL ± 13); (Fig. 6B). Thus, aldosterone uses mineralocorticoid receptors to enhance VEGF-A mRNA and protein production in HL-60 cells.

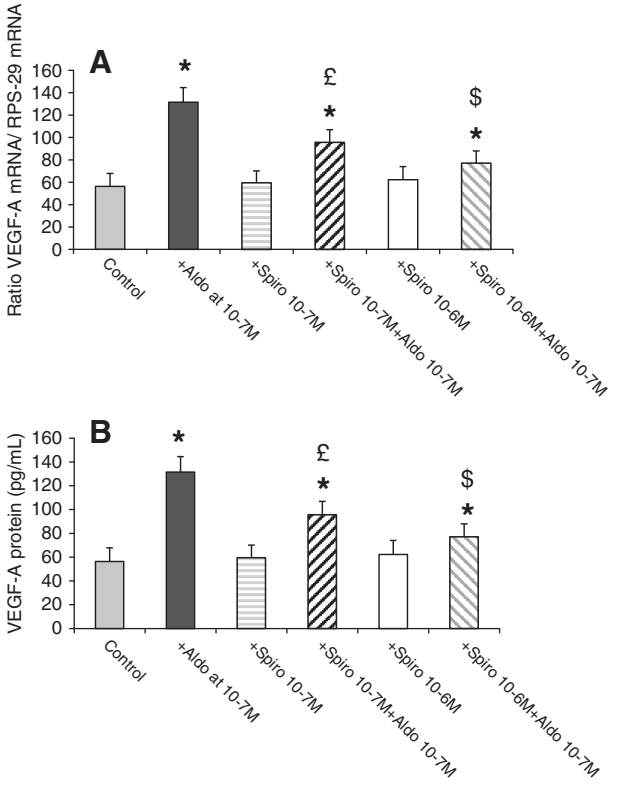

Fig. 6. Effect of spironolactone on VEGF-A mRNA and protein upregulation orchestrated by aldosterone. HL-60 cells were incubated with spironolactone (Spiro) at 10^{-6} M or 10^{-7} M and 30 min later aldosterone at 10^{-7} M was added or not to cell culture medium. (A): For RNA analysis, 6 h after, HL-60 cells were lysed, RNAs were extracted, reverse transcribed and submitted to semi quantitative real-time PCR. The results are expressed as ratios of VEGF-A mRNA/RPS-29 mRNA and representative of a minimum of three separate experiments. Arbitrary units were used and the mean value of 1 was attributed to the duration 0 min. The error bars are S.D. and "*"represents a significant difference ($P<0.05$) between control and aldosterone-treated cells, $^{£}P<0.05$ spironolactone at 10^{-7} M + aldosterone at 10^{-7} M vs aldosterone at 10^{-7} M and $^{\$}P<0.05$ spironolactone at 10^{-6} M + aldosterone at 10^{-7} M vs aldosterone at 10^{-7} M. (B): For VEGF-A protein analysis, cell culture supernatants were taken out 24 h later after aldosterone addition. The error bars are the S.D. and "*"represents a significant difference ($P<0.05$) between control and aldosterone-treated cells, $^{£}P<0.05$ spironolactone at 10^{-7} M + aldosterone at 10^{-7} M vs aldosterone at 10^{-7} M and $^{\$}P<0.05$ spironolactone at 10^{-6} M + aldosterone at 10^{-7} M vs aldosterone at 10^{-7} M.

4. Discussion

Over the past few years, it has become clear that aldosterone/mineralocorticoid receptors possess hypertensive, fibrotic effects and can exert important inflammatory effects in the vasculature system and in organs such as the kidneys and the heart. Moreover, different data have demonstrated that the hormone exerts proangiogenic effects on damaged vascular wall [15,16]. Immune cells are intimately linked to all these phenomena and they communicate with stressed vascular cells to repair the injured tissue. Nevertheless, little data exist about aldosterone/mineralocorticoid receptors and leukocytes. The purpose of our study was to investigate if aldosterone exerts a potential proangiogenic role in leukocytes and especially in neutrophils which possess mineralocorticoid receptors and are present in the altered tissue from the beginning to the inflammation resolution. In fact, in this process angiogenesis plays a crucial role in tissue regeneration. Neutrophils are able to produce different (pro)angiogenic molecules, notably VEGF-A in large amounts. We therefore investigated if aldosterone was able to affect neutrophil VEGF-A production. We were thus able to demonstrate that aldosterone upregulates VEGF-A production in the granulocyte-differentiated HL-60 cells and in human blood polymorphonuclear leukocytes. This cell line constitutes also a good model for this type of study. This increase occurred both at the mRNA and at the protein level and was dose- and time-dependent. For both cell types, the VEGF-A mRNA increase had started by 30 min after aldosterone addition and peaked around 6 h later. Twenty-four hours after, VEGF-A excretion was still increased in cell culture supernatants. Studies with transduction signal inhibitors demonstrated that PI3K and ERK1/2 activation pathways were implicated in this up-regulation and to a lesser extent the p38 MAP kinase pathway. Western-blotting analyses showed that ERK1/2 and p38 MAPK pathways were activated. Nevertheless, ERK1/2 activation seemed to be the most potent. Interestingly, this powerful ERK1/2 activation is shared by different cell types such as renal cells and myocytes [30,31]. We have remarked that ERK1/2 activation was biphasic: a first activation at 2 min, fugacious, and a second which began approximately 30 min after aldosterone addition and which endured. The p38 MAP kinase activation pathway by aldosterone has also been shown in myocytes and in smooth muscle cells [31,13]. As these two pathways are activated, and for ERK1/2 on both occasions, we cannot exclude that different events arise. For example, at first, mineralocorticoid receptors could be activated by aldosterone and then superoxide anion and/or pro-inflammatory cytokines could be released that in turn would activate cells by the bias of these MAP kinase pathways. These molecules have been shown to activate ERK1/2 and p38 MAPK pathways in neutrophils [32,33]. One other possibility (which does not exclude the first) is that in neutrophils, as in other cells, epidermal growth factor and mineralocorticoid receptors could be colocalized at the plasma membrane and could be able to transactivate signals [34]. An argument in favour of this is that when transactivation occurs, the ERK1/2 pathway is activated. We have shown that actinomycin and cycloheximide, respectively RNA and protein synthesis inhibitors, diminished significantly the VEGF-A increase mediated by aldosterone but the inhibition was only partial. As an increase in inhibitor concentration had consequences on cell viability, we do not know if this VEGF-A upregulation was due entirely to de novo synthesis. It is possible that part of the released VEGF-A comes from an increase in mRNA half-life, direct neutrophil degranulation and/or membrane VEGF-A cleavage [35,36]. It should be noted that a discrepancy exists in both cell types between the

VEGF-A mRNA rate increase (about ×10 at 6 h) and the excreted VEGF-A protein rate (about ×2.5 at 24 h). Different possible explanations exist: an important part of VEGF-A mRNA is non-translated and/or neoformed VEGF-A proteins are stored in neutrophil granules and/or remain bound to neutrophil membranes. Further, we investigated an eventual requirement of mineralocorticoid receptors on VEGF-A upregulation. The use of an aldosterone antagonist, spironolactone, permitted us to show that the VEGF-A level increase obtained with aldosterone in HL-60 cells was dose-dependently diminished but not entirely abrogated in the presence of the spironolactone. So, a non-negligible part of the VEGF-A increased production is mediated by mineralocorticoid receptor activation. Now, it remains to be determined if aldosterone is able to increase other pro-angiogenic molecules like VEGF-C and/or VEGF-D which can be produced by polymorphonuclear cells. Moreover, a number of studies have demonstrated that aldosterone potentiates the angiotensin II-induced proinflammatory role now that neutrophils are known to possess AT1 receptors [37]. Pupilli et al. have shown that mesangial cells treated with angiotensin II enhanced their VEGF production [38]. The study by Zhao et al. demonstrated the role of VEGF in vascular inflammation mediated by angiotensin II [39]. More recently, it has been reported that aldosterone needs angiotensin type 1a receptors to active signalling pathways in vascular smooth muscle cells [40]. So, it will be very important to investigate if (and how) angiotensin II exerts any effect on VEGF-A regulation, and whether or not it cooperates with aldosterone in neutrophils. Other immune cells such as monocytes/macrophages are also involved in wound healing, inflammation and neoangiogenesis. They possess mineralocorticoid receptors and produce large amounts of VEGF-A, so it will be important to investigate if aldosterone is able to regulate also their VEGF-A production.

In conclusion, we have shown that aldosterone, as well as being implicated in different phenomena such as fluid homeostasis, fibrotic and hypertrophic effects in organs, could also favour neoangiogenesis via its effects on immune cells such as neutrophils. Aldosterone could play a positive role by favouring immune cell adhesion and angiogenesis in “normal” cases of tissue repair, and could promote neovessel uncontrolled growth in deleterious cases, detrimental to the organism.

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