Aldosterone Abrogates Nuclear Factor κB–Mediated Tumor Necrosis Factor α Production in Human Neutrophils via the Mineralocorticoid Receptor

Astrid Bergmann, Claudia Eulenberg, Maren Wellner, Susanne Rolle, Friedrich Luft, Ralph Kettritz

Abstract—Mineralocorticoid receptor (MR) activation by aldosterone controls salt homeostasis and inflammation in several tissues and cell types. Whether or not a functional MR exists in polymorphonuclear neutrophils is unknown. We investigated the hypothesis that aldosterone modulates inflammatory neutrophil responses via the MR. By flow cytometry, Western blot analysis, and microscopy, we found that neutrophils possess MR. Preincubation with aldosterone (10^{-11} to 10^{-6} M) dose-dependently inhibited nuclear factor κB activation in interleukin (IL)-8– and granulocyte/macrophage colony-stimulating factor–treated neutrophils on fibronectin by IkBα Western blotting, electrophoretic mobility shift assay, and RT-PCR for IkBα mRNA. Aldosterone had no effect on tumor necrosis factor α– and lipopolysaccharide-mediated nuclear factor κB activation or on IL-8– and granulocyte/macrophage colony-stimulating factor–induced extracellular signal-regulated kinase, p38 mitogen-activated protein kinase, or phosphatidylinositol 3-kinase/Akt activation. Spironolactone prevented nuclear factor κB inhibition, indicating an MR-specific aldosterone effect. By RT-PCR, we found that neutrophils have 11β-hydroxysteroid dehydrogenase. Tumor necrosis factor α, which is controlled by nuclear factor κB, increased in the cell supernatant with IL-8 treatment. Aldosterone completely prevented this effect. RT-PCR showed a strong tumor necrosis factor α mRNA increase with IL-8 that was blocked by aldosterone, excluding the possibility that the tumor necrosis factor α increase was merely a consequence of secretion. Finally, conditioned medium from IL-8–treated neutrophils increased intercellular adhesion molecule–1 expression on endothelial cells and subsequently the adhesion of IL-8–treated neutrophils to endothelial cells. These effects were reduced when conditioned medium from aldosterone-pretreated neutrophils was used, and spironolactone blocked the aldosterone effect. Our data indicate that a functional MR exists in neutrophils mediating antiinflammatory effects that are at work when neutrophils interact with endothelial cells. These data could be relevant to MR-blockade treatment protocols. (Hypertension. 2010;55:370-379.)

Key Words: aldosterone ■ mineralocorticoid receptor ■ endothelial cells ■ polymorphonuclear neutrophils ■ inflammation ■ NF-κB ■ adhesion

The pivotal role of aldosterone in extracellular volume control is well established. Aldosterone specifically binds to the cytoplasmic mineralocorticoid receptor (MR), resulting in nuclear translocation of the ligand-receptor complex and subsequently in transcription of genes involved in sodium reabsorption and potassium excretion. Studies have revealed a host of blood pressure–independent MR-mediated aldosterone effects. Some of these effects do not require novel gene transcription. These nongenomic effects involve rapid activation of signaling pathways such as extracellular signal-regulated kinase (ERK), c-Raf, and nuclear factor κB (NF-κB). Compared with the extensive data on aldosterone in nonblood cells, its role in mononuclear and polymorphonuclear leukocytes is largely unknown. Mononuclear cells, such as monocytes and lymphocytes, possess an MR that binds aldosterone with high specificity, regulating sodium and potassium flux, as well as plasminogen activator inhibitor-1 and p22 phox expression in these cells.1–6 Aldosterone administration in rats resulted in increased peripheral blood mononuclear cell activation.7–8 In contrast to the sparse data in mononuclear cells, actually no data on the MR exist in polymorphonuclear cells (neutrophils). Interaction between the renin-angiotensin-aldosterone system and neutrophils may occur in a variety of conditions, including cardiovascular diseases. For example, neutrophils participate in acute coronary syndrome and are exposed to renin-angiotensin-aldosterone system components, as well as to cytokines, such as granulocyte/macrophage colony-stimulating factor (GM-CSF), interleukin (IL) 8, and tumor necrosis factor α (TNF-α).9 Several investigators found a decreased myeloperoxidase content in neutro-
phi of patients with unstable angina, indicating neutrophil activation. In fact, neutrophil number and cardiovascular death are positively correlated in ischemic vascular disease. We investigated the hypothesis that aldosterone modulates inflammatory neutrophil responses via the MR. Our data unequivocally show that the MR exists in human neutrophils. Aldosterone abrogated NF-κB–mediated TNF-α production in IL-8– and GM-CSF–stimulated neutrophils. This effect was inhibited by the MR blocker spironolactone. Finally, we show functional consequences for neutrophil adhesion to endothelial cells.

Methods

Materials

TNF-α, IL-8, GM-CSF, and the TNF-α ELISA were obtained from R&D Systems (Wiesbaden-Nordenstedt, Germany). Histopaque, propidium iodide, lipopolysaccharide (LPS), aldosterone, spironolactone cycloheximide, actinomycin D, and dexamethasone were from Sigma (Deisenhofen, Germany). Hanks’ balanced salt solution (HBSS), PBS, and trypan blue were from PAA (Cölbe, Germany), and dextran was purchased from Roth (Karlsruhe, Germany). Annexin V was from BD Bioscience (Heidelberg, Germany). The anti-MR monoclonal antibodies (mAbs) (clone ID5 and clone 6G1) were a generous gift from Dr. Gomez-Sanchez (Jackson, Miss), and were described elsewhere; the anti-CD4 was from Beckman Coulter (Heidelberg, Germany); the anti-proteinase 3 (anti-PR3) mAb CLB12.8 was from Hiss (Freiburg, Germany); horseradish peroxidase–labeled donkey anti-rabbit IgG was from GE Healthcare; fluorescein isothiocyanate (FITC)-conjugated F(ab′)2 fragments of goat anti-mouse IgG and IgG1 isotype control were from Dako (Hamburg, Germany); phosphospecific antibodies to Akt (S473), ERK, p38, and IκBα were from Cell Signaling (Frankfurt/Main, Germany); the mouse mAb to intercellular adhesion molecule-1 (ICAM-1) antibody (Ab) was from ebioscience (Hatfield, UK); and the actin antibody was from Santa Cruz (Heidelberg, Germany). LY294002 and SB202190 were from Calbiochem (Schwalbach, Germany); the mouse mAb to intercellular adhesion molecule-1 (ICAM-1) antibody (Ab) was from ebioscience (Hatfield, UK). Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western blotting were performed as described. Total RNAs were isolated according to a Qiagen protocol including poly(dI-dC) and 20 mmol/L Hepes, containing 60 mmol/L KCl, 4% Ficoll, 5 mmol/L dithiothreitol, and 0.5 μL/μL nuclease-free BSA. Probes were subjected to electrophoresis on native 5% polyacrylamide gels and autoradiographed. The oligonucleotides for H2K were as follows: forward primer, 5′-GATCC-AGGGCTGGGGATTTCCCATCTTCACAGG-3′; reverse primer, 5′-GATCCTTGAGAGTGGAGATCCGACCCCTG-3′.

Confocal Microscopy

Neutrophils were fixed in paraformaldehyde and permeabilized in 0.2% saponin before being stained with antibodies to the MR (mAb rMR1-18 clone 1D5 and clone 6G1). After cells were washed, a FITC-labeled secondary anti-mouse Fab2 was added for 20 minutes. Microscopy was performed using a Leica TCS SP2 microscope equipped with a ×63 phase contrast plan-apochromat oil objective. Acquisition settings for all images were UV/488/543, and specific parameters for the fluorophores were as follows: FITC excitation at 488 nm light, detected with a 500 to 550 band-pass filter. Image acquisition was done sequentially in the line modus to allow for detection of the intracellularly located proteins. For analysis, results from at least 5 independent experiments were evaluated using MacBiophotonics ImageJ.

Flow Cytometry

MR expression on and in human neutrophils was determined by incubation with antibodies to MR (mAb rMR1-18 clone 1D5 and clone 6G1), CD4, and PR3, respectively, for 25 minutes on ice. Neutrophils were either left untreated or were permeabilized using 0.5% paraformaldehyde and 0.2% saponin. Cells were washed and resuspended in saponin/PBS and FITC-conjugated F(ab′)2 fragments of goat anti-mouse IgG for a further 25 minutes. Endothelial cells (EC) were stained with the ICAM-1 antibody for 25 minutes on ice, washed, and stained with the FITC-conjugated F(ab′)2 fragments of goat anti-mouse IgG for a further 25 minutes. After being washed, samples were assayed using a FACScan instrument (Becton Dickinson).

Quantitative RT-PCR

Total RNAs were isolated according to a Qiagen protocol including DNase treatment. Quantitative RT-PCR was performed as described previously using TaqMan technology (Applied Biosystems, Wiesbaden, Germany). Reverse transcription was carried out according to the Superscript protocol (Invitrogen, De Schelp, The Netherlands). The following oligonucleotides were used for TNF-α: forward primer, 5′-GTCGCTTGTTCTCCACAGGTC-3′; reverse primer, 5′-GCCATAGGAAAGCCTTGTTGC-3′; and the probe Fam 5′-TCTTCCTGTACGTGCGCCGAG-3′. For human 18S, we used the forward primer 5′-CCCTGTAATGGCGCGGACTG-3′, the reverse primer 5′-AGGAGTGAACACCAGGTCAAGGA-3′, and the probe Fam 5′-CCCTACCCCTGGAGCTGACTG-3′ (Tama). For human IL-8, we used the forward primer 5′-CCCTGTAATGGCGCGGACTG-3′, the reverse primer 5′-AGGAGTGAACACCAGGTCAAGGA-3′, and the probe Fam 5′-CCCTACCCCTGGAGCTGACTG-3′ (Tama). We used the SYBR Green method for the detection of 11β-hydroxysteroid dehydrogenase (11β-HSD2). We used the following oligonucleotides: forward primer, 5′-GCATCATACCCGGTCTGAC-3′; reverse primer, 5′-AAGGATGAATAGGACCTGTTGGTGC-3′; and the probe Fam 5′-CTTCCCTGTACGGGCTTGGAC-3′-Tama. For human IL-8, we used the forward primer 5′-CCCTGTAATGGCGCGGACTG-3′, the reverse primer 5′-AGGAGTGAACACCAGGTCAAGGA-3′, and the probe Fam 5′-CCCTACCCCTGGAGCTGACTG-3′ (Tama). For the verification of 11β-HSD2, the RT-PCR product was cloned, sequenced, and used as a positive control. RT-PCR and quantification were performed using an Applied Biosystems 7900 sequence detector and qPCR MasterMix Plus (Eurogentec). Each sample was measured in triplicate, and expression levels were normalized to human GAPDH housekeeper expres-
sion. Results were imported in an Excel spreadsheet and analyzed according to the standard curve method.

**EC Culture**

The human umbilical vein EC line EAhy926 was cultured in RPMI medium 1640 supplemented with 10% FCS, 4 mmol/L glutamine, 100 U/mL penicillin, and 10 μg/mL streptomycin at 37°C and 5% CO₂.

**TNF-α ELISA**

Neutrophils were incubated with the indicated stimuli in HBSS+/+ or FCS for 6 hours. Cells were spun down at 300g for 5 minutes. Supernatants were harvested and stored at −20°C until they were subjected to the TNF-α ELISA. Samples were diluted 1:20, and the ELISA was performed as suggested by the manufacturer (R&D Systems).

**Apoptosis**

After either annexin V staining or ethanol permeabilization and PI staining, flow cytometry was used to assess neutrophil apoptosis. Cells were analyzed using a FACScan instrument, and 10,000 events per sample were collected in list mode using CellQuest Pro software (BD Bioscience).

**Adhesion**

For adhesion experiments, EC were seeded at a density of 2×10⁴ cells per well in 96-well flat-bottomed microtiter plates (TPP, Trasadingen, Switzerland) 2 days before the assay was performed, and cells were grown to confluence. The RPMI medium 1640 was replaced by the neutrophil-conditioned medium (CM), which was incubated under the same conditions for 15 hours. Freshly prepared neutrophils were stained using 5-chloromethylfluorescein diacetate Celltracker Green (Molecular Probes) in HBSS++ for 15 minutes at 37°C, washed, and incubated with 100 nM IL-8 for a further 30 minutes at 37°C. The EC monolayer was gently washed three times with prewarmed HBSS++ before the addition of 2×10⁵ activated neutrophils. Cells were allowed to adhere to EC for 60 minutes at 37°C. After incubation, nonadherent cells were removed by 2 gentle washes with prewarmed HBSS++. The detection of adherent neutrophils was performed by fluorescence microscopy using a Nikon-Diaphot instrument (Tokyo, Japan). For each experimental condition, two different view fields per triplicate well were assessed using a ×10 phase-contrast objective. The images were quantitatively evaluated using the MacBiosphotonics ImageJ program.

**Statistical Analysis**

Results are given as mean±SEM. Comparisons between 2 groups were done using paired t tests. Comparisons between multiple groups were done using 1- or 2-way ANOVA as indicated. Specific differences between multiple groups were then determined by use of a Bonferroni post hoc test. Differences were considered significant at *P*<0.05.

**Results**

The first set of experiments focused on the detection of the MR in human neutrophils. We performed flow cytometry using 2 different MR-specific antibodies and 2 appropriate controls. Figure 1A shows that neither anti-MR antibodies nor anti-CD4 or an isotype control bound to viable nonpermeabilized neutrophils. An anti-PR3 antibody served as a positive control showing the expected bimodal surface staining. In contrast, after cell permeabilization, both anti-MR antibodies detected intracellular MR, whereas negative findings were obtained with the anti-CD4 and isotype control. Anti-PR3 staining showed a single positive peak, indicating complete permeabilization, because both the membrane-positive and membrane-negative neutrophil populations contain intracellular PR3. We performed Western blot analysis to further confirm the flow cytometry data. Figure 1B shows that the anti-MR antibody (MR1D5) detected the characteristic 107-kDa band. Vascular smooth muscle cells were analyzed in parallel and served as a positive control. Next, we performed fluorescence microscopy using an anti-MR-specific antibody and using an anti-CD4 antibody as a negative control. Figure 1C indicates intracellular staining with the anti-MR antibody that was not present with the unrelated control antibody. Together, these experiments show unequivocally that neutrophils possess the MR.

Next, we tested the effect of aldosterone pretreatment in the activation of major neutrophil signaling pathways in response to important cytokines, such as GM-CSF, IL-8, TNF-α, and LPS. We found previously that GM-CSF and IL-8 require integrin costimulation to activate NF-κB, whereas TNF-α and LPS do not need such costimulation. We first evaluated NF-κB activation by assessing the degradation of the NF-κB inhibitor (IκBα) using Western blot analysis. Our data show that aldosterone abrogates IκBα degradation in IL-8- and GM-CSF-treated neutrophils on fibronectin in a dose-dependent manner over a range of 10⁻¹¹ to 10⁻⁶ M, spanning the physiological and pathological range of aldosterone levels (Figure 2A and 2B). On the basis of these data and data from several published studies that used concentrations between 10 to 100 nM for in vitro experiments, we selected 10 nM aldosterone for further study. The results from 5 independent experiments with the corresponding statistics are shown in Figure 2C. To investigate whether or not the observed aldosterone effects occurred via the MR, we pretreated neutrophils with spironolactone. Figure 2D indicates that spironolactone blocked the aldosterone effect, but neither the p38 mitogen-activated protein kinase inhibitor SB202190 nor the phosphatidylinositol 3-kinase/Akt inhibitor (IκB) using Western blot (Figure 2F). An IκB Western blot time course study, using GM-CSF as an example, demonstrated that IL-8 has already induced IκB degradation in neutrophils on fibronectin after 30 minutes and that the subsequent NF-κB-dependent transcription results in new IκBα protein at later time points (Figure 2G). Finally, we performed electrophoretic mobility shift assay studies showing that IL-8 and GM-CSF result in increased NF-κB activation that is blocked by aldosterone.
In previous studies, we had carried out supershifts that established the bands as p50/p50 and p50/p65 complexes.\(^{15}\) With respect to cytokine and pathway specificity, we found that aldosterone did not block I\(\kappa\)B degradation in response to TNF-\(\alpha\) and LPS (Figure 3A). In contrast, when aldosterone-pretreated neutrophils, either in suspension or on fibronectin, were stimulated with GM-CSF or IL-8 and assayed for ERK, p38, and Akt phosphorylation, no inhibitory effect was found (Figure 3B). Spironolactone specifically blocks the MR. These data indicate that the aldosterone effects were cytokine and pathway specific and were mediated by the MR.

The next set of experiments compared the mineralocorticoid aldosterone and the glucocorticoid dexamethasone in parallel. Figure 4A depicts a typical Western blot analysis for I\(\kappa\)B degradation. The data show that aldosterone, but not dexamethasone, blocked NF-\(\kappa\)B activation in GM-CSF– and IL-8–treated neutrophils. In contrast, dexamethasone, but not aldosterone, affected neutrophil apoptosis. Our data indicate delayed constitutive neutrophil apoptosis with dexamethasone, but not with aldosterone, in 2 independent apoptosis assays (Figure 4B). We performed additional experiments investigating IL-8– and GM-CSF–delayed apoptosis in the absence or presence of aldosterone. The data show that both cytokines showed the well-known delay of neutrophil apoptosis and that aldosterone had no effect (Figure 4C). These data indicate that glucocorticoids and mineralocorticoids exhibit distinct effects in neutrophils, suggesting a high degree of MR and glucocorticoid receptor specificity.

When we assayed neutrophils for 11\(\beta\)-hydroxysteroid dehydrogenase 2 by RT-PCR, we obtained a polymerase chain reaction product that showed the typical 66-bp band in an agarose gel (Figure 4D).

Finally, we studied functional consequences of aldosterone-mediated NF-\(\kappa\)B inhibition, concentrating on IL-8 treatment. TNF-\(\alpha\) generation is controlled by NF-\(\kappa\)B, and we performed TNF-\(\alpha\) ELISAs in cell supernatants. The data...
Figure 2. The aldosterone effect on NF-κB activation in IL-8– and GM-CSF–treated neutrophils on fibronectin was studied by Western blot analysis of IκBα degradation. Neutrophils were pretreated for 30 minutes with increasing concentrations of aldosterone (Aldo; 10^{-11} to 10^{-6} M) before a 30-minute stimulation with IL-8 (A) or GM-CSF (B). Five independent experiments were performed using an aldosterone concentration at 10^{-8} M, and densitometric analysis of the IκBα expression is depicted as mean±SEM. Bu indicates buffer control; n.s., not significant; Aldo +, 10^{-8} M aldosterone; Aldo −, buffer (C). The effect of spironolactone pretreatment (10^{-6} M) on IκBα degradation was assessed in parallel with the p38 mitogen-activated protein kinase inhibitor SB202190 (SB) and the phosphatidylinositol 3-kinase/Akt inhibitor LY294002 (LY). A typical example of 3 experiments is depicted (D). Neutrophils were preincubated with Bu, 5 μg/mL actinomycin D (Act), or 2.5 μg/mL cycloheximide (CHX). After 30 minutes, Aldo + or Aldo − was added before stimulation with IL-8 or GM-CSF. IκBα degradation was not affected by Act or CHX (E). RT-PCR for IκBα was performed to assess the aldosterone effect on IL-8– and GM-CSF–induced NF-κB activation by an independent method (F). Total RNA was isolated from neutrophils pretreated for 30 minutes with 10^{-8} M aldosterone and subsequently cytokine-stimulated for 30 minutes. The values represent mean±SEM of 3 experiments. Neutrophils were stimulated with either GM-CSF or Bu, and IκBα protein was assessed by Western blotting for 240 minutes. A typical example of 2 experiments is given (G). Neutrophils were preincubated with 10^{-8} M aldosterone or buffer before GM-CSF and IL-8 treatment. After 30 minutes, NF-κB activation was assessed using electrophoretic mobility shift assay. A typical result of 2 experiments is shown. B indicates buffer; A, 10^{-8} M aldosterone (H).
demonstrate a strong increase of TNF-α in the supernatant of cytokine-treated cells and almost complete blockade by aldosterone treatment (Figure 5A). To exclude the possibility that this result was merely a consequence of TNF-α secretion, we performed RT-PCR experiments. We observed a 37-fold increase of TNF-α mRNA with IL-8 that was significantly decreased by aldosterone (Figure 5B).

We then used CM obtained from IL-8–stimulated neutrophils to treat EC and assayed the EC for ICAM-1 expression. EC that were incubated for 20 hours with CM from IL-8–treated neutrophils strongly upregulated ICAM-1 expression, as shown by flow cytometry. This effect was significantly reduced when CM was prepared from IL-8–stimulated neutrophils that were pretreated with aldosterone (Figure 6). In the last set of experiments, we coincubated EC that were treated overnight with CM from IL-8–stimulated neutrophils and assessed neutrophil adhesion. The data demonstrate that more neutrophils adhered to EC that were preincubated with CM from IL-8–stimulated neutrophils than to EC preincubated with CM from unstimulated cells. Moreover, when CM was obtained from IL-8–stimulated neutrophils that were pretreated with aldosterone, adhesion was significantly reduced (Figure 7). When spironolactone was added before aldosterone incubation and IL-8 treatment, the resulting CM was no longer able to promote neutrophil adhesion. We observed 55 ± 22 adherent neutrophils per field when EC were incubated with CM from cells treated with buffer, 57 ± 22 with aldosterone, and 47 ± 4 with spironolactone. Neutrophil adherence increased to 482 ± 97 when EC were incubated with CM from neutrophils treated with IL-8 and decreased to 63 ± 32 when aldosterone was added before IL-8. Spironolactone blocked aldosterone, resulting in 510 ± 62 adherent cells per field (n = 2). Together, these data indicate that IL-8–treated neutrophils on fibronectin generate a TNF-α–rich milieu and that this milieu results in ICAM-1 upregulation, allowing more IL-8–treated neutrophils to adhere. Aldosterone blocks this effect in an MR-dependent manner.

**Discussion**

Our study is the first to show that human neutrophils possess a functional MR. Neutrophils interact with extracellular matrix proteins during emigration from the bloodstream, and we found that aldosterone inhibits NF-κB activation in IL-8– and GM-CSF–stimulated neutrophils that interact with fibronectin. This effect was abrogated by spironolactone. Aldosterone strongly diminishes NF-κB-dependent TNF-α production in IL-8– and GM-CSF–stimulated neutrophils on fibronectin, resulting in reduced endothelial ICAM-1 expression and neutrophil adhesion to EC.

Several cytokines are upregulated during vascular inflammation, including in patients with acute myocardial infarction. Increased local and circulating levels of TNF-α, IL-8, and GM-CSF were reported, and the IL-8 blood concentration was even a significant predictor of subsequent heart failure. Neutrophils participate in vascular inflammation and therefore are exposed to these cytokines. We demonstrated previously that suspension neutrophils activate NF-κB in response to TNF-α and LPS, but not to IL-8 and GM-CSF. However, we found in subsequent studies that under conditions of activated integrins, genuine CD11b/CD18 and acquired GPIIb/IIIa cooperate with IL-8 and GM-CSF receptors, ultimately allowing neutrophil NF-κB activation. In the current study, we considered the possibility that cytokine-induced NF-κB activation, stimulating the generation of additional cytokines (eg, TNF-α) and thereby accelerating inflammation, was modified by the classical cardiovascular mediator aldosterone.

It was not known whether or not an MR exists in human neutrophils, and we undertook several approaches to detect the receptor. Using 2 different MR antibodies that have been extensively characterized by Gomez-Sanchez et al., we unequivocally show the presence of an intracellular MR by
flow cytometry, Western blot, and confocal microscopy. These new findings add an old receptor to the neutrophil armamentarium. In fact, the MR is ancient, and its affinity for aldosterone probably existed long before the hormone evolved.26 In contrast, the glucocorticoid receptor was long known to exist in neutrophils, and a variety of functions, such as delayed apoptosis and antiinflammatory effects, are well documented.15,27–30

Our next step was to investigate whether or not the MR was functional. Our data indicate that the natural ligand aldosterone and dexamethasone on IκBα degradation and apoptosis were compared in parallel. A, Neutrophils on fibronectin were preincubated with buffer (Bu), 10^{-6} M dexamethasone (D), or 10^{-8} M aldosterone (A) for 30 minutes before GM-CSF or IL-8 was added for another 30 minutes. A Western blot for IκBα was performed. Actin is shown as a loading control. A typical example of 2 independent experiments is shown. B, Neutrophils were preincubated with Bu, 10^{-6} MD, 10^{-8} MA. Cells were cultured for 20 hours, and apoptosis was estimated by annexin V and by analysis of the DNA content using the propidium iodide assay in ethanol-permeabilized cells. C, Neutrophils were preincubated with buffer (Aldo -) or 10^{-8} M aldosterone (Aldo +). After 30 minutes, Bu, IL-8, or GM-CSF (CSF) was added. Cells were cultured for 20 hours, and apoptosis was estimated by analysis of the DNA content using propidium iodide. Means±SEM of 5 experiments are depicted. *P<0.05: significantly different from buffer-treated controls (Bu). D, Neutrophils were assayed for 11β-hydroxysteroid dehydrogenase by RT-PCR using specific primer. The polymerase chain reaction product was then loaded onto an agarose gel, electrophoresed, and visualized by ethidium bromide. The 11β-hydroxysteroid dehydrogenase containing plasmid was used as a positive control and was assayed together with mRNA from 2 neutrophil preparations (PMN) and a negative control from which cDNA was omitted. A typical example of 2 experiments is depicted.

Figure 5. The aldosterone effect on TNF-α production in IL-8-treated neutrophils on fibronectin. A, Neutrophils were incubated with 10^{-8} M aldosterone for 30 minutes and subsequently stimulated with IL-8. After 6 hours, supernatants were harvested and assessed by TNF-α ELISA. Means±SEM of 5 experiments are depicted. B, Neutrophils were incubated with 10^{-8} M aldosterone for 30 minutes and subsequently stimulated with IL-8 for 30 minutes. Total RNA was isolated, and RT-PCR was performed using TNF-α-specific oligonucleotides. The values represent the mean±SEM of 5 experiments.

Flow cytometry, Western blot, and confocal microscopy. These new findings add an old receptor to the neutrophil armamentarium. In fact, the MR is ancient, and its affinity for aldosterone probably existed long before the hormone evolved.26 In contrast, the glucocorticoid receptor was long known to exist in neutrophils, and a variety of functions, such as delayed apoptosis and antiinflammatory effects, are well documented.15,27–30

Our next step was to investigate whether or not the MR was functional. Our data indicate that the natural ligand aldoste-
ronone completely blocked NF-κB activation in IL-8– and GM-CSF–treated neutrophils interacting with fibronectin. Interestingly, this effect was cytokine specific in that LPS and TNF-α still induced NF-κB activation in the presence of aldosterone. MR blockers such as spironolactone and eplerenone are widely used in cardiovascular medicine for their specific MR blocking effect in patients. We used spironolactone in our experiments to show that aldosterone indeed uses the MR for NF-κB abrogation. Thus, it is conceivable that treatment with spironolactone and eplerenone has side effects regarding neutrophil function that were heretofore not suspected. Aldosterone could help to “keep the neutrophils quiet,” and this effect might be inhibited by MR blockade. We are not aware of any studies that investigated this issue in clinical settings; however, future investigations should seriously consider this possibility. Spironolactone and eplerenone are considered vasculoprotective as a result of 2 large heart failure clinical trials.31,32 We have no evidence that such treatment compromises the host defense in an older population exposed to various challenges. Nonetheless, this possibility should be pursued on the basis of our data.

The new findings indicate that neutrophils possess, in addition to a glucocorticoid receptor, a functional MR. We compared aldosterone and dexamethasone treatment to show that both receptors mediate distinct functions. In contrast to aldosterone, dexamethasone had no effect on NF-κB activation. However, dexamethasone is known to delay neutrophil apoptosis by an NF-κB-independent mechanism.15 According to our data, the apoptosis-delaying effect of the glucocorticoid is not shared by the mineralocorticoid aldosterone. Therefore, both steroids may have distinct functions in controlling neutrophil responses.

Interestingly, the aldosterone effect was specific within a given cytokine pathway in that IL-8 and GM-CSF were able to activate ERK, p38 mitogen-activated protein kinase, and phosphatidylinositol 3-kinase/Akt. NF-κB controls transcription of a variety of cytokines, which in turn have profound effects on neighboring cells. One pivotal cytokine is TNF-α, which activates several cell types, including EC. Neutrophils and EC are intimately connected in that neutrophils adhere to and migrate through EC, and cytokines produced by the
neutrophils may participate in EC activation, thereby accelerating inflammation. We show that IL-8–treated neutrophils on fibronectin upregulate the transcription of TNF-α mRNA, resulting in strongly increased TNF-α concentrations in the supernatant. We and others have shown that TNF-α generation in neutrophils is controlled by NF-κB.13 Aldosterone almost completely prevented NF-κB activation and TNF-α generation. More importantly, this TNF-α–containing neutrophil supernatant caused strong ICAM-1 expression up-regulation in EC and subsequently augmented neutrophil adhesion. Aldosterone again significantly prevented both effects. Data from the literature strongly suggest that aldosterone differentially affects leukocyte subtypes. In 1 study, aldosterone stimulated the ICAM-1 expression on coronary artery EC and promoted mononcytic U937 cell adhesion.33 This effect was inhibited by spironolactone. However, in another report, MR blockade in deoxycorticosterone-treated rats reduced interstitial renal fibrosis but not all markers of inflammation.34 Considering our results in this context, it is tempting to speculate that neutrophil-generated cytokines participated under these conditions in the ongoing inflammation.

Perspectives

Aldosterone effects are detrimental in a variety of cardiovascular and renal diseases. As a consequence, the use of MR blocker has increased strongly over the years. However, our data suggest at least some caution with respect to these interventions when it comes to inflammation. Aldosterone may provide antiinflammatory effects that were pivotal centuries ago, when death from inflammation outweighed deadly cardiovascular events. Conceivably, by keeping the neutrophil quiet, aldosterone provides protection from tissue injury in noninfectious conditions, including autoimmunity. Future studies should explore these heretofore unappreciated aspects of the aldosterone-MR relationship in neutrophil inflammation.

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Disclosures

None.

References


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