Alterations in the profile of blood neutrophil membrane receptors caused by in vivo adrenocorticotropic hormone actions

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Submitted 16 May 2014; accepted in final form 21 August 2014

The mature pool of granulocytes in the bone marrow is CXCR4high, which is decreased during the delivery process into the blood (19). In the circulation, senescent neutrophils undergo progressive CXCR4 expression with increased responsiveness to bone marrow stromal-derived factor-1α (SDF-1α), which drives CXCR4+ senescent neutrophils back to the bone marrow to be cleared by local macrophages (19, 54). The relevance of the CXCR4-SDF-1α axis in neutrophil trafficking is evident in CXCR4-knockout mice (14) and individuals with WHIM (warts, hypogammaglobulinemia, infections and myelokathexis) congenital syndrome, which is caused by persistent CXCR4 expression in bone marrow neutrophils. The resulting severe neutropenia detected in WHIM subjects reflects the retention of mature granulocytes together with augmented apoptosis in the bone marrow (1, 11, 23, 59). In agreement with this model, treatment with the CXCR4 antagonist plerixafor induces neutrophilia in humans and mice (10, 33).

The molecular mechanisms involved in the delivery of mature neutrophils into the blood during physiological or stress conditions are complex and not completely understood. Nonetheless, strong evidence has supported the involvement of the hypothalamic-pituitary-adrenal (HPA) axis. Chemical mediators secreted at the site of inflammation reach the hypothalamus and pituitary tissues, leading to the release of corticotropin-releasing hormone and adrenocorticotropic hormone (ACTH), respectively. ACTH stimulates peripheral tissues to release glucocorticoids, which will halt the acute inflammatory process (4, 5, 16, 24, 25, 39, 49). Nevertheless, ACTH might exert direct anti-inflammatory activities in an arthritis-induced model in rats (20) by acting selectively on tissue melanocortin-3 receptor, inhibiting proinflammatory mediator release and neutrophil migration (21, 22, 38).

The traffic of neutrophils in body compartments is pivotal to homeostasis. A continuous delivery of mature granulocytes from the bone marrow yields about 1011 and 107 neutrophils per day into the blood in humans and mice, respectively (19, 31). Although recent data have suggested that a specific population of neutrophils may circulate for longer periods of time (42), the majority of these cells circulate for 6–8 h, becoming senescent and migrating into different terminal tissues (19). The bone marrow blood trafficking of neutrophils is mediated by membrane adhesion molecule expression on neutrophils, sinusoids, and postcapillary venules. Although the role of Cd62L, Cd18, and Cd49d expressed on neutrophils has been proposed in bone marrow/blood traffic, the data are controversial (2, 3, 8). In contrast, it is well established that expression of chemokine (C-X-C motif) receptor 4 (CXCR4) mediates the maintenance of mature granulocytes in the bone marrow as well as the homing of circulating senescent neutrophils back to the bone marrow.

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Neutrophilia caused by stimulation of the HPA axis is supposedly centered on endogenous glucocorticoids, as the platelet-activating factor-induced neutrophilia was accomplished by elevated levels of corticosterone and abolished in mice treated with RU-38486, a cytosolic glucocorticoid receptor antagonist (25, 39). A downstream effector of glucocorticoids for the control of neutrophil reactivity is the protein annexin A1 (ANXA1) (40), yet the role of this protein on ACTH-induced neutrophil mobilization has not been shown.

HPA activation-induced neutrophilia is dependent mainly on the mobilization of the bone marrow pool of mature granulocytes (27, 32, 47). Interestingly, neutrophils that migrate into the blood following HPA activation during ongoing inflammation present a lower ability to migrate into inflamed tissues (27, 47, 56). Therefore, we hypothesize that the population of blood neutrophils after HPA activation presents different and possibly unique phenotypes that support their rapid activity in the blood, with moderating effects on the extent of tissue-specific migration, and subsequent removal by clearance tissues. To challenge this hypothesis, we investigated the profile of neutrophils in the blood after administration of ACTH, focusing on membrane receptors involved in the migratory processes to inflamed and clearance tissues.

MATERIALS AND METHODS

Animals

Male wild-type (WT) and ANXA1-null (ANXA1−/−) (24) 5- to 6-wk-old BALB/c mice were obtained from the Animal House at the School of Pharmaceutical Sciences, University of São Paulo (Brazil). Chow and water were available ad libitum. During the experimental procedures, the animals were anesthetized with ketamine-xylazine (77:7 mg/kg) to avoid any stress conditions. All animal procedures were performed according to protocols approved by the Ethics Committee on Animal Use of the School of Pharmaceutical Sciences, University of São Paulo, (CEUA/FCF/285), for the proper use and care of experimental animals.

Pharmacological Treatments

The glucocorticoid receptor antagonist mifepristone (RU-38486, 10 mg/kg; Sigma, St. Louis, MO) was dissolved in carboxymethyl cellulose (2 mg/ml), polysorbate 80 (50 μl/ml), ethanol (40 μl/ml), and phosphate-buffered saline (PBS; pH 7.2-7.4) and administered intraperitoneally (ip) 24 and 2 h before ACTH (vehicle or PBS). ACTH (Synacthen Depot; Novartis, Basel, Switzerland) was administered at 5 μg/animal (ip), and experiments were carried out 4 h later. Control animals received the same volume of the vehicle used to dissolve the drugs via the same route.

Hematological Parameters

Blood. Animals were anesthetized and whole blood samples obtained via abdominal aorta punctures using EDTA (2 mg/ml; Sigma). Total cells were quantified using a Neubauer chamber. Differential leukocyte counts were performed on blood smears stained with May Grumwald-Giemsa solution (Sigma).

Bone marrow. Bone marrow cells were obtained by flushing the femoral cavity with 2 ml of McCoy’s 5A medium (Sigma). The number of total cells was quantified in a Neubauer chamber. Differential counts were performed on the basis of 500 cells/slide smear stained with May Grumwald-Giemsa.

Enzyme-Linked Immunosorbent Assay

Corticosterone (Cayman, Ann Arbor, MI) levels were quantified in the plasma obtained from the circulating blood of animals. Granulocyte colony-stimulating factor (G-CSF; R & D Systems, Minneapolis, MN) concentrations were determined in the supernatant of bone marrow macrophage culture during the phagocytosis of senescent neutrophils, as described in Preparation of Senescent Neutrophils and In Vitro Senescent Neutrophil Phagocytosis by Bone Marrow Macrophages. Samples obtained from each experiment were analysed in duplicate, and experiments were carried out according to the manufacturers’ protocols.

Flow Cytometry

Leukocytes were isolated from blood collected from the abdominal aorta or from the bone marrow to evaluate Ly6G, CXCR4, CD18, CD49d, CD62L, and formyl peptide receptor-1 (FPR-1) expressions. In brief, erythrocyte lysis was performed using an ammonium chloride solution (0.13 M) in blood samples, and leukocytes were recovered after washing with PBS. Leukocytes (1 × 10⁶) from blood or bone marrow were incubated for 20 min at 4°C in the dark with 100 μl of monoclonal antibody against Ly6G (PECy7-conjugated rat anti-mouse; 50 μg/ml), CXCR4 (PE-conjugated rat anti-mouse CXCR4; 50 μg/ml), CD18 (FITC-conjugated rat anti-mouse CD18; 50 μg/ml), CD49d (PE-conjugated rat anti-mouse CD49d; 50 μg/ml), or FPR-1 (FITC-conjugated rabbit anti-mouse FPR1). All antibodies were diluted 1:100. Immediately after incubations, cells were analysed on a FACS Canto flow cytometer (Becton and Dickinson, San Jose, CA). Data from 10,000 cells were obtained, and results were expressed as the percentage of positive cells and mean of fluorescence intensity (MFI). Ly6G, CXCR4, and adhesion molecule antibodies were purchased from BD Pharmingen Technical, and FPR-1 was purchased from Abcam (London, UK).

In vivo neutrophil migration

Animals were anesthetized, and 3 ml of sterile air was injected subcutaneously into the dorsal region. After 6 days, the pouch was refilled with 3 ml of air. On the 10th day following the first air injection, animals were treated with ACTH (5 μg/animal) or vehicle. After 4 h, LPS from E. coli (serotype 026:B6, 100 μg/ml PBS, 2 ml/animal; Sigma) was injected directly into the pouches. Four hours later, the animals were reanesthetized and euthanized. The pouches were washed with 3 ml of ice-cold PBS, and the total leukocyte number was determined using a Neubauer chamber.

In Vitro Neutrophil Chemotaxis Assay

Samples from whole blood were obtained, and neutrophils were separated by a modified Percoll method (15). A Percoll gradient was made by slowly adding 3 ml of 70% Percoll solution above 3 ml of 60% Percoll solution (Sigma), both of which diluted in Hanks’ balanced salt solution (HBSS; Gibco, Grand Island, NY). Subsequently, 3 ml of whole blood was added slowly above the Percoll gradient and centrifuged at 500 g for 35 min at 22°C, with acceleration/braking of 2 m/s². After centrifugation, a halo between the 70 and 60% Percoll solutions containing the neutrophils was observed and collected. Then, the neutrophils were washed with HBSS by centrifugation at 400 g for 10 min at 22°C. After washing, neutrophils were resuspended at 2 × 10⁵ cells/ml in HBSS supplemented with Ca²⁺. In the next step, 25 μl of cell suspension from blood was loaded into the top well of a commercially available Neutrophor Chemotaxis Assay (ChemoTx-101-8 96-well plate equipped with an 8-μm pore size filter (Neutrophor; Leamington Spa). As a chemoattractant, formyl-methionyl-leucyl-phenylalanine (MLP; 10⁻⁶ M) (Sigma) was added in the bottom well. The chemotaxis assay with neutrophils from the blood was incubated for 2 h, and a cell suspension from the bone marrow was incubated for 1 h at 37°C and 5% CO₂. Migrated neutrophils from the blood were counted using a Neubauer chamber.
Preparation of Senescent Neutrophils

To evaluate the effect of ACTH on aging, neutrophils were isolated from peripheral blood (see Blood) of male Balb/C mice treated with ACTH (5 μg·animal−1·h−1) or vehicle by Percoll gradient (15). Neutrophils (1 × 10⁶ cells/ml) were incubated for 18 h in RPMI 1640 medium (Sigma, St. Louis, MO) with 10% FBS (Vitrocell, Campinas, Sao Paulo, Brazil) at 37°C and 5% CO₂. After the incubation period, neutrophils with a senescent profile (7) were characterized by CXCR4/annexin V labeling (ANXV APC conjugated and CXCR4 PE conjugated, 50 μg/ml; BD PharMingen). Immediately after incubation, cells were analyzed on a FACS Canto flow cytometer (Becton Dickinson). Data from 10,000 cells were obtained, and results are expressed as means ± SE of 6 animals in each group. Statistical analysis was performed using ANOVA, followed by Tukey’s test. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. respective control; #P < 0.05 vs. wild-type (WT) vehicle-treated mice; §P < 0.05 vs WT ACTH-treated animals. KO, knockout.

In Vitro Senescent Neutrophil Phagocytosis By Bone Marrow Macrophages

After bone marrow cells were obtained, as described in Bone marrow, 1 × 10⁶ cells were incubated in a 24-well cell culture plate prepared previously by placing a sterile 13-mm round glass coverslip into each well containing 500 μl RPMI 1640 with 10% FBS and incubating for 2 h at 37°C and 5% CO₂. After incubation, nonadherent cells were removed by three washes with ice-cold PBS. As described in Preparation of Senescent Neutrophils, mouse apoptotic neutrophils (1 × 10⁶ cells/well) were added to bone marrow macrophages and incubated for 1 h at 37°C and 5% CO₂. Noningested cells were removed by three washes with PBS, and phagocytosis was assayed on a coverslip stained with May Grumwald-Giemsa dye. For each experiment, the number of macrophages containing one or more neutrophils in at least five fields (minimum of 400 cells counted) was expressed as a percentage of the total number of macrophages (7).

Intravitral Microscopy

Mice received saline or ACTH (5 μg/animal) and 4 h later underwent an intravitral microscopy study. The animals were anesthetized, the cremaster muscle was surgically exteriorized, and animals were maintained on a special board that was thermostatically controlled at 37°C, which included a transparent platform on which the tissue was placed. The preparation was kept moist and warmed by irrigating the tissue with a warmed (37°C) Ringer-Locke solution (pH 7.2–7.4). The rate of solution outflow onto the exposed tissue was controlled to keep the preparation in continuous contact with a film of the solution. Trans-illuminated images were captured with a video camera and simultaneously transmitted to a computer. Interaction between leukocytes and vessel walls was analyzed by determining the number of rolling and adherent leukocytes on the postcapillary venule wall (20–30 μm in diameter, 200 μm in length) of the cremaster muscle. Leukocytes moving in the periphery of the axial stream in contact with the endothelium were considered to be rollers, and their number was determined in 5-min periods. The number of leukocytes adhered to the endothelial wall was determined in the same vascular segment at the end of 5 min. Data were considered the average for three fields in each animal (18).

ANXA1 Expression in Neutrophils from Bone Marrow and Blood by Immunofluorescence

Blood and bone marrow leukocytes were prepared on 0.1% gelatine-coated glass coverslips. After 30 min, adherent cells were fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.4% Tween-20-PBS for 15 min, blocked with 3% goat serum (Abcam) containing 0.1% bovine serum albumin (Sigma-Aldrich) in PBS for 1 h, and then incubated with rabbit polyclonal anti-ANXA1 antibody (1:300; Invitrogen, Zymed Laboratories, Carlsbad, CA) in PBS containing 1.5% goat serum overnight at 4°C. After three rinses with PBS, the samples were incubated with PE-conjugated goat anti-rabbit secondary antibody (1:100; Abcam) in PBS containing 1.5% goat serum for 1 h at room temperature in the dark. The slides were then washed in PBS and stained with 300 nM 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for 10 min, followed by three rinses with PBS. After mounting with fluorescent mounting medium, the slides were observed under a Zeiss-Axioskop 2 fluorescence microscope (Carl Zeiss).

Fig. 1. Plasma corticosterone levels. Balb/c mice were pretreated with RU-38486 (RU; 10 mg/kg ip) 24 and 2 h before ip injection of ACTH or vehicle injection (5 μg/animal ip). Annexin A1 (ANXA1)−/− animals were treated with ACTH or vehicle injection (5 μg/animal ip). After 4 h, peripheral blood was collected from the abdominal aorta. Results are expressed as means ± SE of 6 animals in each group. Statistical analysis was performed using ANOVA, followed by Tukey’s test. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. respective control; #P < 0.05 vs. wild-type (WT) vehicle-treated mice; §P < 0.05 vs WT ACTH-treated animals. KO, knockout.

Fig. 2. ACTH effects on the number of neutrophils in the bone marrow and blood. Balb/c WT mice pretreated or not with RU (10 mg/kg ip, 24 and 2 h before) or ANXA1−/− received peritoneal injection of ACTH (5 μg/animal) or vehicle. Four hours later, animals were anesthetized and peripheral blood from abdominal aorta and medullar perfusate collected. No. of mature granulocytes in the bone marrow (A) and neutrophils in the peripheral blood (B). Statistical analysis was performed using ANOVA, followed by Tukey’s test. Data are expressed as means ± SE. Mean values obtained in 6 animals for each group. *P < 0.05 and ***P < 0.001 vs. WT vehicle; #P < 0.05 vs. KO vehicle-treated animals; **P < 0.01 vs. WT ACTH-treated animals.
Meditec, San Antonio, TX). Densitometric analysis for the ANXA1 immunostaining on neutrophils was performed to an arbitrary scale ranging from 0 to 255 using AxioVision software (Carl Zeiss). Images represent the overlap of DAPI plus ANXA1 staining from bone marrow and blood neutrophils.

**Statistical Analysis**

Means ± SE of data are presented and were compared using Student’s t-test or analysis of variance, followed by Tukey multiple comparison test, with a significant probability of <0.05.

**RESULTS**

**In Vivo ACTH Injection mobilizes ANXA1+, CD62L+, CD18+, CD49d+, and CXCR4+ Neutrophils, Depending on Glucocorticoid Receptor Activation and ANXA1**

In vivo treatment with ACTH significantly increased the concentration of corticosterone in the plasma of animals treated with vehicle or RU-38486 as well as ANXA1−/− mice. However, ANXA1−/− animals presented lower corticosterone levels than those found in WT animals either in basal conditions or after ACTH administration (Fig. 1), corroborating in vitro data about the action of ANXA1 on cells of the HPA axis (29, 36, 41). In addition, the ACTH-induced enhancement of plasma corticosterone levels in RU-38486-treated mice was lower than that observed in vehicle-treated mice (Fig. 1), which reinforces the role of glucocorticoid actions on HPA axis (30, 41, 55) and shows the genomic pathway on the effect. Four hours after ACTH injection, mature neutrophils were mobilized from the bone marrow into the blood, as detected by the reduced numbers of mature granulocytes in the bone marrow (Fig. 2A) and the parallel increase in circulating neutrophils (Fig. 2B). Pretreatment of mice with RU-38486 did not modify the number of granulocytes in the bone marrow but caused neutrophilia (Fig. 2, A and B); in the presence of RU-38486 pretreatment, ACTH injection did not modify these profiles of neutrophils in both compartments (Fig. 2, A and B). In addition, ANXA1−/− mice presented an elevated number of neutrophils in both bone marrow and blood, and ACTH treatment reduced the number of neutrophils in the bone marrow but failed to modify the number of cells in the blood (Fig. 2, A and B).

To characterize the phenotype of blood neutrophils, expressions of membrane receptors involved in adhesion and migratory processes were quantified. ACTH injection caused mobilization of ANXA1high from bone marrow into blood, as reduced or elevated numbers of ANXA1high neutrophils were detected in the bone marrow and blood, respectively (Fig. 3). In addition, the population of neutrophils in the blood 4 h after ACTH injection was Ly6G+CD11b+, CD49dhigh, CD62Lhigh, and CXCR4high (Fig. 4, A–H). Neutrophils collected from RU-38486-treated mice or ANXA1−/− did not present alterations on CD18, CD49d, and CXCR4 expressions compared with age-matched WT mice; in these animals, ACTH treatment...
Fig. 4. Adhesion molecules and chemokine (C-X-C motif) receptor 4 (CXCR4) expression in neutrophils obtained from peripheral blood of WT and ANXA1−/− mice treated with ACTH. Balb/c mice pretreated with RU (10 mg/kg ip, 24 and 2 h before the experiment) or ANXA1−/− mice received peritoneal injection of ACTH (5 μg/animal ip) or vehicle. Animals were anesthetized, and peripheral blood was collected from the abdominal aorta. Adhesion molecules and CXCR4 expressions were quantified by flow cytometry. A and B: CD18 expression. C and D: CD49d expression. E and F: CD62L expression. G and H: CXCR4 expression. Results are expressed as means ± SE of 6 animals in each group. Statistical analysis was performed using ANOVA, followed by Tukey’s test. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. vehicle; #P < 0.05 and ###P < 0.001 vs. WT ACTH-treated animals. MFI, mean of fluorescence intensity.
failed to elevate the expression of these molecules (Fig. 4, A–D, G, and H). Analysis of the histograms for each receptor indicates that ACTH treatment leads to specific population of neutrophils stained with CD18, CD49d, and CXCR4 (Fig. 4 B, D, and H). On the other hand, neutrophils stained with CD62L were detected in ACTH or RU-38386-treated mice and in ANXA1/H11002/H11002 animals (Fig. 4 F).

In agreement with previous studies (4, 5, 9), enhanced expressions of CD62L on neutrophils from RU-38486 or ANXA1/H11002/H11002 mice could be quantified, and this was efficiently reversed by ACTH treatment (Fig. 4, E and F).

**In vivo ACTH Injection Enhances Leukocyte-Endothelium Interactions in the Microvasculature**

To evaluate the behavior of neutrophils in the circulation after ACTH administration, in vivo observations of the microcirculation of the cremaster muscle were performed by intravital microscopy. In noninflammatory conditions, ACTH-treated animals showed an increased number of rollers and adherent leukocytes to the vascular endothelium compared with vehicle-treated animals (Fig. 5).

**In Vivo ACTH Injection Impairs Chemotaxis of Neutrophils Into Inflamed and Clearance Tissues**

Inflammation induced by the injection of LPS into the subcutaneous tissue was characterized by reduced numbers of neutrophils in the exudate collected from ACTH-treated animals compared with vehicle-treated mice (Fig. 6A).

Moreover, in vitro chemotactic response to fMLP was reduced if neutrophils were purified from ACTH-treated animals (Fig. 6B), which may be dependent on the reduced expression of FPR1 (Fig. 6C).

In addition, blood neutrophils obtained from ACTH-treated mice also presented reduced chemotactic response to SDF-1α (Fig. 7A), even if they were CXCR4high (Fig. 4G). These data infer that CXCR4high neutrophils could remain in the circulation after ACTH injection, and lower numbers of CXCR4high cells would be in the bone marrow. Therefore, subsequent flow cytometry was carried out in bone marrow cells to investigate the fate of CXCR4high neutrophils. Data obtained showed that the percentage of CXCR4high bone marrow neutrophils (Fig. 7B) and levels of CXCR4 per cell (Fig. 7C) were equivalent in ACTH- or vehicle-treated animals.

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Fig. 5. Effect of ACTH administration on leukocyte-endothelial interactions after ACTH treatment. Balb/c WT mice were treated with ACTH (5 μg/animal ip) or vehicle 4 h before exposure of cremaster muscle to intravital microscopy study. A: no. of leukocytes in rolling process. B: no. of leukocytes adhering to the endothelium. Results are expressed as means ± SE of 6 animals in each group. Statistical analysis was performed using Student’s t-test. *P < 0.05 and **P < 0.01 vs. corresponding vehicle.

Fig. 6. Effect of ACTH administration on in vivo and in vitro neutrophil migration and formyl peptide receptor-1 (FPR-1) expression. Balb/c WT mice were treated with ACTH (5 μg/animal ip) or vehicle 4 h before the experiment. A: total no. of leukocytes migrating into the air pouch induced by LPS (100 μg/ml, 2 ml/animal). B: in vitro chemotaxis of neutrophils obtained from peripheral blood in response to formyl-methionyl-leucyl-phenylalanine (fMLP). C: FPR-1 expression on neutrophils obtained from peripheral blood. Results are expressed as means ± SE of 6 animals in each group. Statistical analysis was performed using Student’s t-test and ANOVA, followed by Tukey’s test. *P < 0.05 and **P < 0.01 vs. corresponding vehicle.
CXCR4

the blood after ACTH injection, nonaltered numbers of
in response to SDF-1

Phagocytosis by Bone Marrow Macrophages

In Vivo ACTH Reduces Neutrophil Senescence and their Phagocytosis by Bone Marrow Macrophages

According to the decreased chemotaxis of blood neutrophils in response to SDF-1α and elevated numbers of CXCR4high in the blood after ACTH injection, nonaltered numbers of CXCR4high neutrophils in the bone marrow were not expected. Senescent neutrophils homing to the bone marrow are CXCR4high (2, 19, 45). Phagocytosis by bone marrow macrophages is a mechanism of clearance of senescent and apoptotic neutrophils, a process often associated with secretion of G-CSF by macrophages to stimulate granulopoiesis (19, 45).

Therefore, the effect of in vivo ACTH treatment on the phagocytosis of senescent/apoptotic neutrophils by macrophages was assessed ex vivo, and the levels of G-CSF were measured in the supernatant of phagocytosing macrophages.

Neutrophils collected from ACTH- or vehicle-treated mice were incubated for 18 h to become senescent, as confirmed by the elevated percentage of CXCR4 and ANXAV staining on the cell (Fig. 8, A and B). Nevertheless, the percentage of CXCR4+/ANXAV+ neutrophils and the levels of CXCR4 per cell were reduced in senescent cells obtained from ACTH-treated mice (Fig. 8, A–C). ACTH-senescent/apoptotic neutrophils were less phagocytosed by bone marrow macrophages compared with vehicle-senescent/apoptotic neutrophils (Fig. 8, D and E). Finally, levels of G-CSF were reduced in the supernatant of macrophages phagocytosing senescent/apoptotic neutrophils collected from ACTH-treated animals (Fig. 8F).

DISCUSSION

This study reports, likely for the first time, that the blood population of neutrophils after ACTH injection presents a different profile of membrane receptor expression, a phenotype associated with higher neutrophil interaction into the vessel wall, but impaired subsequent migration into inflamed or clearance tissues.

A direct action of ACTH on inflammation has been proposed (21, 22, 38); nevertheless, the majority of data suggest a fundamental role by its main mediators, the adrenal secreted glucocorticoids, as rapid ACTH secretion and subsequent glucocorticoid release in the circulation occurs during inflammation. In fact, here we show that ACTH injection elevates plasma corticosterone levels and causes neutrophilia, which is due, at least in part, to the elevated migration of mature ANXA1high bone marrow neutrophils into the peripheral blood. Because RU-38486 treated and ANXA1−/− mice presented neutrophilia, which was not altered by ACTH treatment, the dependence on glucocorticoid interaction with the cytosolic receptor and ANXA1 actions on ACTH-induced neutrophilia could not be established.

It has been shown that ANXA1high neutrophils are anti-inflammatory cells, which do not migrate into inflamed tissues (27, 47, 56). Because ACTH-recruited neutrophils in the blood are ANXA1high, an inverse expression of ANXA1 and inflammatory proteins on the neutrophil membrane could be expected. Conversely, here we observed that circulating neutrophils from ACTH-treated mice are also CD62Lhigh, CD18high CD49dhigh, and CXCR4high. CD62L mediates the neutrophil rolling behavior, and CD18 and CD49d mediate the firm adhesion of neutrophils to the vessel wall, which are the initial and fundamental processes of neutrophil recruitment into inflamed tissues (31); also, CXCR4 is especially involved in the homing of neutrophils into clearance tissues, including the bone marrow (19). Intriguingly, enhanced leukocyte-endothelial interactions in the post-capillary venules of the mesentery network were observed after ACTH treatment. These data also could not also be expected, as increased numbers of roller neutrophils are reported in statuses like glucocorticoid deficiency (16) or in ANXA1−/− mice (9, 13). In both cases, the increased rolling behavior is dependent on the elevated expression of CD62L on blood neutrophils (4, 5, 9). In fact, glucocorticoids, via ANXA1, detach neutrophils from the vessel wall by inducing CD62L shedding from the membrane (9, 13, 26, 53). By showing that ACTH-treated mice present elevated levels of circulating corticosterone and CD62Lhigh blood neutrophils and that ACTH injection reverted the elevated CD62L...
levels in RU-38486-treated or ANXA1−/− mice, we hypothesize that ACTH might have a direct action on CD62L expression. The role of glucocorticoids on CD18 and CD49d expression is controversial (3, 4, 16, 17, 56); in contrast, ANXA1 modulates CD18 expression, as shown by elevated and reduced expression in neutrophils from ANXA1−/− and in vitro WT neutrophils treated with ANXA1, respectively (6, 35, 48). Then, we showed that both CD18 and CD49d expression following ACTH injection rely on cytosolic glucocorticoid receptor activation, as well as ANXA1, thus describing a likely genomic glucocorticoid action on in vivo integrin expression.

Together, the data presented here show that the profile of membrane receptors on neutrophils after ACTH injection is different from those observed during homeostasis, suggesting that ACTH administration leads to a unique population of circulating neutrophils, with an adhesion molecule phenotype sufficient to allow release from the bone marrow, yet it preserves an anti-inflammatory outcome. This hypothesis was corroborated by the impaired neutrophil migration into the inflamed tissue in ACTH-treated animals and by in vitro neutrophil-reduced chemotaxis to fMLP and SDF-1α. fMLP binds to the FPR1, a G protein-coupled transmembrane receptor that was found at lower levels in cells taken from ACTH-treated mice. Although the reduced fMLP-induced chemotaxis may be due to lower levels of the receptor, differences in FPR-1 sensibility or the downstream intracellular activation may not be excluded. In addition, the elevated membrane expression of CXCR4 in the presence of a reduced chemotactic response to SDF-1α by neutrophils from ACTH-treated mice favors the hypothesis that these neutrophils may present altered downstream activation and reactivity, and this may be stimulus independent, indicating interference with fundamental processes on chemotaxis that remains to be elucidated.

The equilibrium of granulopoiesis in the bone marrow is maintained by the efficient clearance of CXCR4high senescent/apoptotic neutrophils by local macrophages, which in turn secrete G-CSF to enhance granulopoiesis (2, 19). Several receptors on the apoptotic neutrophil membrane have been proposed as signals for macrophage identification and phagocytosis (37), and phosphatidylserine (PS) exposure on the outer membrane of apoptotic cells is the best-known determinant (46). Here, we show that blood neutrophils from ACTH-treated mice in a senescent profile presented lower levels of CXCR4 and ANXAV staining to quantify exposed PS on the outer membrane and were less phagocytosed by bone marrow macrophages. Together, these data show that ACTH injection inhibits the apoptosis of neutrophils and their consequent clearance by bone marrow macrophages. This effect may be responsible for the normal number of CXCR4high neutrophils in the bone marrow; even ACTH-injected mice presented elevated numbers of blood CXCR4high neutrophils. The ACTH effect may be indirect in view of the action of glucocorticoids, as they enhance the lifespan of neutrophils (28, 52, 58). On the other hand, ANXA1 is a proapoptotic protein in neutrophils by virtue of activating caspase-3 and the inhibition of Mcl-1, ERK 1/2, and NF-κB-mediated survival signals (15a, 50, 57). These apparently contradictory observations might reflect differences in the functions of intracellular and extracellular ANXA1 during the apoptotic process, which may counterbalance the glucocorticoid actions (15a).
Recent data in the literature have broken some paradigms on neutrophil trafficking in the body compartments and have shown that different phenotypes of neutrophils exert functions other than those related to the innate immune response (42–44, 51). Alterations in the profile and lifespan of neutrophils contribute to the genesis of diseases and can be targeted as a viable therapeutic approach for inflammatory diseases (34, 48). Therefore, herein we expand our knowledge of the profile of circulating neutrophils in stress conditions elicited by ACTH injection and also highlight the influence of the cytosolic glucocorticoid receptor and ANXA1 in some specific readouts of this process. We conclude by proposing that neutrophil plasticity and phenotype, with associated functional responses, could be rapidly yet differently attained in stress settings, with the ultimate outcome varying, whether in the presence or absence of an inflammatory status. Future studies will be essential to extend the molecular mechanisms and effectors of this fascinating area of science that crosses endocrinology with immunology and cell biology.

GRANTS

We thank Fundação de Amparo a Pesquisa do Estado de São Paulo for Grant No. 2010/16828-0 and the following fellowships: 2010/08402-2 (to I. D. Machado), 2010/17175-0 (J. R. Santin), and 2010/19802-1 (C. C. Drewes). Also, S. H. P. Farsky and S. M. Oliani have Conselho Nacional de Desenvolvimento Científico e Tecnológico research fellowships.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


