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Prevention of Neutrophil Extravasation by α2-Adrenergic Receptor–Mediated Endothelial Stabilization

Ada María Herrera-García,* María Jesús Domínguez-Luis,† María Arce-Franco,‡ Estefanía Armas-González,* Diego Álvarez de La Rosa,§ José David Machado,¶ Martina K. Pec,* Manuel Feria,¶ Olga Barreiro,‖ Francisco Sánchez-Madrid,‖# and Federico Díaz-González**†‡

Adrenergic receptors are expressed on the surface of inflammation-mediating cells, but their potential role in the regulation of the inflammatory response is still poorly understood. The objectives of this work were to study the effects of α2-adrenergic agonists on the inflammatory response in vivo and to determine their mechanism of action. In two mouse models of inflammation, zymosan air pouch and thioglycolate-induced peritonitis models, the i.m. treatment with xylazine or UK14304, two α2-adrenergic agonists, reduced neutrophil migration by 60%. The α2-adrenergic antagonist RX821002 abrogated this effect. In flow cytometry experiments, the basal surface expression of L-selectin and CD11b was modified neither in murine nor in human neutrophils upon α2-agonist treatment. Similar experiments in HUVEC showed that UK14304 prevented the activation-dependent upregulation of ICAM-1. In contrast, UK14304 augmented electrical resistance and reduced macromolecular transport through a confluent HUVEC monolayer. In flow chamber experiments, under postcapillary venule-like flow conditions, the pretreatment of HUVECs, but not neutrophils, with α2-agonists decreased transendothelial migration, without affecting neutrophil rolling. Interestingly, α2-agonists prevented the TNF-α–mediated decrease in expression of the adherens junctional molecules, VE-cadherin, β-catenin, and plakoglobin, and reduced the ICAM-1–mediated phosphorylation of VE-cadherin by immunofluorescence and confocal analysis and Western blot analysis, respectively. These findings indicate that α2-adrenergic receptors trigger signals that protect the integrity of endothelial adherens junctions during the inflammatory response, thus pointing at the vascular endothelium as a therapeutic target for the management of inflammatory processes in humans. The Journal of Immunology, 2014, 193: 3023–3035.

Adrenergic receptors, also referred to as adrenoceptors, are expressed on the surface of a wide range of target cells and mediate the diverse biological effects of the endogenous catecholamines, epinephrine and norepinephrine. To date, nine subtypes of these G protein–coupled, transmembrane receptors have been cloned, as follows: α1A, α1B, α1D, α2A, α2B, α2C, β1, β2, and β3 (1). The precise physiological functions of many of them are not fully known, and although sufficiently selective ligands are not yet available, a number of α2-receptor agonists, such as clonidine, medetomidine, and brimonidine, have been used to treat different human disorders (2).

Two stress pathways, the hypothalamus-pituitary-adrenal axis and the sympathetic nervous system, regulate the immune response via the release of glucocorticoids and norepinephrine, respectively. Cortisol and norepinephrine act synergistically, increasing glucocorticoid receptors, β-adrenoceptors, and intracellular mediators in different cell types (3, 4), thereby inducing an anti-inflammatory response (5, 6). Norepinephrine recognizes membrane receptors (α- and β-adrenoceptors) on cells implicated in the immune response. Anti-inflammatory effects in the lung were evident during the β-adrenergic response (7), which seems to inhibit interaction between human neutrophils and HUVECs in vitro (8). However, less is known about the role of α-adrenoceptors in the inflammatory response. The presence of α2-adrenergic receptors has been posited for both leukocytes and endothelial cells, based mainly on pharmacologic evidence (9–12); in addition, significant experimental data support the view that these receptors act as inflammation modulators. The production of proinflammatory cytokines has been shown to be inhibited by the α2-agonist, in both an animal lung model (13) and a canine model of inflammation (14). More recently, intrathecal administration of clonidine, a central antihypertensive α2-agonist, was reported to suppress leukocyte migration in a mouse air pouch model, suggesting that α2-adrenoceptors exert, at least in part, a central anti-inflammatory effect (15). Nevertheless, the potential implications of α2 receptors, the subtypes involved in the peripheral regulation of the inflammatory response, and the molecular mechanisms by which these receptors

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Two stress pathways, the hypothalamus-pituitary-adrenal axis and the sympathetic nervous system, regulate the immune response via the release of glucocorticoids and norepinephrine, respectively. Cortisol and norepinephrine act synergistically, increasing glucocorticoid receptors, β-adrenoceptors, and intracellular mediators in different cell types (3, 4), thereby inducing an anti-inflammatory response (5, 6). Norepinephrine recognizes membrane receptors (α- and β-adrenoceptors) on cells implicated in the immune response. Anti-inflammatory effects in the lung were evident during the β-adrenergic response (7), which seems to inhibit interaction between human neutrophils and HUVECs in vitro (8). However, less is known about the role of α-adrenoceptors in the inflammatory response. The presence of α2-adrenergic receptors has been posited for both leukocytes and endothelial cells, based mainly on pharmacologic evidence (9–12); in addition, significant experimental data support the view that these receptors act as inflammation modulators. The production of proinflammatory cytokines has been shown to be inhibited by the α2-agonist, in both an animal lung model (13) and a canine model of inflammation (14). More recently, intrathecal administration of clonidine, a central antihypertensive α2-agonist, was reported to suppress leukocyte migration in a mouse air pouch model, suggesting that α2-adrenoceptors exert, at least in part, a central anti-inflammatory effect (15). Nevertheless, the potential implications of α2 receptors, the subtypes involved in the peripheral regulation of the inflammatory response, and the molecular mechanisms by which these receptors...
can influence the accumulation of leukocytes into inflamed tissues have not been studied.

Extravasation of both fluid and leukocytes across endothelial barriers is a key event in the inflammatory response. In order to exit the bloodstream, leukocytes need to establish complex, adhesive interactions with endothelial cells, a process termed the adhesion cascade (16). Adhesion molecules selectins, integrins, and members of the Ig superfamily participate sequentially in this process (17–19). Several adhesion molecules located at the junctions between endothelial cells participate in endothelial stability; among these, vascular endothelial (VE)-cadherin plays an essential role both in vascular permeability and in controlling the final stage of leukocyte extravasation (20). Each leukocyte–endothelial cell adhesion event is essential for successful leukocyte extravasation. Thus, if one becomes impaired, leukocyte emigration from blood vessels to tissues does not occur.

We aimed to study the potential involvement of the α2-adrenoceptor in the regulation of neutrophil recruitment into inflammatory sites. Our data demonstrate that α2-adrenergic agonists exert an in vivo anti-inflammatory effect, thus rendering the endothelium more resistant to leukocyte extravasation during the inflammatory response. This effect was apparently due to the prevention of TNF-mediated disruption of VE-cadherin–dependent junctions between endothelial cells.

**Materials and Methods**

**Abs and reagents**

The following mAbs were used: anti-human adrenergic α2A (C-19), α2B (AR C-19), and α2C (AR C-19), and the monoclonal anti-human CD11b (activation epitope) CBRM1/5 from Santa Cruz Biotechnology (Dallas, TX); anti-human α-tubulin, anti-human β-actin, and rabbit polyclonal anti-α-catenin from Sigma-Aldrich (St. Louis, MO); Leu-8, anti-β1-selectin/CD62L mAb, Dreg-56, anti-β-1-selectin functional blocking mAB, and anti-human ICAM-1/CD54 from BD Biosciences (San Jose, CA); anti-CD11b FITC anti-mouse (M1/70.15) and anti-CD45, anti–L-selectin rat anti-mouse (San Francisco, CA). Anti-human CD11b (Bear 1) (21), anti-CD45 (D3/9) and anti–phospho-y-actin, and rabbit poly- and anti-rabbit Abs were supplied by Molecular Probes (Eugene, OR). Human α5 and rabbit polyclonal α-actin were obtained from Zymed Laboratories (San Francisco, CA). Anti-human CD11b (Bead 1) (21), anti-CD45 (D3/9) (22), anti-CD31 (TP1/15.1.), anti–VE-cadherin (TEA 1/31.1) (23), anti–VCAM-1 (4B9), and PX63 were drawn from myeloma culture supernatants (negative control) and have been described elsewhere. Goat anti-rabbit-HRP and rabbit anti-mouse HRP were purchased from Dako (Saltrup, Denmark), and Alexa 488–labeled goat anti-rat IgG and IgM and anti-rabbit Abs were supplied by Molecular Probes (Eugene, OR).

**Materials and Methods**

**Cell isolation and treatments**

HUVECs were obtained, as previously described (24). HUVECs were grown on gelatin (Sigma-Aldrich)- precoated culture plastic and cultured in a 5% CO2, humidified atmosphere at 37˚C in medium 199, supplemented with 10% FBS (PAA), 50 μg/ml streptomycin, 50 μg/ml penicillin, 5 μg/ml gentamicin (PAA), 50 μg/ml endothelial cell growth supplement (BD Biosciences), 5% heparin (Chiesi), and 20% FCS (PAA). Confluent HUVECs were activated with 20 ng/ml TNF-α for 6 h at 37˚C in the absence (versus the presence) of 100 μM α2-agonist UK14304, 100 μM α2-antagonist RX821002, or both (both solubilized in DMSO and further diluted to a working concentration of 1:1000 in PBS), except as indicated otherwise. In all experiments, TNF-α was added to cells 5 min before drugs.

Human neutrophils were isolated from the peripheral blood of healthy volunteers by Biocoll (Biochrom AG) density-gradient centrifugation for 20 min at 600 × g, followed by sedimentation at 1 × g in 1.3% (w/v) dextran (Sigma-Aldrich) for 20 min at room temperature. The neutrophil-enriched fraction was further purified by hypotonic lysis of erythrocytes, yielding a purity >95% as assessed by CD11b immunoreactivity in flow cytometry analyses. Except as otherwise indicated, neutrophils were resuspended at a concentration of 106 cells/ml in HBSS; treated with the α2-agonist, the α2-antagonist, or both (as above); and incubated at 37˚C for 20 min. Activation of neutrophils was performed by incubation in the presence of 20 μg/ml IL-8 for 20 min at 37˚C. All experiments with neutrophils were carried out in 5-mL disposable polystyrene tubes (Falcon Labware, Oxnard, CA).

Control data for α2-agonists and the antagonist were obtained in the presence of 0.1% DMSO in HBSS.

**Animal models of inflammation**

Experiments were conducted using 3-mo-old male CD-1 mice weighing between 24 and 30 g. The air pouch was prepared, as previously described (25). Six days after the initial injection of sterile air into the back, animals were treated i.m. with 1 mg/kg UK14304 or xylazine at 2, 4, or 16 mg/kg. A group of animals treated with α2-agonists was simultaneously injected i.m. with 5 mg/kg RX821002. All chemicals had been solubilized previously in DMSO at 500 mg/ml and diluted to a final concentration of 5 mg/ml in saline buffer. Control animals were treated i.m. with 50 μl control vehicle, a saline buffer solution containing 1% DMSO in saline buffer. One hour after i.m. treatments with the different compounds, air pouches were injected with 0.5 ml saline buffer solution to induce a local inflammation. Animals were maintained at room temperature or under an external heat source. Four hours after zymosan administration, the animals were sacrificed by cervical dislocation and the pouch exudates were collected with 10 ml cold saline buffer. The total number of leukocytes in the exudate fluid was assessed by flow cytometry in an Epics XL flow cytometer (Beckman Coulter) using a known concentration of fluorescent beads (BD Biosciences) as a control.

Peritonitis was induced in mice as previously described (26, 27). Animals were treated i.m. with 16 mg/kg xylazine or vehicle, as described above. After 1 h, mice were injected i.p. with 2 ml 4% w/v thioglycollate. After 4 h, animals were sacrificed by cervical dislocation, and the peritoneal cavity was harvested by injecting 10 ml cold saline buffer through the peritoneal wall. RBCs were eliminated from the lavage fluid by hypotonic lysis. The number of exudate neutrophils was counted by flow cytometry, as described above.

Rectal temperature was measured in conscious animals with a pediatric digital thermometer. The tip of the probe was coated with sterile lubricant and inserted to the length of the probe (~2 cm). Temperature was recorded every 20 min during the first hour and then every hour.

**Assessment of total CAMP**

Resting neutrophils from healthy donors (107 cells/ml) were preincubated for 15 min in HBSS containing 500 μM 3-isobutyl-1-methylxanthine. Then the cells were incubated at 37˚C in 3-isobutyl-1-methylxanthine-containing HBSS in the presence of 100 μM UK14304, with or without 100 μM RX821002. As a positive control, cells were incubated with 50 μM forskolin. After 20 min, the cells were lysed, and neutrophil cAMP levels were determined with a direct enzyme immunoassay kit (Amersham, GE Healthcare), according to the manufacturer’s instructions.

**Analysis of apoptosis in human polymorphonuclear cells**

Human neutrophils from healthy donors (5 × 106/ml) were incubated in HBSS in the presence of UK14304 (100 μM), with or without RX821002 (100 μM), under orbital shaking at 75 rpm (Intraper-Eviro-Genie from Scientific Industries, Bohemia, NY). Control cells were cultured in the presence of either DMSO (0.1%) or LPS (1 μg/ml), the latter being known to inhibit apoptosis (28). After 24 h at 37˚C, cells were washed and resuspended in 5 ml hypotonic buffer (140 mM NaCl, 2.5 mM CaCl2, and 10 mM HEPES at pH 7.4) to which 50 μg/ml propidium iodide was added just before sample uptake by the flow cytometer (Epics XL; Coulter). The proportion of cells in the late stage of apoptosis was estimated by the relationship between cells in the sub-G1 stage (hypodiploid cells) and cells in the G0/G1 phase (cells with diploid DNA content).

**Gene expression**

Cell RNA was extracted with an RNeasy Midi Kit (Qiagen) following the manufacturer’s instructions. HUVECs and neutrophil samples were treated with DNase I to avoid contamination with genomic DNA. Reverse transcription was carried out using SuperScript III reverse transcriptase (Life Technology) with random hexamers. The primer and probe sequences, designed using Primer Express software (Applied Biosystems, Foster City, CA), were as follows: α2A, forward, 5’-GCTGCGCAACAGGAGTCAT-3’ and reverse, 5’-CGCCCTTCTCTCAGGGA-3’ (260 bp); α2B, forward,
were quantified using the NIH Image J1.36 software developed at the National
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was followed by incubation with primary Abs (anti-human α2A, α2B, and α2C; 1:100, anti-human VE-cadherin 1:2; anti-mouse α-tubulin. Sapi anti-
human-β-actin 1:2000, and anti–phospho-VE-cadherin [pY658] 1:200), which were detected with HRP-conjugated anti-mouse IgG or HRP-
conjugated anti-rabbit IgG, respectively, using an ECL Western blotting
substrate (Pierce). Protein expression of VE-cadherin was calculated in rela-
tion to intracellular control cultures diluted to DMSO (set to 100%), using a
Kodak Imaging System (Rochester, NY) for data collection and analysis.
Band intensities were quantified using the NIH Image J1.36 software developed at the

Flow cytometry analysis

Human neutrophils were treated, as described above, and incubated with the
different mAbs at 4˚C for 30 min. HUVECs were incubated at 37˚C for 6 h
in the presence of UK14304 at 100 μM. After washing with PBS, cells were
labeled with FITC-labeled, rabbit anti-mouse Ig (Dako) and then incubated at
4˚C for 30 min. At least 10,000 cells from each sample were analyzed in an
Epics XL flow cytometer (Beckman Coulter), and the data were quan-
tified using a logarithmic scale. Fluorescence resulting from irrelevant myel-
oma P3X63 Ab was considered background fluorescence. Neutrophils
isolated from the peripheral blood of mice, treated with the different com-
ounds as described above, were incubated with Mel-14 or anti-CD11b FITC
Abs at 37˚C for 30 min. After washing with PBS, cells labeled with Mel-14
or Mel-14 were incubated at 4˚C for 30 min in the presence of Alexa
Flour 488-labeled goat anti-rat IgG and IgM Abs. Because fluorescence
conditions varied between experiments, data were normalized to express the
relative mean fluorescence intensity (rMFI) according to the following
equation: rMFI = (MFIcompound − MFIbackground)/MFIbackground − MFIbackground × 100.
In addition, a few blood samples from xylazine-treated mice and control
animals were labeled with FITC or PE-conjugated mAbs as described
above with respect to the fluorescent beads, as described above.

Leukocyte transmigration assays

The capability of neutrophils to migrate through the activated endothelium was
assessed with a Transwell assay using IL-8 as a chemottractant. Briefly,
a confluent HUVEC monolayer was cultured in the gelatin-coated (20 μg/ml,
37˚C, 1 h) Transwell insert (5 μm; Costar, Cambridge, MA) and then acti-
vated with TNF-α for 6 h in the absence or presence of UK14304, RX821002,
or both. Medium was then removed by gently washing with HBSS. A total
of 2 × 10⁸ freshly isolated neutrophils in 100 μl HBSS, supplemented with
0.05% FCS, was added to the upper compartment and stimulated by adding
IL-8 (22.5 ng/ml) to effect migration into the BSA-precoated (1%, at 37˚C,
1 h) lower compartment. Similarly, neutrophils were treated with the
α2-agonist and the antagonist prior to migration (as described above) through
the Transwell insert. After 4 h, the cytokine was removed from the upper
compartment of the HUVEC monolayer, and the neutrophils were assayed for
the adhesion of mAbs against CD31, VE-cadherin, and the

Flow chamber experiments

HUVECs (2 × 10⁵ cells/plate) were seeded in 35-mm cell culture plates
(Nalgene Nunc) and then precoated with 20 μg/ml gelatin for 1 h at 37˚C.
Confluent endothelial cell monolayers were incubated with TNF-α at 37˚C
for 6 h, and then the TNF was removed by washing and endothelial cells
were incubated in the flow chamber set up (1 h). Neutrophils, freshly isolated from
normal donor peripheral blood, were preincubated with or without
UK14304 and RX821002 for 20 min at 37˚C. After washing, a neutrophil
suspension containing 1 × 10⁶ cells/ml in HBSS was injected at 4 dynes/cm²
with an infuse/withdraw pump (Harvard Apparatus) in a two-parallel
plate flow chamber (Glycotech), which was maintained at 37˚C (Warner
Instrument, Hamden, CT). In similar experiments, HUVECs were activated with TNF-α at 37˚C for 6 h in medium alone or in the presence of UK14304, RX821002, or both.
Neutrophil–HUVEC interaction was recorded with a CCD camera (Ham-
matsu) connected to an inverted microscope (Axiovert SI07V; Zeiss, Jena,
Germany) for a period of 15 min, starting when the first flowing cell was
observed. Neutrophil movement on the HUVEC monolayer was tracked with
Metamorph software (Molecular Devices). Cells were regarded as rolling
when they met the following two conditions: 1) when they moved on en-
thodelial cells for at least 2 s while remaining in the field of view, and 2) when their average velocity was <50% of that calculated for noninteracting
neutrophils (29) during the first 9 min of recording. Cells that had observable
phase-bright and phase-dark periods at any time during the 6 min after the
rolling period were considered transmigrated.

Permeability assay

Endothelial permeability was analyzed in vitro by diffusion of 70-kDa FITC-
dextran (Invitrogen) through the endothelial monolayer. HUVECs were
grown to confluence on fibronectin-coated (20 μg/ml) Transwell inserts of
0.4-μm pore width (Costar) and were then placed in 24-well tissue culture
dishes (Costar) containing HUVEC culture medium. Endothelial cells were
TNF-α activated in plain medium for 6 h at 37˚C in the presence of
UK14304 or UK14304 plus RX821002. Medium with FITC-dextran was
then loaded into the upper compartment of the Transwell. After 1 h at 37˚C,
the amount of FITC-dextran that had diffused through the endothelial
monolayer was measured in the lower compartment using the microplate
fluorometer SpectraFluor Genios (Tecan, Grödig, Austria). Data are pre-
sented as the percentage of fluorescence in the lower compartment of the
differently treated cells with respect to that resulting from TNF-α–acti-
vated HUVECs in medium alone.

Assessment of electrical resistance

The movement of ions across the endothelial barrier was studied by
evaluating transendothelial electrical resistance. We used a DVC-1000
amplifier with Ag/AgCl electrodes (World Precision Instruments) to
determine the electrical resistance of the HUVEC monolayer, cultured and
treated in the same manner as was the permeability assays. Data are pre-
sent as the variation of electrical resistance in the HUVEC monolayer
analyzed with the different compounds with respect to the TNF-α–activated
monolayer maintained in medium alone.

Ab-coated beads and assessment of tyrosine-phosphorylated
VE-cadherin

Three-micron polystyrene beads (Polysciences, Warrington, PA) were
pretreated overnight with 8% glutaraldehyde, washed five times with PBS,
and incubated with 300 μg/ml mAb anti–ICAM-1 (BD) Systems, ac-
cording to the manufacturer’s protocol. To study VE-cadherin phosphor-
ylation, TNF-α–activated HUVECs were incubated in the presence of
10 μg/ml control beads or Ab-coated beads in the presence or absence of
100 μM UK14304 for 10 min at 37˚C. Cells were then washed twice
gently with ice-cold HBSS and lysed in 35 μl sample buffer. Cells were
scraped from the dishes, and samples were boiled for 10 min. Sample
analysis was performed by Western blot using anti-VE-cadherin and anti–
phospho-VE-cadherin (pY658) Abs, as described above.

Immunofluorescence analysis

Immunofluorescence experiments were performed using HUVECs grown in
fibronectin-precoated, 8-well culture chambers (Nalgene Nunc). The
confluent cell monolayer was activated with TNF-α and subsequently
treated with UK14304 and RX821002, as described above. Cells were
fixed with 4% paraformaldehyde in PBS for 3 min at room temperature
(RT), rinsed with PBS, permeabilized with Triton X-100 (0.2% in PBS) for
3 min at RT, and then blocked with 1% goat serum for 60 min. Incubation
with mAbs was carried out in PBS with 1% goat serum for 30 min at RT,

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followed by five rinse steps with PBS and incubation with Alexa 568–labeled goat anti-mouse, Alexa 488–labeled goat anti-mouse, or anti-rabbit IgG for 45 min at RT. Finally, samples were covered with mounting medium (Testog) and examined using a W Plan/Apochromat 20×/1.0 DIC (UV/visible/Infra Red) objective coupled to an upright microscope (Examiner Z1; Zeiss) equipped with a confocal laser-scanning unit (LSM 780; Zeiss). Image analysis was performed using ZEN Black 2012 Software (Zeiss). VE-cadherin fluorescence of the cell membrane–adjacent cytoplasmic region was analyzed, and its mean fluorescence plus 3 times the SD was considered background. Cell membrane sections with a higher fluorescence than background were considered VE-cadherin positive.

Statistical analysis

Results are expressed as the arithmetic mean ± SD of the mean, as indicated. Statistical analysis of Western blot quantifications was performed using a Student’s t test. In general, Wilcoxon rank sum tests or Wilcoxon signed rank tests were used to determine significant differences, as indicated. The p values <0.05 were considered significant.

Study approval

Written, informed consent for research use was obtained from all sample donors. Protocols were previously approved by the Hospital Ethics Committee. Experimental procedures involving animals were approved by the Ethics Committee of the University of La Laguna in agreement with institutional and European guidelines and regulations.

FIGURE 1. Human neutrophils and HUVECs express α2-adrenoceptors. (A) RT-PCR amplicons from α2-adrenoceptor subtypes in resting HUVECs and human neutrophils run in agarose gel electrophoresis. A representative experiment of four is shown. (B) Western blot of protein extracts from resting HUVECs and neutrophils. Immunolabeling of α-tubulin was used as a loading control. A representative experiment of three is shown. (C) Bar graph showing the total concentration of cAMP in human neutrophils. Cells were incubated in medium alone (control), in the presence of the α2-adrenoceptor agonist, UK14304, and with UK14304 plus the α2-adrenoceptor antagonist RX821002. The adenylyl cyclase activator forskolin was used as a positive control. Data represent the means ± SD of the absolute cAMP concentration in pM/10^6 cells from seven independent experiments. *p < 0.05 by Wilcoxon signed rank test.

Results

Human neutrophils and HUVECs express α2-adrenoceptors

The expression of α2-adrenoceptors has been described, mostly by indirect methods, for a variety of cell types (12, 30–32), including mononuclear leukocytes (33). To assess the potential role of α2-adrenoceptors in the inflammatory response, we first studied the expression of α2-adrenoceptor subtypes in both resting human neutrophils and HUVECs by RT-PCR. Fig. 1A shows the presence of α2A, α2B, and α2C receptor transcripts in both cell types. The three isoforms were detected by quantitative RT-PCR in four independent samples of human neutrophils. However, Western blots revealed exclusively immunoreactive α2A protein in neutrophils and α2B protein in HUVECs, respectively. Protein from the α2C receptor subtype was not detected in the lysates of either cell type (Fig. 1B).

In contrast to endothelial cells, in which the presence of functional α2-adrenoceptors is well established (12, 34), there exist contradictory data about the expression and function of α2-adrenoceptors in human neutrophils (9, 35, 36). To verify the presence of such functionally active α2-adrenoceptors in this cell type, we assessed cAMP production by neutrophils upon treatment with UK14304, a well-characterized α2-adrenoceptor agonist (37),
both in the presence and absence of the α2-antagonist RX821002 (38). UK14304 treatment resulted in a ∼50% reduction in cAMP concentrations compared with cells maintained in medium alone. Pretreatment of neutrophils with RX821002 prevented this effect. Cell exposure to forskolin, an adenylyl cyclase activator used as a positive control, led to a significant increase in cAMP concentrations in human neutrophils (Fig. 1C) within the range previously described (39). Taken together, these data demonstrate that resting human neutrophils and HUVECs express the α2A and the α2B adrenergic receptor, respectively.

α2-Adrenoceptor agonists prevent the accumulation of neutrophils in inflammatory foci in vivo

To evaluate the potential role of α2-adrenergic receptors in the inflammatory response, different α2-agonists were assayed in two mouse models of acute inflammation. In the air pouch model, animals treated i.m. with either xylazine or UK14304 had a significantly lower number of recruited neutrophils in their air pouches compared with controls. The pretreatment of animals with the α2-adrenergic antagonist RX821002 completely blocked the inhibitory effects of either agonist (Fig. 2A). In this model, the effect of xylazine on neutrophil recruitment was dose dependent (Fig. 2B). Similar results were obtained in the thioglycolate-induced model of peritonitis, in which neutrophils in the peritoneum of animals treated with xylazine were reduced by ∼50–60% compared with those treated with vehicle alone (Fig. 2C).

Agonists of the α2-adrenoceptor are known to reduce body temperature (40). Therefore, we studied the potential relationship between hypothermia and the anti-inflammatory effect of α2-adrenoceptor agonists in vivo. As expected, the body temperature of animals treated with xylazine was lower than in untreated animals (Fig. 3A, inset). However, even when the body tempera-

FIGURE 2. α2-adrenergic agonists exert an anti-inflammatory effect in two mouse models of acute inflammation. (A) Box plot showing the concentration of neutrophils in the air pouches of mice treated i.m. with either UK14304 (1 mg/kg) or xylazine (16 mg/kg) in the presence or absence of the α2-antagonist RX821002 (5 mg/kg), and in control animals. Data represent concentrations of neutrophils in pouch exudates from five independent experiments involving three animals each. **p < 0.01 by Wilcoxon rank sum test. (B) Box plot showing the dose-response relation of xylazine in neutrophil concentrations in dorsal air pouches of mice. Data are pooled from five independent experiments involving three animals per experimental condition. ***p < 0.01, **p < 0.001 by Wilcoxon rank sum test. (C) Effect of xylazine (16 mg/ml) on neutrophil accumulation in the peritoneum in a mouse model of thioglycolate-induced peritonitis. Data represent neutrophil concentrations from three independent experiments and three animals per experimental condition. The 25th and 75th percentile (boxes), the median (line within the boxes), the 90th and 10th percentile (error bars), and the outlying points (dots) are plotted. *p < 0.05 by Wilcoxon rank sum test.
assessed, as described in Materials and Methods. Presence of either vehicle (control), LPS, or UK14304. Hypodiploidy was determined for healthy donors and then subsequently treated with UK14304. The percentage of neutrophils at a sub-G$_1$ peak level after 24 h of incubation with this $\alpha_2$-agonist did not differ significantly from cells maintained in buffer alone. As shown in other studies (28), the control proinflammatory bacterial LPS strongly protected neutrophils from programmed cell death (Fig. 3B).

Altogether, these data demonstrate that $\alpha_2$-adrenoceptor occupancy by agonists exerts a significant anti-inflammatory effect in vivo by a mechanism independent of both apoptosis and changes in body temperature.

$\alpha_2$-Adrenoceptor agonists desensitize human neutrophils and HUVECs to cytokine activation

During cell extravasation, constitutively expressed L-selectin in neutrophils is downregulated by shedding, whereas CD11b rapidly increases both its surface expression level (17, 18) and the affinity for its endothelial counter-receptor (45). Intramuscular administration of xylazine or UK14304 did not modify the basal expression of L-selectin or CD11b, as determined by flow cytometry (data not shown), in neutrophils isolated from the heart blood of animals. However, taking into account the key roles played by these two adhesion molecules in neutrophil extravasation, we decided to examine the influence of $\alpha_2$-agonists on surface expression levels during neutrophil activation. Neutrophils isolated from healthy donors were incubated in HBSS in the presence or absence of 100 $\mu$M UK14304 for 10 min and then activated for 20 min with IL-8, a chemokine widely implicated in neutrophil activation during the adhesion cascade (46). Quantification of L-selectin cell surface expression by flow cytometry revealed that the $\alpha_2$-agonist significantly inhibited L-selectin shedding in IL-8–activated neutrophils (Fig. 4A). Conversely, UK14304 prevented any increase in CD11b surface expression in IL-8–activated neutrophils (Fig. 4A).

The adhesive action of integrins depends not only on their surface expression levels, but also on dynamic conformational changes in their structure, which allow them to switch from a low- to a high-affinity state (45). Therefore, we studied the effect of UK14304 on the activation state of CD11b in IL-8–activated neutrophils. To this end, we used CBRM1/5, a mAb that recognizes an activation-specific neoepitope in CD11b (47). In flow cytometry experiments, the presence of UK14304 did not modify the mean fluorescence intensity of CBRM1/5 in IL-8–activated neutrophils (data not shown), which ruled out the possibility that the $\alpha_2$-agonist interferes with the affinity state of CD11b.

The endothelium plays a crucial role in inflammation by providing the key signals for leukocyte extravasation. In their initial response to inflammatory mediators, endothelial cells strongly upregulate the surface expression of the ICAM-1, thereby enhancing adhesion of those cells expressing $\beta_2$ integrins, such as CD11b (48). Bearing in mind that HUVECs express at a minimum the adrenoceptor subtype $\alpha_2B$, we decided to study the potential...
implication of α2-adrenoceptors in the regulation of both ICAM-1 and VCAM-1 surface expression in activated HUVECs. Surface expressions were assessed by flow cytometry in HUVECs activated with TNF-α for 12 h in the absence or presence of different doses of xylazine. A dose-dependent decrease in activation-induced ICAM-1 overexpression was observed in xylazine-treated HUVECs (Fig. 4B). Similar effects were obtained when TNF-α–activated HUVECs were treated with the α2-agonist UK14304 (data not shown).

α2-Adrenoceptor activation affects late-stage endothelial transmigration, but not neutrophil rolling

The foregoing results suggested that α2-agonists might exert their in vivo anti-inflammatory effects by affecting the neutrophil–endothelium adhesion cascade. These compounds might interfere with either of the following: 1) neutrophil rolling, by modifying the expression profile of L-selectin; or 2) firm adhesion, by interfering with the upregulation of either CD11b in neutrophils or ICAM-1 in endothelial cells; or, possibly, 3) with both phases simultaneously.

To clarify these potential explanations, we analyzed the effect of α2-adrenoceptor agonists on the transendothelial migration of neutrophils by using a Transwell migration assay under static conditions. When HUVECs were stimulated with TNF-α, the number of transmigrated neutrophils highly increased compared with unstimulated endothelial cells. The presence of UK14304 reduced neutrophil transendothelial migration by ~50%. However, when TNF-α–activated HUVECs were preincubated with the α2-agonist RX821002, the inhibitory effect of UK14304 was abrogated (Fig. 5A). Resting neutrophils were then preincubated with the α2-antagonist RX821002, the inhibitory effect of UK14304 was abrogated (Fig. 5A). Resting neutrophils were then preincubated with the α2-agonist UK14304 (data not shown).

Using a flow chamber to reproduce postcapillary venule-like conditions, the dynamic interactions between neutrophils and HUVECs were assessed in a more physiological setting. Similar to the Transwell experiments, neutrophils or TNF-α–activated HUVECs were treated independently with the α2-agonist UK14304, or with a combination of agonist and the antagonist RX821002. Fig. 5B shows that neutrophil rolling on TNF-α–activated endothelial cells was not affected by treating either HUVECs or neutrophils with the α2-agonist. Under these experimental conditions, L-selectin played a key role in neutrophil rolling, as revealed in its inhibition by Dreg-56, a blocking anti–L-selectin mAb (49). In contrast, neutrophil transmigration through a UK14304-treated HUVEC monolayer was drastically reduced, but was then restored by additional treatment with RX821002 (Fig. 5C). Blockade of
FIGURE 5. UK14304 reduces transmigration of human neutrophils through the endothelial barrier. (A) Bar graphs showing the effect of UK14304 on the capability of HUVECs and neutrophils to transmigrate under static conditions. The left graph depicts control HUVECs maintained either in medium alone or activated with TNF-α (20 ng/ml, at 37°C for 12 h), HUVECs in the presence of UK14304 (100 μM), and HUVECs pretreated with RX821002 (100 μM) and then stimulated with UK14304. As described in Materials and Methods, the lower Transwell compartment contained IL-8 (20 ng/ml) as a chemoattractant. Data represent means ± SD from three independent experiments (percentage of migrated neutrophils) with respect to TNF-α–activated HUVECs, considered 100%. The right graph shows data from resting neutrophils, incubated in medium alone (control), in the presence of UK14304 (100 μM), and pretreated with RX821002 (100 μM) and then stimulated with UK14304. Data represent means ± SD from three independent experiments (percentage of migrated neutrophils) with respect to control neutrophils, which were considered 100%. (B) Bar graphs showing the effect of UK14304 on HUVECs and on rolling neutrophils, under dynamic conditions. The left graph shows data from control HUVECs, either maintained in medium alone or activated with TNF-α (20 ng/ml, at 37°C for 12 h), in the presence of UK14304 (100 μM), and pretreated with RX821002 (100 μM) and then stimulated with UK14304. Data represent means ± SD from three independent experiments (percentage of migrated neutrophils) with respect to TNF-α–activated HUVECs, considered 100%. The right graph presents data from resting neutrophils, incubated in medium alone (control), in the presence of UK14304 (100 μM), and pretreated with RX821002 (100 μM) and then stimulated with UK14304. Data represent means ± SD from three independent experiments (percentage of migrated neutrophils) relative to control neutrophils, considered 100%. As a control, neutrophil rolling was also determined on TNF-α–activated HUVECs in the presence of Dreg-56, a blocking anti–L-selectin mAb. (C) Bar graph showing the effect of UK14304 on HUVECs in neutrophil transmigration under dynamic conditions. HUVECs were treated as described in (B). The effect of the supernatant of (Figure legend continues)
VE-cadherin and, to a lower extent, of CD31 significantly decreased neutrophil transmigration, confirming the role of both molecules in preserving the endothelial barrier.

Taken together, these observations strongly suggest that α2-adrenoceptor agonists may exert a stabilizing effect on the endothelial barrier in such a way that it interferes with the capability of neutrophils to pass across it.

**UK14304 reduces the transport of ions and macromolecules through the endothelial barrier**

To further analyze the phenomenon of reduced neutrophil transmigration across α2-agonist–treated endothelial cells, we analyzed the effect of UK14304 on vascular permeability, a process that occurs concomitantly with endothelial activation. The passage of ions and macromolecules across the endothelium was assessed by Transwell experiments. HUVEC confluent monolayers were activated in the presence of α2-agonist and antagonist, as described in Materials and Methods. The presence of UK14304 caused electrical resistance in the endothelial monolayers to become significantly elevated compared with controls. This effect was abolished by RX821002 (Fig. 6A). Transendothelial macromolecular permeability was assessed in the same setting by examining the ability of fluorescent dextran (added to the upper compartment of the Transwells) to pass through the endothelial cell monolayer. As shown in Fig. 6B, dextran passage through the TNF-α–activated endothelial monolayer was significantly reduced in the presence of UK14304. Parallel incubation with the α2-agonist partially restored the amount of dextran permeating to the lower compartment of the Transwells (Fig. 6B).

**UK14304 interferes with ICAM-1–mediated VE-cadherin phosphorylation in HUVECs, thereby preserving the localization of adherens junction protein complexes at endothelial cell–cell contacts**

The results above indicated that α2-adrenoceptors convey signals to endothelial cells that increase endothelial barrier resistance to cells, macromolecules, and even ions. We therefore decided to study the effect of α2-agonists on those proteins present at the endothelial cell–cell lateral junctions. By means of immunofluorescence and confocal analysis, we analyzed the effect of UK14304 on the distribution of adherens junction and tight junction proteins in a monolayer of activated HUVECs. Fig. 7 shows that, upon α2-agonist treatment, TNF-activated HUVECs expressed significantly higher amount of VE-cadherin, plakoglobin, and β-catenin, proteins of the adherens junctions, than did control cells over a range similar to that of resting HUVECs (data not shown). However, the cell distribution of ZO-1 and claudin-5, proteins of the tight junctions, as well as α-catenin, was not modified by UK14304 in activated HUVECs. Because VE-cadherin is a major adhesive component of endothelial cell–cell junctions (20), we decided to further study the observed preservation of this molecule at endothelial cell–cell contacts upon α2 agonist treatment. This effect of UK14304 on VE-cadherin cell distribution in TNF-activated HUVECs was prevented by the α2-agonist RX821002 (Fig. 8A).

In the course of leukocyte transmigration, endothelial cell junctions are transiently disassembled (50). Engagement of endothelial ICAM-1 by neutrophils leads to phosphorylation of the cytoplasmic domain of VE-cadherin, an essential process for efficient neutrophil extravasation (51). When endothelial cells were incubated with anti–ICAM-1 mAb-coupled microspheres, which are known to induce VE-cadherin tyrosine phosphorylation (51), we observed enhanced phosphorylation of the tyrosine residue Y658, which corresponds to the p120-catenin binding site on VE-cadherin. Interestingly, VE-cadherin tyrosine phosphorylation was

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The clone P3X63 was used to control anti-CD31 and anti–VE-cadherin specificity. Data represent mean ± SD from three independent experiments (percentage of migrated neutrophils) compared with cell migration in TNF-α–activated neutrophils, which was considered 100%. *p < 0.05, **p < 0.01 by Wilcoxon signed rank test.

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FIGURE 6. The α2-adrenoceptor agonist UK14304 increases electrical resistance and reduces transendothelial macromolecular transport in HUVECs. (A) Effect of UK14304 (100 μM) on the electrical resistance of a confluent HUVEC monolayer, activated with TNF-α, TNF-α was added 5 min before the drug and left for 6 h. Data represent means ± SD (percentage change in electrical resistance) from six independent experiments with regard to control HUVECs (TNF), considered 100%. **p < 0.01 by Wilcoxon rank test. (B) Transendothelial passage of fluorescent dextran (70,000 m.w.) under the same conditions as in (A). Data were assessed in the lower Transwell compartments in six independent experiments and represent means ± SD (percentage of fluorescence) with regard to control HUVECs (TNF), considered 100%. *p < 0.05 by Wilcoxon rank test.
prevented when TNF-α–activated HUVECs were pretreated with UK14304 and then exposed to anti–ICAM-1–coupled microspheres (Fig. 8B). Because α2-adrenoceptor agonists induce a drop in intracellular cAMP levels, and taking into account that increment in cAMP has been linked to the enhancement of endothelial barrier function (52), we studied the effect of UK14304 in cAMP levels in TNF-activated HUVECs. The results in Supplemental Fig. 1 show that UK14304 reduces intracellular cAMP in 6-h TNF-activated HUVECs, an effect that is prevented by the α2-antagonist RX821002.

These data strongly suggest that α2-adrenergic agonists prevent the activation-dependent reduction of adherens junction protein complexes, as a consequence of reduced ICAM-1–mediated VE-cadherin phosphorylation. This effect would stabilize the endothelial barrier, thus hampering neutrophil extravasation.

Discussion

The major findings of this study are as follows: 1) endothelial cells and human neutrophils constitutively express α2B and α2A subtype adrenoceptors, respectively; 2) α2-adrenergic agonists cause an anti-inflammatory effect in animal models of acute inflammation by increasing the resistance of endothelial cells to neutrophil extravasation; and finally, 3) this effect is consistent with the interference in endothelial adherens junction instability that physiologically causes TNF during the inflammatory response. This seems to be produced by the reduction in ICAM-1–mediated phosphorylation of VE-cadherin induced by α2-adrenergic agonists.

The α2-adrenoceptor subtypes α2A, α2B, and α2C mediate some of the biological effects induced by endogenous catecholamines, both in the CNS (53, 54) and in a variety of peripheral tissues and cell types, including platelets (30) and leukocytes (35, 55, 56). Research on genetically deficient animals has clarified the biological roles of these receptors, which seem to be centered in the control of nociception and blood pressure (2). Pharmacological studies suggest the presence of α2-adrenergic receptors in different animal blood vessels (11, 12, 57). Although pharmacological data indicate the presence of functional α2-adrenoceptors in HUVECs (12), there are no published reports about subtype protein expression levels in this specific cell type. In this study, the three α2-adrenoceptor isotypes were detected by RT-PCR in HUVEC mRNA, whereas the subunit α2B was detected only at the protein level. However, different types of human endothelial

FIGURE 7. UK14304 prevents the downregulation of adherens junction protein complexes in activated endothelial cells. TNF-α–activated HUVECs were either maintained in medium or treated with UK14304. TNF-α was added 5 min before the drug and left for 6 h. They were then fixed, permeabilized, and stained using Abs against prototypical proteins of adherens junctions (left) or tight junctions (upper right). Stained with Alexa 488–labeled goat anti-mouse or anti-rabbit Igs. Original magnification ×400. Bar graph (lower right) shows data in terms of means ± SD of the percentage of fluorescence intensities with respect to cells maintained in the presence of UK14304, which were considered 100% for each condition (n = 4 random fields of view). *p < 0.05, **p < 0.005 by Wilcoxon rank test.
cells may well express different α2-adrenoceptor subtypes and exhibit differential reactions to natural and synthetic agonists.

In contrast to the well-established expression of β-adrenoceptors in lymphocytes (58), only a few studies have postulated, using indirect methods, the presence of α-adrenoceptors in mononuclear peripheral blood cells (10, 33, 55, 56). Their presence has also been suggested, similarly by indirect evidence, in human neutrophils (35). Given the absence of a biological response to α2-adrenoceptor agonists, however, a number of studies were unable to find any evidence supporting the presence of these receptors in neutrophils (9, 36, 44, 59). Our study demonstrates that resting human neutrophils, in a manner similar to HUVECs, constitutively express transcripts of all three α2-adrenoceptor subtypes, although only the α2A subtype was detectable at the protein level.

The α-adrenoceptors induce intracellular signaling through the Gi/Go complex, which in turn inhibits adenyl cyclase and reduces cAMP levels (60). We tested the well-characterized α2-adrenergic agonist UK14304 (37) and found significantly decreased cAMP concentrations in human neutrophils, indicating that this cell type expressed functional α2-adrenoceptors.

Several reports suggest that α2-adrenoceptor agonists might exert both peripheral (13, 14) and central (15) anti-inflammatory actions. In our study, data obtained from animal models of inflammation demonstrated that xylazine and UK14304 significantly reduce neutrophil recruitment and accumulation in the inflammatory focus. Treatment of these animals with the α2-adrenergic antagonist RX821002 prevented the anti-inflammatory action of both α2-agonists. Hypothermia (40) and neutrophil apoptosis (44) are two in vivo effects that have been attributed to α2-agonists, both of which might have contributed to the observed decrease in neutrophil transmigration (41). Controlling α2-agonist–induced hypothermia by an external heat source, however, did not significantly interfere with the anti-inflammatory action of xylazine. In addition, UK14304 neither caused a noticeable reduction in circulating neutrophils in animals nor induced apoptosis in human neutrophils under our in vitro experimental conditions. Therefore, these data suggested that the α2-adrenoceptors were transducing signals that interfered with the recruitment of neutrophils to the inflammatory foci, very likely through a peripheral effect on circulating neutrophils and/or vascular endothelial cells.

During the inflammatory response, the initial contact between circulating neutrophils and the endothelium is mediated by selectins. These interactions reduce neutrophil velocity, and circulation is converted into rolling on the endothelium. Next, endothelial ICAM-1 is engaged by neutrophil integrins CD11b, and mainly, CD11a, which switches from rolling to firm neutrophil adhesion to the endothelium. During the last phase of the cascade, neutrophils navigate their way between the endothelia cells (diapedesis) to
reach the inflammatory tissue focus (19). This final stage is mediated by a number of endothelial adhesion molecules, such as CD31 (PECAM-1), CD99, VE-cadherin, and members of the junctional adhesion molecule family (61). We did not detect relevant differences when we studied the effects of the α2-adrenergic agonist UK14304 on L-selectin and CD11b neutrophil expression levels. Nonetheless, when UK14304-pretreated human neutrophils were activated with IL-8, a chemokine widely implicated in neutrophil activation during the adhesion cascade (46), these cells reduced sensitivity to this chemokine in terms of L-selectin shedding and increased CD11b expression. This finding is consistent with the response of L-selectin and CD11b, which has been observed when neutrophils are activated with IMLP in the presence of epinephrine (62). Thus, our data suggest that signaling via the α2-adrenoceptor renders neutrophils less sensitive to proinflammatory mediators. Part of the anti-inflammatory effects of several nonsteroidal anti-inflammatory drugs, such as piroxicam, phenylbutazone, and meloxicam, has been attributed to a similar mechanism in neutrophils (63).

The endothelium plays an essential role in inflammation, providing the key signals for leukocyte migration to extravascular tissues. It does so by regulating the level of ICAM-1 and VCAM-1 expression in response to proinflammatory signals (64). Interestingly, UK14304 and xylazine reduced, in a dose-dependent manner, the capability of HUVECs to upregulate ICAM-1 in response to TNF-α. This decreased endothelial ICAM-1 expression, along with the reduced response of neutrophils to augment CD11b (which serves to maintain the expression of L-selectin), suggests that the anti-inflammatory mechanisms underlying α2-adrenergic agonist activity most likely affect the later steps of the adhesion cascade—either during firm adhesion or extravasation. In addition, our data demonstrate that UK14304 increases endothelial resistance to the ion as well as macromolecular passage. In similar fashion, both adrenaline and clonidine, two other α2-agonists, significantly reduced the paracellular transport of macromolecules in an epithelial model of Caco-2 cells (65). These results, together with our observations of neutrophil transmigration through endothelial monolayers, strongly point to endothelial cells as the main target for the anti-inflammatory effect of α2-adrenergic agonists in vivo.

The final step of leukocyte extravasation during the inflammatory response requires that leukocytes squeeze between adjacent endothelial cells to pass across the endothelium. VE-cadherin is a specific component of homophilic, calcium-dependent, endothelial junctions and plays an important role in endothelial stability (66). Most permeability-inducing molecules promote an increase in VE-cadherin tyrosine phosphorylation and a decrease of VE-cadherin/catenin binding, which results in the opening of endothelial cell–cell junctions (67). Phosphorylation of VE-cadherin is regulated by signaling initiated through the binding of the endothelial ICAM-1 to its neutrophil ligand (51). During transendothelial migration, VE-cadherin disappears transiently from the cell surface, facilitating leukocyte extravasation. Thereafter, the extra-cellular domain reappears rapidly, reconstituting the endothelial junctions (20). Our initial observations indicated that activated endothelial cells respond to α2-agonists by avoiding the upregulation of ICAM-1, the molecule that, in combination with the cathersins, plays a key role in regulating leukocyte extravasation (68). Data on the cell distribution of proteins from adherens junctions revealed that the downregulation of VE-cadherin, plakoglobin, and β-catenin induced by TNF-α was prevented by the presence of UK14304 in confluent HUVEC monolayers. However, the α2-agonist did not modify the expression of either ZO-1, claudin-5, two tight junction proteins, or α-catenin, an actin-binding protein, in activated HUVECs. Most importantly, treatment of endothelial cells with UK14304 resulted in a reduction in the ICAM-1–mediated phosphorylation of VE-cadherin at the p120-catenin binding site, which may explain the permanence of VE-cadherin at the interendothelial junctions during cell activation. This decline in ICAM-1 expression in α2-agonist–treated HUVECs may also contribute to the decrease in VE-cadherin phosphorylation. However, this is not likely to represent the main mechanism of action within the anti-inflammatory spectrum of UK14304, as the incubation periods necessary to reduce ICAM-1 surface expression in HUVECs are considerably longer than those required to lessen VE-cadherin phosphorylation. Data regarding the changes in cAMP levels induced by UK14304 in HUVECs suggest that the effect of α2-adrenergic agonists interfering with the TNF-α–mediated endothelial adherens junction instability cannot be explained by variations in the intracellular cAMP levels.

Taken together, our results demonstrate that α2-adrenergic agonists modulate the inflammatory response at the endothelial level. They interfere with the final phase of the adhesion cascade by maintaining the integrity of endothelial adherent junctions, thereby rendering the endothelium more resistant to TNF activation, and consequently to leukocyte transmigration. These findings point to the vascular endothelium as a potential therapeutic target for the development of new anti-inflammatory compounds, which could improve management of inflammatory diseases in humans.

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Disclosures
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