British Journal of Pharmacology (2004) 143, 1033–1041
www.nature.com/bjp

β_2-Adrenergic receptor regulation of human neutrophil function is sexually dimorphic

[1,2,5]Catherine de Coupade, [1]Robert W. Gear, [3]Paul F. Dazin, [4]Herve Y. Sroussi, [1]Paul G. Green & *,[1]Jon D. Levine

[1]Department of Medicine, NIH Pain Center, CA, U.S.A.; [2]Department of Oral and Maxillofacial Surgery, NIH Pain Center, CA, U.S.A.; [3]Howard Hughes Medical Institute, CA, U.S.A. and [4]Oral Biology, UCSF, San Francisco, CA 94143-0440, U.S.A.

1 While the mechanisms underlying the marked sexual dimorphism in inflammatory diseases are not well understood, the sexually dimorphic sympathoadrenal axis profoundly affects the inflammatory response. We tested whether adrenergic receptor-mediated activation of human neutrophil function is sexually dimorphic, since neutrophils provide the first line of defense in the inflammatory response.

2 There was a marked sexual dimorphism in β_2-adrenergic receptor binding, using the specific β_2-adrenergic receptor ligand, [^{3}H]-dihydroalprenolol, with almost three times more binding sites on neutrophils from females (20,878 $\pm$ 2470) compared to males (7331 $\pm$ 3179).

3 There was also a marked sexual dimorphism in the effects of isoprenaline, a β-adrenergic receptor agonist, which increased nondirected locomotion (chemokinesis) in neutrophils obtained from females, while having no effect on neutrophils from males.

4 Isoprenaline stimulated the release of a chemotactic factor from neutrophils obtained from females, but not from males. This chemotactic factor acts on the G protein-coupled CXC chemokine receptor 2 (CXCR2) chemokine receptor, since an anti-CXCR2 antibody and the selective nonpeptide CXCR2 antagonist SB225002, inhibited chemotaxis produced by this factor. While interleukin- (IL-) 8 is a principal CXCR2 ligand, isoprenaline did not produce an increase in IL-8 release from neutrophils.

5 IL-8-induced chemotaxis was inhibited in a sexually dimorphic manner by isoprenaline, which also stimulated release of a mediator from neutrophils that induced chemotaxis, that was inhibited by anti-CXCR2 antibodies.

6 These findings indicate an important role for adrenergic receptors in the modulation of neutrophil trafficking, which could contribute to sex-differences in the inflammatory response.
British Journal of Pharmacology (2004) **143,** 1033–1041. doi:10.1038/sj.bjp.0705972

Keywords: Cell recruitment; chemotaxis; chemokinesis; β-adrenergic receptor; inflammation; sex differences; chemokine; polymorphonuclear leucocytes

Abbreviations: CXCR2, CXC chemokine receptor 2; DHA, dihydroalprenolol; ELISA, enzyme-linked immunosorbent assay; HEK, human embryonic kidney; IL, interleukin; n.s., nonsignificant; PBS, phosphate-buffered saline; RT–PCR, reverse transcriptase–polymerase chain reaction; TNF-α, tumor necrosis factor-α

Introduction

Inflammation is a physiological process that protects against infection and promotes tissue repair. Neutrophils, which constitute 70% of circulating leucocytes, provide the first line of defense against infection by being rapidly recruited to sites of infection and inflammation where they enhance the inflammatory response by increasing tissue concentrations of proinflammatory cytokines like tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6 and IL-8 (Cassatella, 1995). However, prolonged neutrophil recruitment and activation can lead to tissue damage and eventually to inflammatory diseases (Feldmann *et al*., 1996; Barnes, 1998; Ajuebor *et al*., 2002). Many inflammatory diseases, such as rheumatoid arthritis and systemic lupus erythematosus, have a much greater incidence in women (Green, 1992; Da Silva, 1995; Gaillard *et al*., 1998; Castagnetta *et al*., 2002), and there is a similar sexual dimorphism in animal models of inflammatory disease (Wilder *et al*., 1982; Allen *et al*., 1983; Griffiths *et al*., 1994). We have shown that sex differences in the inflammatory response in rats are adrenal medulla dependent (Green *et al*., 1999), and that activation of the sympathoadrenal system by stress or noxious stimulation inhibits the inflammatory response (Green *et al*., 1997; Strausbaugh *et al*., 1999). It is likely that adrenaline plays an important role, since it is a principal adrenal medulla mediator and the inflammatory response can be regulated through β_2-adrenergic receptor activation (Coderre *et al*., 1990; Ottonello *et al*., 1996; Barnes, 1999; Mills *et al*., 2000). While the cellular site of action of adrenaline on the inflammatory response is currently unknown, β_2-adrenergic receptors in humans are constitutively expressed in a wide variety of tissues including immune cells,

*Author for correspondence at: NIH Pain Center, C522 Box 0440, UCSF, 521 Parnassus Avenue, San Francisco, CA 94143-0440, U.S.A.; E-mail: levine@itsa.ucsf.edu
[5]Current address: Diatos SA, Paris BioPark, 3-5 Impasse Reille, 75014 Paris, France
Advance online publication: 11 October 2004

where activation of these receptors leads to anti-inflammatory actions, for example, by regulation of cytokine production in leucocytes, reduction of plasma exudation and cell adhesion to activated endothelial cells (Barnes, 1999; Johnson, 2002). Furthermore, there is evidence that neutrophils may play a role in the sexual dimorphism of the inflammatory response (Spitzer, 1999). For example, administration of progesterone produces an increase in the number of β_2-adrenergic receptors on lymphocytes in healthy women (Marchetti *et al*., 1994), but decreases them in asthmatic women (Tan *et al*., 1997). While β_2-adrenergic receptor agonists have also been implicated in regulating migration of neutrophils (Elferink *et al*., 1996) and monocytes (Straub *et al*., 2000), it is not known whether neutrophil function is sexually dimorphic.

The purpose of this study was to investigate sexual dimorphism in immune functions of human neutrophils after β_2-adrenergic receptor activation. Since crosstalk between receptors and downstream signaling pathways could influence the migratory response of leucocytes (Campbell *et al*., 1997; Struyf *et al*., 2001), we also studied the regulatory role of β_2-adrenergic receptor in cytokine-induced neutrophil locomotion.

Methods

Drug treatments

Isoprenaline (10^{-10}, 10^{-8} or 10^{-6} M; Sigma, St Louis, MO, U.S.A.) and the human recombinant IL-8, now known as CXCL8 (10, 30 or 50 ng ml^{-1} from R&D System, Minneapolis, MN, U.S.A.) were used in the neutrophil migration assays. ICI-118,551 hydrochloride (10 μM from Tocris, Ellisville, MO, U.S.A.) was incubated with neutrophils for 30 min at 37°C prior to the migration assay.

For neutralizing experiments, neutrophils were incubated 15 min on ice with a monoclonal anti-human CXC chemokine receptor 2 (CXCR2) (IL-8 RB) antibody (1 μg ml^{-1}; R&D System, Minneapolis, MN, U.S.A.) before stimulating neutrophils with isoprenaline. The selective nonpeptide CXCR2 antagonist, SB225002 (300 nM; Calbiochem, San Diego, CA, U.S.A.), was added to the upper chamber and the selective CXCR2 ligand GROα (10 nM; R&D System, Minneapolis, MN, U.S.A.) was added to the lower chamber.

Cell lines

HEK-293 and stably transfected HEK-293 cells with wild-type human β_2-adrenergic receptor gene were generous gifts of Dr M. Von Zastrow (UCSF).

Neutrophil isolation

Peripheral blood was collected between 08:00 and 09:00 h from nonsmoking healthy donors (18 male, age range 27–61 years, and 12 female, age range 25–45 years) in heparinized tubes, loaded on a Histopaque-1119/1077 gradient (Sigma) and centrifuged at $700 \times g$ for 30 min at 25°C. The Committee on Human Research at UCSF approved this procedure. Neutrophils were recovered at the interface of histopaque 1119/1077 and washed in a cold nonfixing lysing solution (1500 mM NH_4Cl, 100 mM $NaHCO_3$ and 10 mM EDTA) for 10 min at room temperature. Cells were centrifuged at 4°C for 10 min at $250 \times g$ and washed three times in cold PBS. Cell viability (>95%) was assessed by trypan blue exclusion.

RT–PCR

Total RNA from neutrophils was extracted with RNeasy (Qiagen, Valencia, CA, U.S.A.), according to the manufacturer's instructions. Total RNA (3 μg) was used in each reverse transcriptase–polymerase chain reaction (RT–PCR) reaction. The internal primers for the β_1-adrenergic receptor were 5′-ATGGGCGCGGGGGTGCTCGTCCTGGGCGCC-3′ and 3′-GGGCCGAAGCGGAGCCTTAGGTTCCACATC-5′ and primers for the β_2-adrenergic receptor were 5′-ATGGGG CAACCCGGGAACGGCAGCGCCTTC-3′ and 3′-AATG TCGTCACTCAGTAAACATGATGTTAAGGA-5′. GAPDH was used as an internal control of the RT–PCR.

Western blot analysis

Neutrophils (10^7) were incubated in a lysis buffer (10 mM Tris, pH 7.2, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1% Nonidet P-40) for 30 min on ice in the presence of protease inhibitors (100 mM PMSF, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} aprotinin and 1 μg ml^{-1} pepstatin). For membrane fraction preparation, cells were collected and hand homogenized in a Dounce homogenizer in 0.32 M sucrose pH 7.4 buffered with HEPES and supplemented with protease inhibitors. After centrifugation (5 min at $4000 \times g$), supernatants were resuspended in sucrose–HEPES solution and centrifuged 15 min at $75{,}000 \times g$. The pellets were then resuspended in Tris–HCl + 5 mM EDTA buffer supplemented with protease inhibitors and spun for 30 min at $75{,}000 \times g$. Pellets were used as the membrane protein fraction and 30 μg were separated on 10% polyacrylamide gels (Laemmli, 1970) and electroblotted onto nitrocellulose membranes (Biorad, Hercules, CA, U.S.A.). Immunostaining was performed using a polyclonal β_2-adrenergic receptor antibody (1/200, H-20 from Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). The immunoreactive bands were detected with an anti-rabbit IgG-horseradish peroxidase secondary antibody (1/10,000; Sigma) and peroxidase reaction was developed using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, U.K.).

Neutrophil mobility assays

Chemotaxis, chemokinesis and fugetaxis were performed as previously described (Campbell *et al*., 1997) with modifications. Human neutrophils were resuspended in migration medium at a density of 10×10^6 cells ml^{-1} (0.2 μm filtered RPMI-1640 + 25 mM HEPES + 0.5% BSA (Fraction V, Sigma)). The wells were set-up ahead of time with 600 μl dilutions of chemoattractants and uniform-field agonist. Transwell inserts (Fisher, Santa Clara, CA, U.S.A.) were placed in each well of 24-well tissue culture plates forming an upper and lower chamber separated by a polyester membrane having 3 μm pores. Neutrophils (100 μl) added to the chamber, were incubated at 37°C for 3 h. The inserts were carefully removed and the migrated cells were stained with Hoechst 33342 (5 μg ml^{-1}, Sigma) for nucleated cells separation from red blood cells and propidium iodide (1 μg ml^{-1}, Sigma) to assess nucleated cell viability. Leucocyte classification was performed using nuclear content, forward scatter and side scatter patterns

obtained from excitation at 488 and 354/63 nm wavelengths. Log fluorescence was measured for 30 s at constant pressure for each sample, using a triple laser Vantage SE cell sorter (Becton Dickinson, San Jose, CA, U.S.A.). Data acquisition and analysis were performed using CellQuest Pro software, version 4.01 (Becton Dickinson).

Neutrophil β_2-adrenergic receptor binding assay

β_2-adrenergic receptor agonist binding to human neutrophils was performed as previously described (Sandnes *et al*., 1987). Briefly, binding assays were performed on whole cells using [^{3}H] di-hydroalprenolol (DHA) (Amersham Corp., 97 Ci mmol^{-1}). Neutrophils (1.5×10^6) were incubated with various concentrations of hot ligand [^{3}H] DHA in binding buffer (50 mM HEPES, 5 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5% BSA) for 3 h at room temperature prior to filtration through glass filters (GF/C, Whatman). DHA binding was measured by scintillation counting of bound ligand, 24 h later (Wallac 1410). Nonspecific binding of DHA was determined in the presence of 10 μM alprenolol. Specific binding represents the total number of DHA contents minus DHA binding in the presence of alprenolol (10 μM, Sigma). Mean saturation binding data from triplicate determinations were analyzed using Prism software in order to determine the number of receptors per cell.

Plasma adrenaline and noradrenaline measurement

Plasma adrenaline and noradrenaline concentrations, taken at the time of leucocyte harvesting, was determined using HPLC with electrochemical detection, as previously described (Green *et al*., 1993). Plasma samples (500 μl) were adsorbed onto alumina at pH 8.5. After washing, catecholamines were eluted by treating the alumina with 100 μl acetic acid. A LC-10ADvp solvent delivery system (Shimadzu Scientific Instruments, Columbia MD, U.S.A., a reverse-phase column, and a coulometric electrochemical detector (ESA, Inc., Chelmsford, MA, U.S.A.) were used.

IL-8 quantification

The level of human IL-8 was measured by an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (Cell Analysis, Beckman Coulter, Inc., Brea, CA, U.S.A.). Briefly, 50 μl of samples or human recombinant IL-8 protein were added to monoclonal anti-IL-8 antibody-coated wells and incubated for 2 h at room temperature. Biotinylated anti-human IL-8 and streptavidin–horseradish peroxidase conjugate were used for detection. The detection limit of the assay was 8 pg ml^{-1}.

Statistical analysis

Significant differences between groups ($P<0.05$). were determined with repeated-measures ANOVAs. In cases where there was a significant interaction between a within-subjects factor (e.g. dose) and sex, the analysis was repeated for each sex individually in order to determine the basis of the interaction. In cases where a between-subjects factor with more than two levels was significant, Tukey *post hoc* comparisons were performed to determine the basis of the difference. The Mauchly criterion was used to determine if the assumption of sphericity for the within-subjects effects was met; if the Mauchly criterion was not satisfied, Huynh–Feldt adjusted *P*-values are presented.

Results

Expression of β_2-adrenergic receptors in human neutrophils

The first step in assessing the role of β_2-adrenergic receptors in neutrophil function was to determine which receptor subtypes are expressed in those cells. We first used RT–PCR and Western blot analysis to evaluate β_2- and β_1-adrenergic receptor expression in neutrophils from males and females (the β_3-adrenergic receptor is not present in immune cells (Berkowitz *et al*., 1995)). Total RNA was extracted from freshly isolated neutrophils from males and females and RT–PCR was performed using primers specific for human β_1- and β_2-adrenergic receptors. No expression of β_1-adrenergic receptor message was detected in either male or female fraction (Figure 1a, left panel). In contrast, significant β_2-adrenergic receptor messenger RNA was detected in neutrophils in a band at the expected location of 1213 bp with

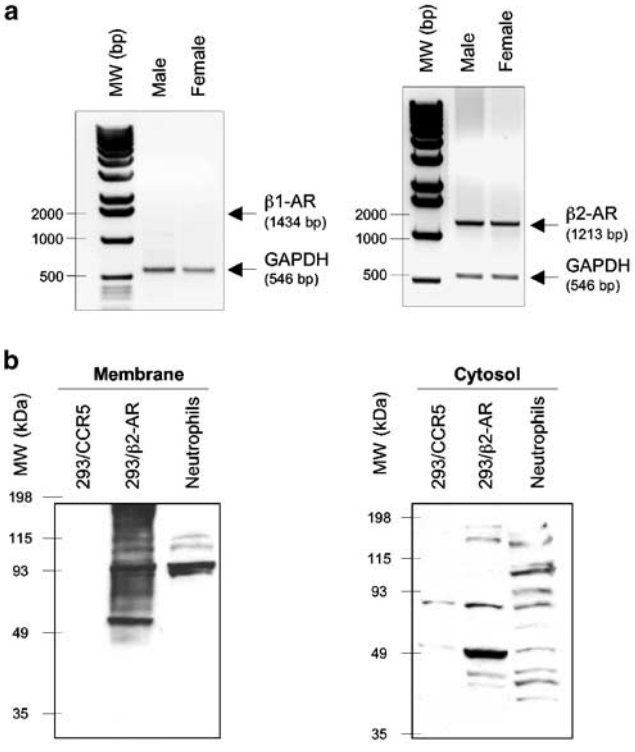


Figure 1 Human neutrophils express β_2-adrenergic receptors. (a) RT–PCR analysis of β_1 and β_2-adrenergic receptor expressions in neutrophils from males and females. (b) β_2-adrenergic receptor protein expression in the membrane fraction (left panel) and the cytosolic fraction (right panel) of male neutrophils. HEK-293 cells stably transfected with the human CCR5 gene (293/CCR5) were used as a negative control and HEK-293 cells stably transfected with the human β_2-adrenergic receptor gene (293/β_2-AR) as positive control. Data shown are representative of three independent experiments.

equal expression in males and females (Figure 1a, right panel). Given the absence of β_1-adrenergic receptor message, it is likely that the effects of β-adrenergic receptor agonists on neutrophils are mediated by β_2-adrenergic receptors.

To determine the levels at which receptors are present in neutrophils, we measured β_2-adrenergic receptor immunoreactivity in cytosolic and membrane protein extracts. Using a specific antibody against human β_2-adrenergic receptors, the cytosolic fraction showed immunoreactive bands with molecular weights between 35 and 115 kDa; similar bands were seen in males and females (Figure 1b, data not shown for females). The specificity of the immunoreaction was confirmed by the negligible background immunoreactivity detected in HEK-293 cells stably transfected with the human gene of the CCR5 chemokine receptor. HEK-293 cells stably transfected with β_2-adrenergic receptor gene were used as a positive control. β_2-adrenergic receptor protein immunodetected around 90 kDa was expressed at high levels in the neutrophil membrane fraction (Figure 1b, left panel). HEK-293/CCR5 showed no reactive band while HEK-293/β_2-adrenergic receptors showed major bands at 50, 90 kDa and greater, as previously reported for these transfected cells (von Zastrow *et al*., 1992).

Sex difference in isoprenaline-induced neutrophil mobility

Previous studies reporting a chemoattractant effect of β-adrenergic receptor agonists, prompted us to investigate the effect of isoprenaline on human neutrophil mobility. For this study, we performed checkerboard analysis of neutrophil migration to different concentrations of isoprenaline on neutrophils from males and females using the Transwell-clear insert model. The effect of isoprenaline (0, 10^{-10}, 10^{-8} and 10^{-6} M) was measured by counting the number of cells (expressed as percent of total) that migrated into the lower chamber from the upper chamber in each of three experimental protocols: (1) chemotaxis (migration toward) was assessed by adding isoprenaline only to the lower chamber (Figure 2a) (2) chemokinesis (random migration) was assessed by adding isoprenaline in equal concentrations to both upper and lower chambers (Figure 2b); and (3) fugetaxis (migration away) was assessed by adding isoprenaline only to the upper chamber (Figure 2c). A three-way repeated measures ANOVA (within-subjects factor: dose) demonstrated a significant dose $\times$ sex $\times$ protocol interaction ($P<0.05$), indicating that responses to increasing doses of isoprenaline were significantly different between neutrophils from males and females and that

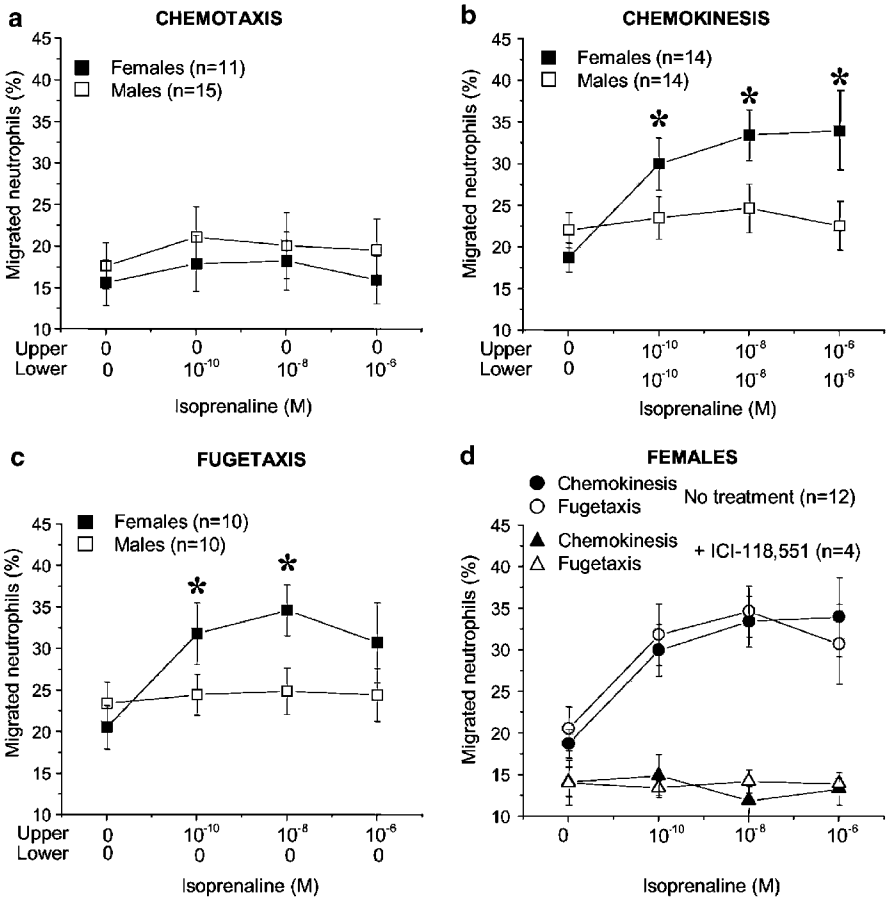


Figure 2 Checkerboard transmigration analysis of human neutrophils in response to serial dilutions of isoprenaline. (a) Chemotaxis analysis with isoprenaline added to the lower chamber; (b) chemokinesis analysis with isoprenaline added to both lower and upper chambers, and (c) fugetaxis with isoprenaline added to the upper chamber. (d) Inhibitory effect of ICI-118,551 on female chemokinesis and fugetaxis. Cells were preincubated with the β_2-adrenergic receptor antagonist before the transmigration assay. Numbers shown represent mean of percentage of transmigrated cells $\pm$ s.e.m. with n, number of donors. Asterisks indicates $P<0.05$ for a pairwise contrasts for each isoprenaline concentration compare to no isoprenaline.

these effects differed among the three protocols. A two-way ANOVA (dose × sex), performed separately for each of the three protocols, showed significant dose × sex interactions for fugetaxis ($P<0.005$) and chemokinesis ($P<0.005$) but not for chemotaxis, indicating significant sex differences in two of the three protocols. Separate one-way repeated measures ANOVA analyses (dose) for sex in the fugetaxis and chemokinesis protocols indicated that only neutrophils from females responded to isoprenaline ($P<0.001$ and 0.005, respectively). The three-way ANOVA also showed significant differences among the protocols (main effect, $P<0.01$); Tukey *post hoc* analysis demonstrated that when isoprenaline was added alone to the lower chamber (to induce chemotaxis), migration was significantly less than when isoprenaline was added either to the upper chamber (to induce fugetaxis, $P<0.05$) or to both the upper and lower chambers (to induce chemokinesis, $P<0.01$). There was no significant difference between fugetaxis and chemokinesis in neutrophils from females. Significant inhibition by the selective β_2-adrenergic receptor antagonist, ICI-118,551 ($P<0.005$) indicated the specificity of β_2-adrenergic receptor effects on neutrophil mobility in females (Figure 2d). ICI-118,551 lowered the baseline level, suggesting a spontaneous activation of the receptor even in the absence of agonist, as reported previously for this receptor (Stevens *et al*., 1998; Zhou *et al*., 2000).

These results indicate that β_2-adrenergic receptor activation induces nondirected neutrophil movement in females but not in males.

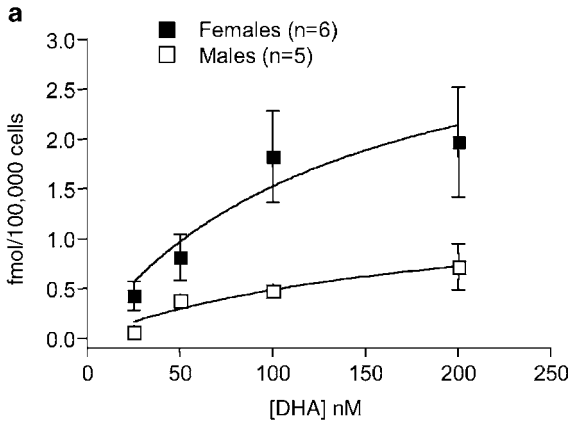
Sex differences in β_2-adrenergic receptor binding in neutrophils

Receptor binding experiments with different concentrations of DHA were undertaken to determine if the sex difference in β_2-adrenergic receptor-mediated enhancement of neutrophil mobility could be explained by a difference in receptor numbers.

These. results showed significant sex × [^{3}H] DHA binding interaction ($P<0.05$; Figure 3a) binding in female neutrophils ($20{,}878 \pm 2470$ binding sites cell^{-1}) was significantly higher than in males (7331 ± 3179 binding sites cell^{-1}), (Figure 3b; $P<0.01$). Since numbers can be regulated by agonist level (Wahle *et al*., 2001), we measured adrenaline and noradrenaline plasma concentrations in the subjects; there was no significant sex difference in adrenaline and noradrenaline (Figure 4).

β_2-adrenergic receptor stimulation reduces IL-8-induced chemotaxis

Our observation that β_2-adrenergic receptor stimulation elicited nondirected cell movement prompted us to investigate the potential interaction this effect might have with chemokine-induced chemotaxis. IL-8, added to the lower chamber in the absence of isoprenaline, induced neutrophil migration from the upper to the lower chamber in a dose-related fashion for both females and males (both $P<0.001$). That is, addition of isoprenaline to the upper chamber significantly attenuated IL-8-induced migration in neutrophils from females ($P<0.001$) but not from males (n.s.) (Figure 5). These results show that β_2-adrenergic receptor stimulation produced sexually dimorphic impairment of IL-8-induced migration.



b

	Bmax
Males (n =5)	7,331 ± 3,179
Females (n =6)	20,878 ± 2,470
P-value (two tailed)	0.009

Figure 3 β_2-adrenergic receptor density on neutrophils is greater in females than males. (a) Saturation curves representing [^{3}H]-DHA binding in neutrophils freshly isolated from males and females. (b) Specific binding values were obtained by subtracting nonspecific binding from total binding. Data were analyzed using a nonlinear regression plot. Data represent mean ± s.e.m. with *n*, number of donors.

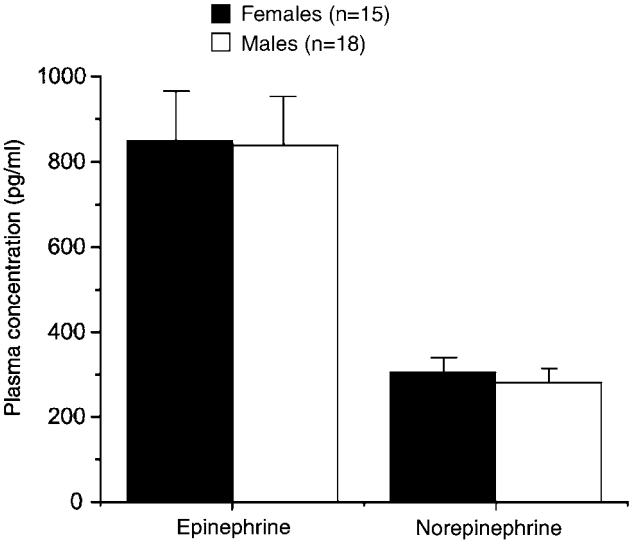


Figure 4 Determination of plasma adrenaline and noradrenaline concentrations in females and males donors. Data represent mean ± s.e.m.; *n*, number of donors.

CXCR2 neutralizing antibodies reduce neutrophil locomotion in response to isoprenaline

One mechanism by which catecholamines might modulate cytokine-induced chemotaxis would be by altering the

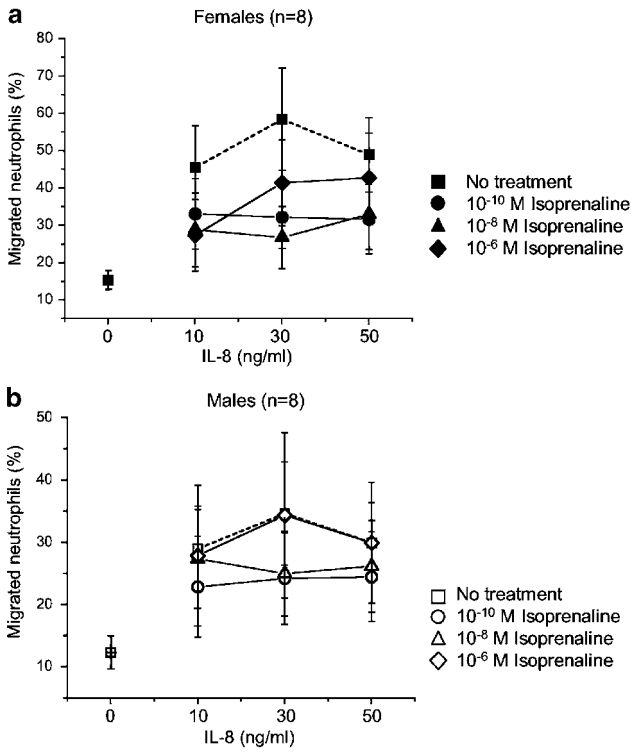


Figure 5 Inhibition of IL-8-induced neutrophil chemotaxis in the presence of isoprenaline. Neutrophil locomotion in females (a) and males (b) is expressed as percentage of migrated cells into the lower chamber. Dose–response to IL-8 is shown as a control curve. Neutrophil migration is shown in the presence of 10^{-10}, 10^{-8} or 10^{-6} M isoprenaline for the three different concentrations of IL-8. Data are expressed as mean ± s.e.m.; *n*, number of donors.

concentration gradient of the inflammatory source of cytokines by autocrine release of chemotactic factors. Thus, IL-8-induced chemotaxis to the lower chamber would be decreased if isoprenaline released a chemotactic cytokine (e.g. IL-8) from neutrophils in the upper chamber, thereby decreasing the chemotactic concentration gradient. If so, then cytokine accumulation in the culture medium of isoprenaline-stimulated cells should itself be capable of inducing neutrophil chemotaxis.

To investigate this hypothesis, neutrophils from females were incubated with different concentrations of isoprenaline for 3 h. Cell-free supernatant from these cultures, placed in the lower chamber with unstimulated neutrophils in the upper chamber, induced chemotaxis that depended on the isoprenaline concentration in the conditioning cultures, in females ($P<0.05$) but not in males (Figure 6a). This finding is compatible with the suggestion that the supernatants contained an activating factor released from neutrophils. Residual isoprenaline that might have remained in the cell-free supernatants should not have contributed to the response since isoprenaline alone does not induce chemotaxis (Figure 2a).

Since β_2-adrenergic receptor agonists are known to stimulate IL-8 release (Linden, 1996; Kavelaars *et al*., 1997), we investigated whether the released factor could be IL-8. Neutralizing antibody for CXCR2 receptors (cytokine receptors for IL-8, present on neutrophils) was added to the upper chamber along with the unstimulated cells. This treatment significantly attenuated female cell migration towards supernatant from isoprenaline-stimulated cells ($P<0.05$), but had no significant effect on neutrophils obtained from males (Figure 6a). To confirm the specificity of this effect, we also evaluated the effect selective nonpeptide CXCR2 antagonist, SB225002, on the migration of cell migration in females, in response to supernatant from isoprenaline-stimulated cells. SB225002 also significantly inhibited cell migration towards the supernatant from isoprenaline-stimulated cells ($P<0.05$), as well as migration towards the selective CXCR2 ligand, GROα ($P<0.05$)

To determine if the unknown mediator released by isoprenaline stimulation was IL-8, cell-free supernatants harvested from neutrophils (1×10^5 cells) were pretreated with different doses of isoprenaline were quantified for IL-8 content by ELISA. IL-8 concentration was not affected by any dose of isoprenaline (Figure 6b), suggesting that the CXCR2 receptor agonist released from neutrophils is a cytokine other than IL-8.

Discussion

Our data show a striking sexual dimorphism in human neutrophil function mediated by β_2-adrenergic receptor activation. This may be a key mechanism underlying sexual dimorphism in the inflammatory response, and moreover, the mechanism by which sympathoadrenal activity modulates inflammation. The control of leucocyte migration is of fundamental importance to the timely resolution of inflammatory response; chronic inflammatory diseases are believed to be linked to a failure to terminate leucocyte recruitment into inflamed tissue (Buckley, 2003). Sexual dimorphism in the incidence and severity of inflammatory diseases may be dependent, at least in part, on differential sensitivity of leucocytes to inflammatory cytokines. We also showed that β_2-, but not β_1-, adrenergic receptors are abundantly expressed in neutrophils.

Although previous investigators concluded that isoprenaline promotes chemotactic migration of human monocytes and macrophages (Straub *et al*., 2000), our data demonstrate that it does not mediate chemotaxis in neutrophils from either males or in females, consistent with previous observations in dendritic (Maestroni, 2000) and HEK-293 cells (Neptune *et al*., 1997). In our study, we specifically tested for chemokinesis, in addition to chemotaxis and showed that, when isoprenaline was incubated with the cells and/or added to both chambers, cell mobility of neutrophils from females but not from males was strongly enhanced, suggesting a sex-dependent role for β_2-adrenergic receptors in initiating nondirected movement. However, it should be noted that chemokinetic effect of isoprenaline was concluded solely from the data obtained in the checkerboard analysis. We cannot, therefore, categorically exclude a chemotactic effect since we did not directly determine the effect of isoprenaline on cell asymmetry or polarization.

There is growing evidence that sex steroids influence the immune response by modulation of β_2-adrenergic receptor density on immune cells. It has been shown that on human leucocytes, female sex steroids upregulate β_2-adrenergic receptor density (Marchetti *et al*., 1994). In our binding assays, we showed a three-fold higher β_2-adrenergic receptor density in neutrophils from females, suggesting that for a similar increase in plasma catecholamine concentration *in vivo*,

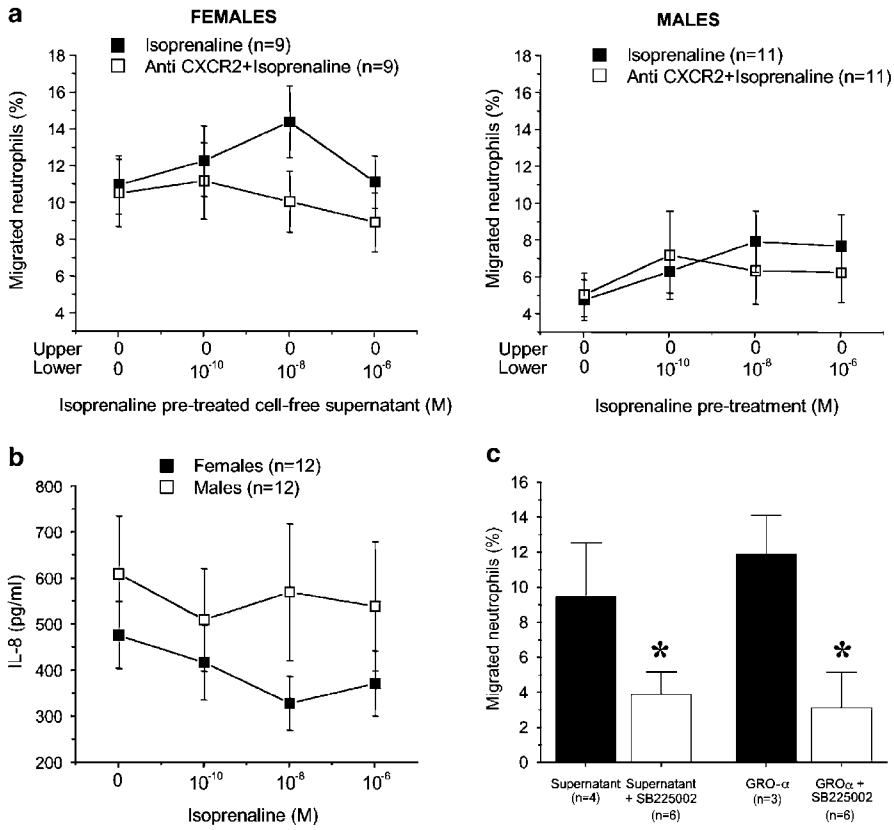


Figure 6 Neutrophil β_2-adrenergic receptor activation stimulates the release of a mediator that enhances chemotaxis. (a) Freshly isolated neutrophils from females (left panel) and males (right panel) were incubated with different concentrations of isoprenaline for 3 h at 37°C. Cell-free supernatants were added to the lower chamber of transwells and tested for their chemoattractive properties with freshly neutrophils added to the upper chamber. Neutralizing antibody against human CXCR2 was incubated on ice for 15 min prior to isoprenaline pretreatment. Note that baseline migration levels of neutrophils were 35% (male) and 65% (females) less than the data shown in Figure 2 (checkerboard analysis). This difference could be due to the fact that in this experiment, neutrophils were used 5 h after blood harvesting, compared to 2 h for the checkerboard analysis. Values are mean ± s.e.m.; *n*, number of donors. (b) Quantification of IL-8 released by isoprenaline-stimulated neutrophils. Level of IL-8 was determined by EIA from cell-free supernatants of isoprenaline-stimulated neutrophils from females and males. The values are mean ± s.e.m.; *n*, number of donors. (c) Freshly isolated neutrophils from females were added to the upper chamber with or without the selective CXCR2 receptor antagonist, SB225002 (300 nM) and either cell-free supernatant from isoprenaline (10^{-8} M)-stimulated cells or the selective CXCR2 agonist GROα (10 nM) was added to the lower chamber. Asterisks indicate $P<0.05$ for a paired one-tailed *t*-test for the effect of SB225002.

β_2-adrenergic receptor signal transduction and neutrophil response is likely to be significantly more pronounced in females. This result is consistent with the sex difference in neutrophil mobility we observed. However, the number of receptors expressed on the membrane does not necessarily reflect the level of receptor signaling activity (Lombardi *et al*., 1999) and therefore, we cannot exclude other mechanisms contributing to the observed sexual dimorphism.

While plasma levels of endogenous catecholamines, such as adrenaline and noradrenaline, are relevant to β_2-adrenergic receptor activity, we found no gender difference in the levels of catecholamines, in agreement with the literature (Davis *et al*., 2000; Geelen *et al*., 2002). Plasma catecholamine concentrations usually range around 1×10^{-9} M for noradrenaline and 0.2×10^{-9} M for adrenaline, but increase in response to stress and anxiety (Swain, 2000), or hypoglycemia (Goldstein *et al*., 2003). In our study, blood samples were obtained from subjects in a normal laboratory setting (and not from supine subjects in a light- and noise-controlled room), and we observed plasma catecholamine concentrations higher than some other reports, consistent with mild stress associated with the venipuncture procedure (Pacak *et al*., 1998). However, it should also be noted that there is a large variation of published catecholamine plasma concentration obtained from healthy humans (Goldstein *et al*., 2003).

Our observation that β_2-adrenergic receptor stimulation initiates nondirected cell movement prompted us to investigate the potential interaction of β_2-adrenergic receptors in the regulation of chemokine-induced neutrophil recruitment. We observed that IL-8 alone caused significant positive chemotactic activity in both sexes, and isoprenaline significantly reduced this IL-8 recruitment of neutrophils only from females. Of note, our findings that β_2-adrenergic receptor stimulation can both induce chemokinesis and also inhibit IL-8-induced chemotaxis, parallel the observation that increased intracellular cGMP concentration, either directly (by electroporation) or by exposure to cGMP-enhancing substances (e.g. nitric oxide), produces neutrophil chemokinesis and inhibits fMLP-induced chemotaxis (Elferink *et al*., 1996). Our results are also consistent with the β_2-adrenergic agonist–induced inhibitory effect on neutrophil chemotaxis induced by other proinflammatory agents, like LPS, C_{5a} (Silvestri *et al*., 1999),

IL-1β or TNF-α (Koyama *et al*., 1999). This effect of a β-adrenergic receptor agonist to attenuate neutrophil recruitment by strong chemoattractants is an important aspect of the anti-inflammatory properties of β_2-adrenergic receptors.

The inhibitory effect of β_2-adrenergic receptors may involve the release of a mediator that would impair chemotaxis. For example, it has been shown that IL-8, which is produced by neutrophils (Ribeiro *et al*., 2003), inhibits neutrophil chemotaxis, *in vitro,* towards a distant source of IL-8 (Foxman *et al*., 1997). We observed that cell culture supernatants collected from isoprenaline-stimulated neutrophils initiated chemotaxis in neutrophils from both males and females. This effect was antagonized, in females, by SB225002, a selective CXCR2 antagonist, and by a CXCR2 antibody, which has been shown to markedly inhibit IL-8-induced neutrophil migration in murine models of inflammation (Garcia-Ramallo *et al*., 2002). However, while β_2-adrenergic receptor agonists can increase IL-8 production (Linden, 1996; Kavelaars *et al*., 1997; Prause *et al*., 2003), this did not appear to be the mechanism of isoprenaline-induced chemotaxis in our study. Rather, our data suggest the possibility that β_2-adrenergic receptor activation induced the release of other cytokines that are ligands for the CXCR2 receptor, such as CXCL1 gene products (e.g. GROα), which stimulates chemotaxis that is also antagonized by SB225002 (Figure 6c).

We observed a loss of cellular response to β_2-adrenergic receptor stimulation at a concentration of isoprenaline (10^{-6} M), a dose equivalent to a pathophysiological adrenaline concentration *in vivo*. Loss of cellular response could reflect a protective mechanism to limit stimulation of receptors in the face of excessive activation of β_2-adrenergic receptor.

Recently, it has been recognized that resolution of the inflammatory response depends on several active processes, with the role of glucocorticoids being the most extensively studied (Goulding *et al*., 1998). Our novel findings suggest an additional inflammatory control system, dependent on release of catecholamines from the sympathoadrenal axis. Our observations of sexual dimorphism in the β_2-adrenergic control of neutrophil function may be a contributing factor to sexual dimorphism in chronic inflammatory diseases. Further understanding of this regulatory mechanism is likely to contribute to understanding the sex differences in neutrophil-mediated immune response and chronic inflammatory diseases.

We thank Dr D. Reichling for critical reading of the manuscript, Dr J. Hislop for advice on binding assays and Ms L. Barker for her technical assistance. This work was supported by NIH-AM AR 32634.

References

AJUEBOR, M.N., SWAIN, M.G. & PERRETTI, M. (2002). Chemokines as novel therapeutic targets in inflammatory diseases. *Biochem. Pharmacol.,* **63,** 1191–1196.

ALLEN, J.B., BLATTER, D., CALANDRA, G.B. & WILDER, R.L. (1983). Sex hormonal effects on the severity of streptococcal cell wall-induced polyarthritis in the rat. *Arthritis. Rheum.,* **26,** 560–563.

BARNES, P.J. (1998). Anti-inflammatory actions of glucocorticoids: molecular mechanisms. *Clin. Sci. (Colch.),* **94,** 557–572.

BARNES, P.J. (1999). Effect of beta-agonists on inflammatory cells. *J. Allergy. Clin. Immunol.,* **104,** S10–S17.

BERKOWITZ, D.E., NARDONE, N.A., SMILEY, R.M., PRICE, D.T., KREUTTER, D.K., FREMEAU, R.T. & SCHWINN, D.A. (1995). Distribution of beta 3-adrenoceptor mRNA in human tissues. *Eur. J. Pharmacol.,* **289,** 223–228.

BUCKLEY, C.D. (2003). Michael Mason prize essay 2003. Why do leucocytes accumulate within chronically inflamed joints? *Rheumatology (Oxford),* **42,** 1433–1444.

CAMPBELL, J.J., FOXMAN, E.F. & BUTCHER, E.C. (1997). Chemoattractant receptor cross talk as a regulatory mechanism in leukocyte adhesion and migration. *Eur. J. Immunol.,* **27,** 2571–2578.

CASSATELLA, M.A. (1995). The production of cytokines by polymorphonuclear neutrophils. *Immunol. Today,* **16,** 21–26.

CASTAGNETTA, L., GRANATA, O.M., TRAINA, A., COCCIADIFERRO, L., SAETTA, A., STEFANO, R., CUTOLO, M. & CARRUBA, G. (2002). A role for sex steroids in autoimmune diseases: a working hypothesis and supporting data. *Ann. NY Acad. Sci.,* **966,** 193–203.

CODERRE, T.J., BASBAUM, A.I., DALLMAN, M.F., HELMS, C. & LEVINE, J.D. (1990). Epinephrine exacerbates arthritis by an action at presynaptic B2-adrenoceptors. *Neuroscience,* **34,** 521–523.

DA SILVA, J.A. (1995). Sex hormones, glucocorticoids and autoimmunity: facts and hypotheses. *Ann. Rheum. Dis.,* **54,** 6–16.

DAVIS, S.N., SHAVERS, C. & COSTA, F. (2000). Differential gender responses to hypoglycemia are due to alterations in CNS drive and not glycemic thresholds. *Am. J. Physiol. Endocrinol. Metab.,* **279,** E1054–E1063.

ELFERINK, J.G. & VANUFFELEN, B.E. (1996). The role of cyclic nucleotides in neutrophil migration. *Gen. Pharmacol.,* **27,** 387–393.

FELDMANN, M., BRENNAN, F.M. & MAINI, R.N. (1996). Rheumatoid arthritis. *Cell,* **85,** 307–310.

FOXMAN, E.F., CAMPBELL, J.J. & BUTCHER, E.C. (1997). Multistep navigation and the combinatorial control of leukocyte chemotaxis. *J. Cell Biol.,* **139,** 1349–1360.

GAILLARD, R.C. & SPINEDI, E. (1998). Sex- and stress-steroids interactions and the immune system: evidence for a neuroendocrine-immunological sexual dimorphism. *Domest. Anim. Endocrinol.,* **15,** 345–352.

GARCIA-RAMALLO, E., MARQUES, T., PRATS, N., BELETA, J., KUNKEL, S.L. & GODESSART, N. (2002). Resident cell chemokine expression serves as the major mechanism for leukocyte recruitment during local inflammation. *J. Immunol.,* **169,** 6467–6473.

GEELEN, G., LAITINEN, T., HARTIKAINEN, J., LANSIMIES, E., BERGSTROM, K. & NISKANEN, L. (2002). Gender influence on vasoactive hormones at rest and during a 70 degrees head-up tilt in healthy humans. *J. Appl. Physiol.,* **92,** 1401–1408.

GOLDSTEIN, D.S., EISENHOFER, G.F. & KOPIN, I.J. (2003). Sources and significance of plasma levels of catechols and their metabolites in humans. *J. Pharmacol. Exp. Ther.,* **20,** 20.

GOULDING, N.J., EUZGER, H.S., BUTT, S.K. & PERRETTI, M. (1998). Novel pathways for glucocorticoid effects on neutrophils in chronic inflammation. *Inflamm. Res.,* **47** (Suppl. 3), S158–S165.

GREEN, M.S. (1992). The male predominance in the incidence of infectious diseases in children: a postulated explanation for disparities in the literature. *Int. J. Epidemiol.,* **21,** 381–386.

GREEN, P.G., DAHLQVIST, S.R., ISENBERG, W.M., STRAUSBAUGH, H.J., MIAO, F.J. & LEVINE, J.D. (1999). Sex steroid regulation of the inflammatory response: sympathoadrenal dependence in the female rat. *J. Neurosci.,* **19,** 4082–4089.

GREEN, P.G., JÄNIG, W. & LEVINE, J.D. (1997). Negative feedback neuroendocrine control of inflammatory response in the rat is dependent on the sympathetic postganglionic neuron. *J. Neurosci.,* **17,** 3234–3238.

GREEN, P.G., LUO, J., HELLER, P.H. & LEVINE, J.D. (1993). Further substantiation of a significant role for the sympathetic nervous system in inflammation. *Neuroscience,* **55,** 1037–1043.

GRIFFITHS, M.M., SAWITZKE, A.D., HARPER, D.S., MCCALL, S., REESE, V.R. & CANNON, G.W. (1994). Exacerbation of collagen-induced arthritis in rats by rat cytomegalovirus is antigen-specific. *Autoimmunity,* **18,** 177–187.

JOHNSON, M. (2002). Effects of beta2-agonists on resident and infiltrating inflammatory cells. *J. Allergy. Clin. Immunol.,* **110,** S282–S290.

KAVELAARS, A., VAN DE POL, M., ZIJLSTRA, J. & HEIJNEN, C.J. (1997). Beta 2-adrenergic activation enhances interleukin-8 production by human monocytes. *J. Neuroimmunol.,* **77,** 211–216.

KOYAMA, S., SATO, E., MASUBUCHI, T., TAKAMIZAWA, A., KUBO, K., NAGAI, S. & ISUMI, T. (1999). Procaterol inhibits IL-1beta- and TNF-alpha-mediated epithelial cell eosinophil chemotactic activity. *Eur. Respir. J.,* **14,** 767–775.

LAEMMLI, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* **227,** 680–685.

LINDEN, A. (1996). Increased interleukin-8 release by beta-adrenoceptor activation in human transformed bronchial epithelial cells. *Br. J. Pharmacol.,* **119,** 402–406.

LOMBARDI, M.S., KAVELAARS, A., SCHEDLOWSKI, M., BIJLSMA, J.W., OKIHARA, K.L., VAN DE POL, M., OCHSMANN, S., PAWLAK, C., SCHMIDT, R.E. & HEIJNEN, C.J. (1999). Decreased expression and activity of G-protein-coupled receptor kinases in peripheral blood mononuclear cells of patients with rheumatoid arthritis. *FASEB. J.,* **13,** 715–725.

MAESTRONI, G.J. (2000). Dendritic cell migration controlled by alpha 1b-adrenergic receptors. *J. Immunol.,* **165,** 6743–6747.

MARCHETTI, B., MORALE, M.C., PARADIS, P. & BOUVIER, M. (1994). Characterization, expression, and hormonal control of a thymic beta 2-adrenergic receptor. *Am. J. Physiol.,* **267,** E718–E731.

MILLS, P.J., GOEBEL, M., REHMAN, J., IRWIN, M.R. & MAISEL, A.S. (2000). Leukocyte adhesion molecule expression and T cell naive/memory status following isoproterenol infusion. *J.Neuroimmunol.,* **102,** 137–144.

NEPTUNE, E.R. & BOURNE, H.R. (1997). Receptors induce chemotaxis by releasing the betagamma subunit of Gi, not by activating Gq or Gs. *Proc. Natl. Acad. Sci. U.S.A.,* **94,** 14489–14494.

OTTONELLO, L., MORONE, P., DAPINO, P. & DALLEGRI, F. (1996). Inhibitory effect of salmeterol on the respiratory burst of adherent human neutrophils. *Clin. Exp. Immunol.,* **106,** 97–102.

PACAK, K., BAFFI, J.S., KVETNANSKY, R., GOLDSTEIN, D.S. & PALKOVITS, M. (1998). Stressorspecific activation of catecholaminergic systems: implications for stress-related hypothalamic–pituitary–adrenocortical responses. *Adv. Pharmacol.,* **42,** 561–564.

PRAUSE, O., LAAN, M., LOTVALL, J. & LINDEN, A. (2003). Pharmacological modulation of interleukin-17-induced GCP-2-, GRO-alpha- and interleukin-8 release in human bronchial epithelial cells. *Eur. J. Pharmacol.,* **462,** 193–198.

RIBEIRO, F.P., FURLANETO, C.J., HATANAKA, E., RIBEIRO, W.B., SOUZA, G.M., CASSATELLA, M.A. & CAMPA, A. (2003). mRNA expression and release of interleukin-8 induced by serum amyloid A in neutrophils and monocytes. *Mediators Inflamm.,* **12,** 173–178.

SANDNES, D., GJERDE, I., REFSNES, M. & JACOBSEN, S. (1987). Down-regulation of surface beta-adrenoceptors on intact human mononuclear leukocytes. Time-course and isoproterenol concentration dependence. *Biochem. Pharmacol.,* **36,** 1303–1311.

SILVESTRI, M., ODDERA, S., LANTERO, S. & ROSSI, G.A. (1999). beta 2-agonist-induced inhibition of neutrophil chemotaxis is not associated with modification of LFA-1 and Mac-1 expression or with impairment of polymorphonuclear leukocyte antibacterial activity. *Respir. Med.,* **93,** 416–423.

SPITZER, J.A. (1999). Gender differences in some host defense mechanisms. *Lupus,* **8,** 380–383.

STEVENS, P.A. & MILLIGAN, G. (1998). Efficacy of inverse agonists in cells overexpressing a constitutively active beta2-adrenoceptor and type II adenylyl cyclase. *Br. J. Pharmacol.,* **123,** 335–343.

STRAUB, R.H., MAYER, M., KREUTZ, M., LEEB, S., SCHOLMERICH, J. & FALK, W. (2000). Neurotransmitters of the sympathetic nerve terminal are powerful chemoattractants for monocytes. *J. Leukoc. Biol.,* **67,** 553–558.

STRAUSBAUGH, H.J., DALLMAN, M.F. & LEVINE, J.D. (1999). Repeated, but not acute, stress suppresses inflammatory plasma extravasation. *Proc. Natl. Acad. Sci. U.S.A.,* **96,** 14629–14634.

STRUYF, S., PROOST, P., LENAERTS, J.P., STOOPS, G., WUYTS, A. & VAN DAMME, J. (2001). Identification of a blood-derived chemoattractant for neutrophils and lymphocytes as a novel CC chemokine, Regakine-1. *Blood,* **97,** 2197–2204.

SWAIN, M.G. (2000). I. Stress and hepatic inflammation. *Am. J. Physiol. Gastrointest. Liver Physiol.,* **279,** G1135–G1138.

TAN, K.S., MCFARLANE, L.C. & LIPWORTH, B.J. (1997). Paradoxical down-regulation and desensitization of beta2-adrenoceptors by exogenous progesterone in female asthmatics. *Chest,* **111,** 847–851.

VON ZASTROW, M. & KOBILKA, B.K. (1992). Ligand-regulated internalization and recycling of human beta 2- adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors. *J. Biol. Chem.,* **267,** 3530–3538.

WAHLE, M., STACHETZKI, U., KRAUSE, A., PIERER, M., HANTZSCHEL, H. & BAERWALD, C.G. (2001). Regulation of beta(2)-adrenergic receptors on CD4 and CD8 positive lymphocytes by cytokines *in vitro*. *Cytokine,* **16,** 205–209.

WILDER, R.L., CALANDRA, G.B., GARVIN, A.J., WRIGHT, K.D. & HANSEN, C.T. (1982). Strain and sex variation in the susceptibility to streptococcal cell wall-induced polyarthritis in the rat. *Arthritis Rheum.,* **25,** 1064–1072.

ZHOU, Y.Y., YANG, D., ZHU, W.Z., ZHANG, S.J., WANG, D.J., ROHRER, D.K., DEVIC, E., KOBILKA, B.K., LAKATTA, E.G., CHENG, H. & XIAO, R.P. (2000). Spontaneous activation of beta(2)- but not beta(1)-adrenoceptors expressed in cardiac myocytes from beta(1)beta(2) double knockout mice. *Mol. Pharmacol.,* **58,** 887–894.

(Received July 14, 2004
Accepted August 11, 2004)