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Urotensin II is a New Chemotactic Factor for UT Receptor-Expressing Monocytes

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Urotensin II (U-II), a vasoactive cyclic neuropeptide which activates the G protein-coupled receptor UT receptor, exerts various cardiovascular effects and may play a role in the pathophysiology of atherosclerosis. In this study, we report that the UT receptor is expressed and functional on human PBMC and rat splenocytes. PBMC surface expression of the UT receptor was mainly found in monocytes and NK cells, also in a minority of B cells, but not in T cells. Stimulation of monocytes with LPS increased UT receptor mRNA and protein expression. Cloning and functional characterization of the human UT receptor gene promoter revealed the presence of NF-κB-binding sites involved in the stimulation of UT receptor gene expression by LPS. Activation of the UT receptor by U-II induced chemotaxis with maximal activity at 10 and 100 nM. This U-II effect was restricted to monocytes. Analysis of the signaling pathway involved indicated that U-II-mediated chemotaxis was related to RhoA and Rho kinase activation and actin cytoskeleton reorganization. The present results thus identify U-II as a chemoattractant for UT receptor-expressing monocytes and indicate a pivotal role of the RhoA-Rho kinase signaling cascade in the chemotaxis induced by U-II.

Materials and Methods

Reagents

Tissue-culture products were obtained from Invitrogen Life Technologies. TNF-α, IL-1β, and IFN-γ were obtained from PeproTech. The PE-conjugated mAbs, anti-CD3 (clone HIT3a), CD19 (clone 4G7), -CD56 (clone B159), and the allophycocyanin-conjugated mAbs anti-CD4 (clone RPA-T4), -CD8 (clone RPA-T8), -CD16 (clone 3G8) were obtained from BD Biosciences. The PE-conjugated mAbs anti-CD14 (clone RM02), anti-TCRαβ (clone BMA03), and anti-TCRγδ (clone IMMYS10) were obtained from Beckman Coulter. The rabbit anti-human UT receptor Ab (Ab-90), the mouse anti-RhoA mAb (RMO52), and the goat anti-MLC2 Ab (A-20) were obtained from Santa Cruz Biotechnology. The rabbit anti-phospho-MLC2 (Ser19) Ab was obtained from Cell Signaling Technology. Human U-II, the inactive U-II analog (Asp-Cys-Asp-Ala-Lys-Tyr-Cys-Val), was provided by Prof. H. Vaudry (Institut National de la Sante´ et le Recherche M´edicale, Unit´e 413, Rouen, France) (24). All other reagents were purchased from Sigma-Aldrich.

Cell isolation

The protocols have been reviewed and approved by our institutional review committee. Human PBMC were isolated from heparinized blood of healthy volunteers by Ficoll density gradient centrifugation and suspended in RPMI 1640 medium supplemented with 2 mM t-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

Polytomorphonuclear cells (PMN) were recovered from the lower phase of Ficoll gradient and separated from RBC by addition of 6% dextran (m.w. 500 000, final concentration: 1% dextran). After sedimentation, the supernatant was centrifuged, then the PMN pellet was suspended in RPMI 1640. The purity of the PMN preparation assessed by Giemsa coloration was >98%.

Monocytes were isolated by elutriation (Aventi J-20; Beckman Coulter). CD3+ T cells and NK cells were purified using the CD3 and NK isolation kits, respectively, according to the manufacturer’s instructions (Miltenyi Biotec). Purity of cell populations exceeded 95% as assessed by flow cytometry.

To generate immature monocyte-derived DCs (iDC), elutriated monocytes were cultured in medium containing 500 IU/ml GM-CSF and 200 IU/ml IL-4 (AbCys) for 6 days. Mature DC (mDC) were obtained by an additional 48-h treatment of iDC with 1 μg/ml LPS.

Splenocytes were isolated from Sprague-Dawley rats as previously described with slight modifications (25). Briefly, the spleen was perfused with the above RPMI 1640 medium and the cell suspension was centrifuged at 1500 rpm for 5 min. The pellet was resuspended and incubated in 0.17 M ammonium chloride for 10 min at room temperature to lyse erythrocytes. Splenocytes were then washed and suspended in RPMI 1640 medium.

Cell culture

To analyze the modulation of UT receptor expression by several stimuli, PBMC (5 × 10^6) were cultured in RPMI 1640 medium supplemented with 10% FCS at 37°C in the absence or presence of LPS (1 μg/ml), TNF-α (10 ng/ml), IL-1β (10 ng/ml), IFN-γ (500 U/ml), or PMA (50 ng/ml) for 6 or 18 h. Cells were then recovered for the analysis of mRNA and protein expressions by real-time RT-PCR and flow cytometry, respectively. Cells were then recovered for RNA isolation. To analyze Rhoa activation and MLC phosphorylation, splenocytes (60 × 10^6) were preincubated at 37°C in 30 ml of RPMI 1640/0.1% BSA containing or not the Rho-kinase inhibitor Y-27632 (10 μM) for 20 min, before addition of 10 nM U-II or 1 μg/ml LPS for 1 more hour. Cells were then centrifuged and washed twice with ice-cold PBS and pellets were rapidly frozen in liquid nitrogen and lysed as described below.

Flow cytometry analysis

Flow cytometry analysis of surface expression of the UT receptor was performed on freshly isolated PBMC by a two- or three-color method using mAbs directed to human leukocyte Ags (i.e., CD3, CD4, CD8, CD14, CD16, CD19, CD56, TCRαβ, and TCRγδ), and an anti-human UT receptor Ab. Cells were also stained with appropriate isotype-matched control Abs. Fcs and nonspecific binding sites were blocked during a saturation step by incubating cells with PBS containing 3% normal donkey serum and 2% human AB serum (Sigma-Aldrich). PBMC were stained for 20 min with the anti-UT receptor Ab. Cells were then washed with PBS-0.1%BSA, and conjugated to FITC (with a donkey anti-rabbit, FluorProbes; Interchim) and with the anti-CD3, -CD4, -CD8, -CD14, -CD19, or -CD56-PE mAb. After washing, cells were resuspended in PBS and analyzed on a FACScan flow cytometer using the CellQuest software (BD Biosciences). Compensation was checked before each acquisition and 10^5 events were collected.

RNA isolation and real-time RT-PCR

Total RNA was isolated using TRizol reagent (Invitrogen Life Technolo
gies) and treated for 45 min at 37°C with RQ1 DNase (Promega). One microgram of RNA was reverse transcribed using random primers and the Superscript III-Reverse Transcriptase (Invitrogen Life Technologies) at 30°C for 45 min, according to the manufacturer’s instructions. The resulting cDNA was subjected to PCR in a Bio-Rad iCyser iQ system using the QuantiTect SYBR Green PCR kit (Qiagen) and specific primers for human UT receptor (ATG AGG ACTA CAG CGG ACC T) and B-actin (GGA GGC TAC TCC GAA T). Expression levels were normalized to B-actin and calculated as 2^-ΔΔCt.

Cloning and site-directed mutagenesis of the human UT receptor promoter

The sequence of 1500 bp upstream of the ATG codon of the human UT receptor gene (chromosome 17 contig. NT-010663) was cloned into the pDRIVE vector (Qiagen) by PCR using the 5’-GACGCTGTGATGCTGTGATGCTTCA TGA-3’ sense primer (up), the 5’-GTTGGAAAAAGACAACTCA-3’ antisense primer (down), and human genomic DNA as matrix. This sequence was fused to the luciferase coding gene in the pGL2-Basic vector (Promega) between KpnI and HindIII restriction sites. The entire sequence was verified by sequencing the insert with pGL1 and pGL2 promoters (Promega).

In vitro site-directed mutagenesis of the four NF-κB-binding sites

The first (distal to ATG), second, third, and fourth (proximal to ATG) NF-κB-binding site of the human UT receptor promoter was mutated to inactive ones according to the QuickChange site-directed mutagenesis kit instruction manual (Stratagene) using the following PAGE-purified primers: N1: up 5’-CCTGACTCTGATTATCTCCTGCTG-3’; down 5’-GCAAACAT GCAAGGAAATGACTCAGGAGG-3’; N2: up 5’-GCTCCTTTTCTATGAGTGCCCGTCCGATCAGGTTTGG-3’; down 5’-CCAAACCTGGACGTCGCAAG-3’; N3: up 5’-TCTGCCTCCAGGATAGCTGCAGTCTCTGTTG-3’; down 5’-CCCCGAACACAGTGGACGACATCTCGG-3’; N4: up 5’-TGGCTTTCAGAGTCTGATGATGATG-3’; down 5’-GCCCTCCAACTCTCTAGACTCTCTGGAAGC-3’. NA-3 to obtain the N1 to N4 mutants, respectively.

Transfections and reporter assays

Splenocytes freshly isolated from rat spleen were electroporated with wild-type (WT) UT receptor or N1 to N4 mutants (Amaxa Nucleofector). One day after transfection, cells were pretreated or not with MEK1/2 (U0126, 10 μM) or NF-κB (cafeic acid phenylester (CAPE), 10 μM) inhibitors and then stimulated with LPS (2 μg/ml) for different times (as indicated). The pBRES-EF-GFP vector was always cotransfected to estimate the level of transfection (fluorescence measure from lysates with Victor2) and to normalize the luciferase activity measured with the luciferase reporter reagent (Promega) in a LB960 luminometer (Berthold Technologies).

Chemotaxis assay

Chemotaxis assays were performed using Transwell cell culture chambers (Costar) with 6.5-μm diameter, 5-μm pore size polycarbonate membranes essentially as described (27). PBMC or splenocytes (0.5 × 10^6) suspended in 100 μl of RPMI 1640/0.1% BSA were added to the upper chamber and U-II or the inactive analog S28981-1 (at the indicated doses) were added to the lower chamber. In checkerboard control experiments, U-II was also added in the upper chamber. When indicated, cells were pretreated with 0.1 ng/ml IL-1β at 37°C for 18 h or with 10 μM Y-27632 or 20 μM ML-7 at 37°C for 30 min. Cells were allowed to migrate for 3 h at 37°C. After this period, migrated cells onto the lower well were counted in four high-power sites.
fields under light microscopy and also recovered and counted with a Malassez counting chamber in triplicates under light microscopy. Cell migration was expressed as the chemotactic index calculated by the following ratio as previously described (number of cells migrating to U-II/cells migrating to vehicle) (7).

**Calcium [Ca^{2+}] measurements**

[Ca^{2+}] was measured fluorometrically in monocytes loaded with the intracellular probe fura 2-AM (Molecular Probes/Invitrogen Life Technologies). Briefly, 3 × 10^5 monocytes were plated on 35-mm diameter glass-bottom culture dishes (MatTek) and incubated with 1 μM fura 2-AM in HBSS at 37°C for 30 min, then washed, and further incubated in fresh HBSS at 37°C for 30 min in the experimental cuvette. Cells were stimulated with 0.1 μM U-II or 10 μM ATP (used as control) and fluorescence was recorded by videoimaging (Leica). Fluorescence intensities (340 and 380 nm) were stored and analyzed using Metafluor software (Roper Scientific).

**Actin staining**

Polymerized (F) actin staining was performed as previously described (28), with slight modifications. PBMC or splenocytes (0.5 × 10^6) were plated in culture chambers (Lab-Tek; Nuncl) and preincubated at 37°C without or with the Rho-kinase inhibitor Y-27632 (10 μM) in 400 μl of RPMI 1640/0.1% BSA, for 30 min. Different concentrations (as indicated) of U-II were then added to the culture medium and cells were maintained at 37°C for 1 h. After washing with PBS, cells were fixed in PBS containing 2% paraformaldehyde, washed, and permeabilized with PBS/0.1% Triton X-100 for 5 min. Cells were then stained with Alexa Fluor 488 phalloidin (Molecular Probes) (1 U/ml in PBS) for 20 min at room temperature. After four to five washes, cells were mounted in Vectashield medium (Vector Laboratories/AbCys) and examined with a fluorescence microscope (Nikon).

**Analysis of RhoA activity and MLC phosphorylation**

Cells were homogenized in ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 10 mM MgCl₂, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, and 1 mM sodium orthovanadate. RhoA activity was assessed by a pull-down assay, using the Rho-binding domain of the Rho effector protein rhotekin as described previously (29). Precipitated GTP-bound RhoA and total RhoA were then analyzed by Western blot using a mouse anti-RhoA mAb. Cell lysates were also analyzed by Western blot with Abs directed to total or phosphorylated MLC. Immunoreactive bands were visualized using HRP-conjugated secondary Abs and subsequent ECL detection (Amersham Pharmacia Biotech).

**Statistical analysis**

The significance of differences was determined by the Mann-Whitney U test and Kruskall-Wallis ANOVA using the Statview software (Abacus Concepts). A difference with $p < 0.05$ was considered significant.

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**FIGURE 1.** Expression of UT receptor mRNA. UT receptor mRNA expression has been analyzed by quantitative real-time RT-PCR in human PBMC and rat splenocytes. Boxes represent the median ± quartiles of the relative UT receptor mRNA expression calculated by the 2^-ΔΔCt method in different cell population (PBMC, $n = 6$; splenocytes, $n = 5$); vertical bars indicate the 10th and 90th percentiles.

**FIGURE 2.** Flow cytometry analysis of UT receptor cell surface expression in human PBMC. Freshly isolated PBMC from three healthy donors were stained with anti-human UT receptor/FluoProbe488-conjugated secondary Abs and with PE/allophycocyanin-anti-CD3, -CD4, -CD8, -CD14, -CD16, -CD19, or -CD56, -TCRαβ, -TCRγδ mAbs and analyzed by flow cytometry. A, Representative histogram of UT receptor expression in total PBMC. B, Panels show representative dot plots of expression of UT receptor vs CD14, CD16, CD3, CD4, CD8, TCRαβ, TCRγδ, and CD19. Percentages of single-positive and double-positive cells among total PBMC are indicated. C, Representative dot plots of expression of CD14 vs CD16. Analysis of UT receptor expression on gated CD14^high^CD16^−^ (R1), CD14^low^CD16^−^ (R2), CD14^high^CD16^+^ (R3), and CD14^low^CD16^+^ (R4) populations is reported in Table II.
Results

Expression of UT receptor mRNA in human and rat mononuclear cells

UT receptor mRNA expression was analyzed by real-time RT-PCR using total RNA extracted from freshly isolated, noncultured human PBMC and rat splenocytes. Expression was detected in both human and rat mononuclear cells, with a higher interindividual variability observed in human subjects (Fig. 1). UT mRNA expression was also detected in RNA extracted from Jurkat U-937 or THP1 leukemia cell lines (data not shown).

Cell surface expression of UT receptor by PBMC

We next wanted to phenotype the subpopulation of PBMC expressing cell surface UT receptor protein by flow cytometry. The analysis of surface expression of UT receptor was performed on freshly isolated human PBMC using a polyclonal Ab directed against the N-terminal domain of the human UT protein. The proportion of UT receptor-positive cells in total PBMC varied from 15 to 30% among healthy individuals (Fig. 2A). This variability among individuals was similar to that observed at the level of mRNA expression (Fig. 1). We next combined the analysis for UT receptor with the analysis of Ags associated with monocytes (CD14), NK cells (CD56), B cells (CD19), and T cells (CD3, CD4, CD8, TCRβ, and TCRγδ). We found that the UT receptor was mainly expressed by monocytes since near 98% of the cells positive for the CD14/LPS receptor were UT receptor positive (Fig. 2B and Table I). The UT receptor was also consistently expressed by NK cells (68% of CD56+ cells) and to a lesser extent by B lymphocytes (10% of CD19+ cells) (Fig. 2B and Table I). Only <2% of CD3+ T lymphocytes were positive for the UT receptor, with similar proportions of CD4+ and CD8+ T cells (Fig. 2B and Table I). In accordance, T cells expressing either αβ or γδ T cell receptors barely expressed the UT receptor (Fig. 2B). The population expressing low levels of CD4 (15% of PBMC) and positive for the UT receptor (Fig. 2B) corresponds to CD14+ cells as confirmed by three-color flow cytometry analysis (data not shown). Similarly, the UT receptor was also found in cells expressing low levels of CD8 (9% of PBMC) that were shown to be CD56+ NK cells (data not shown). Thus, all the monocytes and a high fraction of NK cells present in human peripheral blood express the UT receptor on their cell surface. These two subpopulations account for ~95% of total UT receptor-positive cells in PBMC (calculated from data in Table I). To further characterize monocyte populations expressing the UT receptor, we performed a three-color flow cytometry analysis of PBMC stained for the UT receptor, CD14, and CD16 (FcγRIII) receptors. Indeed, two main populations of human blood monocytes can be distinguished on the basis of CD14 and CD16 expression (30). Four main subpopulations could be identified: CD14highCD16− (region R1), CD14highCD16− (R2), CD14highCD16+ (R3), CD14highCD16+ (R4) cells, representing, respectively, 67, 11, 14, and 8% of the total monocytes (Fig. 2C and Table II). All these monocyte populations express the UT receptor. However, CD16-positive cells (21% of CD14+ cells) expressed higher levels (X3) of the UT receptor, as assessed by the mean fluorescence intensity (MFI), than CD16-negative cells (Table II).

We also analyzed UT receptor expression in PMN and monocyte-derived DC. All PMN and iDC expressed the UT receptor at very low levels in comparison to monocytes (PMN: MFI = 9.23; iDC: MFI = 4.74; representative of three individuals). Maturation of iDC by treatment with LPS stimulated by 2-fold UT receptor expression (mDC: MFI = 9.06, representative of three individuals).

Inflammatory stimuli up-regulate UT receptor mRNA expression

Modulation of membrane receptor function and expression influences immune cell responses (31). We therefore assessed whether stimulation of PBMC with proinflammatory cytokines modulates

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**FIGURE 3.** Up-regulation of UT receptor by inflammatory stimuli. A, UT receptor mRNA expression has been analyzed by quantitative real-time RT-PCR in human PBMC in the absence (control) and the presence of LPS (2 μg/ml), IL-1β (10 ng/ml), TNF-α (10 ng/ml), and IFN-γ (500 U/ml). Results are expressed relative to β-actin expression and normalized to the control as taken as 1. B, Flow cytometry analysis of the effect of LPS (2 μg/ml) on UT receptor expression in monocytes (CD14+), NK cells (CD56+), T lymphocytes (CD3+), and B lymphocytes (CD19+). Results are expressed as mean fluorescence intensity (MFI) ± SEM of three individuals (*, p < 0.05 and **, p < 0.01 vs control).
UT receptor mRNA expression. As shown in Fig. 3A, stimulation for 6 h with LPS (2 μg/ml), IL-1β (10 ng/ml), TNF-α (10 ng/ml), and IFN-γ (500 U/ml) increased UT receptor mRNA expression by 10-, 8-, and 4-fold over control, respectively.

LPS up-regulates UT receptor expression in monocytes

The cell subpopulations responsible for LPS-induced UT receptor up-regulation have been further characterized by flow cytometry analysis of PBMC stained for the UT receptor and, CD14 (monocytes), CD56 (NK cells), CD3 (T lymphocytes), or CD19 (B lymphocytes). Stimulation by LPS (2 μg/ml) for 18 h induced a 2.6-fold-increase in UT receptor expression in monocytes cells but did not affect U-II receptor expression on CD3+ T cells, CD16+ NK cells, and CD19+ B cells (Fig. 3B). In agreement with the stimulatory effect of LPS on UT receptor mRNA, this result indicates that LPS up-regulated UT receptor expression in monocytes. Although TNF-α (10 ng/ml, 6 h) stimulates U-II receptor mRNA expression, we did not observe such an effect at the protein level by flow cytometry analysis after 18 h of stimulation with TNF-α (10 ng/ml; data not shown).

Four NF-κB-binding sites are identified in the human UT receptor promoter sequence

To gain additional insight into the mechanisms of transcriptional regulation of UT receptor expression, we examined the human UT receptor promoter using Genomatix tools (32). A promoter structure was found in a sequence of 1500 bp upstream of the ATG codon of the human UT receptor gene on chromosome 17. Using MatInspector (33, 34), we located potential transcription factor-binding sites for early growth response gene 1 (Egr), Elk1, GATA, Ap1–2–4, RasREBP1 (shaded), or NF-κB (underlined) potential binding sites were found in this sequence. Up and down boxes indicates the primers used to fuse the promoter to the luciferase gene for functional experiments.

LPS induces UT receptor promoter activity through MAPK and NF-κB activation

To identify the transcriptional regulation involved in LPS-induced stimulation of UT receptor mRNA expression, functional analysis of the human UT receptor promoter was performed. Splenocytes were transfected with the 1500-bp fragment upstream of the ATG codon of the human UT receptor gene fused to the reporter gene firefly luciferase. Measurement of luciferase activity showed that
LPS (2 µg/ml) induced a transient 8-fold increase in UT receptor promoter activity after 4–6 h of stimulation. After 24 h of LPS treatment, the promoter activity was nonsignificantly different from that measured under control condition. As MAPK and NF-κB pathways are major signaling pathways involved in the transcriptional effect of LPS, a pharmacological analysis has been performed to assess their involvement in LPS-induced stimulation of UT receptor promoter activity. Treatment of UT receptor promoter expressing splenocytes with the MEK1/2 inhibitor U0126 (10 µM) or the NF-κB inhibitor CAPE (20 µM) suppressed the stimulatory effect of LPS on UT receptor promoter activity (Fig. 5A). These results thus indicate that the stimulation of UT receptor promoter activity by LPS depends on MEK1/2 and NF-κB activation. This observation is in agreement with the presence of four predicted NF-κB-binding sites (N1-N4) in the UT receptor promoter (Fig. 4). To identify the NF-κB-binding sites involved in the LPS induction of UT receptor promoter activity, four UT receptor promoter constructs containing inactivating mutation of each putative NF-κB-binding sequence were transfected to splenocytes. Mutational ablation of the N1 and N3 NF-κB-binding sites did not modify the effect of LPS on UT receptor promoter activity (Fig. 5B). In contrast, in cells expressing UT receptor gene promoter carrying the mutational ablation of the N2 or N4 NF-κB-binding sites, LPS had no effect on promoter activity. These results provide evidence that NF-κB-binding sites N2 and N4 of the UT receptor promoter are necessary to induce UT receptor gene transcription in response to LPS stimulation.

U-II is a chemoattractant for human monocytes

The next question to address was therefore to assess the functional role of UT receptor activation by its ligand U-II in PBMC. In preliminary experiments, the effect of U-II has been tested on several immune functions. Treatment of PBMC with different doses of U-II (0.1 nM to 10 µM) for 18 h did not affect cell viability (trypan blue exclusion test), cell proliferation (colorimetric MTT assay), or cytokine production (TNF-α and IL-8, biological and ELISA, respectively) (data not shown).

FIGURE 5. LPS induces UT receptor promoter activity through ERK and NF-κB activation. Luciferase activity of splenocytes expressing UT receptor gene promoter constructs fused to the luciferase gene. A, Twenty-four hours posttransfection, cells were stimulated with LPS (2 µg/ml) for indicated times with or without pretreatment with of MEK1/2 (U0126, 10 µM) or NF-κB inhibitor (CAPE, 10 µM). B, Splenocytes expressing WT UT receptor promoter or NF-κB-binding site inactive mutants (N1–N4) were stimulated with LPS (2 µg/ml) for 4 h. Luciferase activity was measured as described in Materials and Methods and normalized by measuring the fluorescence of enhanced GFP (EGFP) expressed by the cotransfected pIRES-EGFP vector. The data are the means ± SD of four independent experiments performed in duplicate (A and B). (*, p < 0.01 vs control (t = 0); #, p < 0.005 vs LPS in A and LPS/WT in B).

FIGURE 6. U-II is a chemoattractant for human PBMC. A, Under basal conditions, U-II (1 nM-1 µM; 3 h) induced PBMC migration. B, Pretreatment of PBMC with IL-1β (0.1 ng/ml; 12 h) potentiated U-II-mediated chemotaxis (*, p < 0.01 vs control). C, Effect of U-II (100 nM) on chemotaxis of purified monocytes, NK cells, and T lymphocytes. D, Effect of U-II (100 nM) on chemotaxis of mDC and iDC. E, Effect of U-II (100 nM) on chemotaxis of PMN. The data are the means ± SD of four independent experiments (*, p < 0.01 vs control).
The fact that the UT receptor is structurally related to chemokine receptors, together with studies showing that several neuropeptides have chemoattracting properties (6, 7) prompted us to examine whether U-II could induce chemotaxis of PBMC. We observed that U-II (1 nM-1 μM) induced PBMC chemotaxis with typical bell-shaped dose-response curves, indicating a receptor-mediated effect (Fig. 6A). Notably, the majority of the cells migrated onto the lower well were adherent to plastic (Fig. 6A). The maximum activity obtained with 10 and 100 nM U-II is similar to that obtained in response to 10 nM IL-8 (chemotactic index = 2.18 ± 0.6, n = 3; data not shown). In checkerboard control experiments, when U-II was also added in the upper chamber, PBMC chemotaxis was not observed. The inactive U-II analog had no effect on PBMC chemotaxis (data not shown). Furthermore, pretreatment of PBMC with IL-1β for 12 h potentiated the chemotactic activity of 100 nM U-II (Fig. 6B). This result is thus in agreement with the observed IL-1β-induced increase in UT receptor mRNA expression (Fig. 3).

To further define the cell population that migrated in response to U-II, we next analyzed the chemoattracting properties of U-II on purified monocytes, NK cells, and T lymphocytes (Fig. 6C). U-II (100 nM) had no effect on NK and T cells, but was efficient in monocytes, stimulating monocytes chemotaxis by up to 3-fold over control (Fig. 6C).

The chemoattracting properties of U-II have also been assessed in monocyte-derived DC. Whereas iDC did not respond, U-II strongly stimulated mDC chemotaxis (Fig. 6D), in agreement with the induction of UT receptor expression by LPS treatment. In PMN, in agreement with the low level of UT receptor expression, U-II failed to stimulate migration (Fig. 6E).

U-II induces Rho kinase-dependent actin organization

Actin polymerization and actomyosin contraction are key determinants of monocyte migration (35). Labeling of polymerized F-actin with Alexa 488-conjugated phalloidin has therefore been used to analyze the effect of U-II on actin cytoskeleton organization (Fig. 7). In rat splenocytes, U-II (10 and 100 nM) induced actin polymerization, particularly located at the cell periphery and in cytoplasmic extensions (Fig. 7A). This effect was associated with an increase in cell area. Higher U-II concentration had almost no effect on actin cytoskeleton (Fig. 7A). The RhoA-signaling pathway plays a major role in the regulation of actin polymerization and actomyosin contraction (36). RhoA regulated-actomyosin contraction involves Rho kinase-mediated MLC phosphatase phosphorylation, which leads to inhibition of its phosphatase activity and increased MLC phosphorylation (37–39). To address the role of the RhoA/Rho kinase pathway in the effect of U-II on actin cytoskeleton, we used the Rho kinase inhibitor Y-27632. In the presence of 10 μM Y-27632, the effect of U-II (10 nM) on actin cytoskeleton organization was abolished (Fig. 7B), indicating that U-II-mediated actin organization involved Rho kinase activation.

The RhoA-Rho kinase signaling pathway is involved in U-II-induced chemotaxis

We therefore next examined whether Rho kinase activation was also involved in U-II-induced chemotaxis in human PBMC and in rat splenocytes. As shown in Fig. 8A, inhibition of Rho kinase by Y-27632 (10 μM) abolished U-II-induced chemotaxis in both types of cells indicating that it depends on Rho kinase activation. Direct analysis of RhoA activity by pull-down assay indicated that stimulation of splenocytes by U-II (10 nM) induced a 3- to 4-fold increase in the amount of active, GTP-bound RhoA (Fig. 8B). U-II stimulation also induced a rise in intracellular calcium concentration measured as a 2.6-fold increase in fura-2 fluorescence ratio (F340/F380) (data not shown). U-II-induced RhoA activation was associated with increased phosphorylation of MLC, which was blocked by Y-27632 (10 μM). Similar results were also observed with human PBMC (data not shown). These data thus indicate that U-II-induced chemotaxis is mediated by activation of the RhoA/Rho kinase pathway.

Discussion

Recent immunohistochemical studies have reported the presence of inflammatory cells expressing both U-II and UT receptor in atherosclerotic lesions of the human aorta (23). UT receptor mRNA was mainly detected in monocytes by RT-PCR, suggesting a potential immune function for U-II (23). In the present study, we show that UT receptor is up-regulated by inflammatory stimuli and identify U-II, the UT receptor ligand, as a novel chemoattractant peptide for monocytes.

Analysis of cell surface expression of UT receptor in PBMC isolated from healthy volunteers by flow cytometry indicated an important interindividual variability (15–30% of total PBMC) also observed at the level of the UT receptor mRNA. Three-color flow cytometry analysis revealed that in human peripheral blood, UT receptor is essentially expressed in monocytes and NK cells. Furthermore, we found that among CD14+ (high and low) monocytes,
those expressing the CD16/FcγRIII receptor had the higher levels of UT receptor expression. The CD14\textsuperscript{+}CD16\textsuperscript{−} subset represents 10–15% of monocytes in healthy individuals, whereas the major subset CD14\textsuperscript{low}CD16\textsuperscript{+} accounts for the remaining 30% (30). It has been described, in a model of transendothelial trafficking, that the CD16\textsuperscript{+} subset of human monocytes had a higher potential to become migratory DCs than CD14\textsuperscript{+}CD16\textsuperscript{−} monocytes (40). This population of CD14\textsuperscript{low}CD16\textsuperscript{+} monocytes, which exhibits characteristic of tissue macrophages, is found increased in several inflammatory conditions such as sepsis (30). Accordingly, it has been shown that CD14\textsuperscript{low}CD16\textsuperscript{+} cells also express TLR2 and TLR4 and secrete TNF-α and IL-6 upon stimulation with LPS (41). Of particular interest, an increased proportion of CD14\textsuperscript{low}CD16\textsuperscript{+} monocytes has been detected in the blood of patients with low levels of HDL cholesterol, a risk indicator for the development of atherosclerosis (42). Since the development atherosclerosis also appears to be associated with the hallmarks of a systemic inflammatory reaction (43), it would be interesting to verify whether the proportion of CD14\textsuperscript{low}CD16\textsuperscript{+} monocytes expressing the UT receptor could be increased in PBMC of patients at risk for atherosclerosis. Interestingly, our finding that UT receptor expression in monocytes is up-regulated by LPS could be relevant to the CD14\textsuperscript{low}CD16\textsuperscript{+} monocyte population with increased levels of UT receptor surface expression. After PCR cloning and sequencing of the UT receptor gene promoter, we have identified four NF-κB-binding sites involved in LPS-induced UT receptor gene transcription. Indeed, site-directed mutagenesis revealed that two NF-κB-binding sites mediated LPS-induced stimulation of UT receptor gene promoter activity. NF-κB-dependent up-regulation of UT receptor expression in response to inflammatory stimuli supports an increased role for UT receptor and UT receptor-expressing cells in inflammatory conditions.

The structural relationship between UT and chemokine receptors prompted us to assess the potential chemotactic activity of U-II. Chemokines are a family of small proteins, which plays a critical role in immune and inflammatory reactions by directing leukocyte migration to inflammatory sites (44). Binding of chemokines to G protein-coupled receptor triggers intracellular signal pathways involved in cell migration, in particular Rho protein signaling which regulates the dynamic regulation of actin cytoskeleton organization (35, 39, 45, 46). We demonstrate for the first time that U-II is a chemoattractant for human PBMC and rat splenocytes, acting at concentrations similar to most chemokines. The inactive U-II analog that did not bind the UT receptor (24) did not display chemoattractant activity, indicating that U-II-induced chemotaxis is specific of UT receptor activation. Furthermore, we showed that U-II specifically induced the chemotaxis of CD14\textsuperscript{+} monocytes. Although a great proportion of NK cells express the U-II receptor, we did not observe cell migration of this subset in response to U-II. Thus, the function of the U-II receptor in NK cells remains to be elucidated. The stimulatory effect of U-II on proliferation previously described in vascular smooth muscle cells (20) and renal epithelial cells (14, 21) was not observed in PBMC suggesting that the mitogenic effect of U-II might involve cell-specific signal transduction pathways.

As previously shown in rat smooth muscle cells (20), we found that U-II activates the RhoA/Rho kinase pathway in rat splenocytes and that inhibition of Rho kinase by Y-27632 blocks U-II-induced actin reorganization and chemotaxis. Video-imaging analysis showed that U-II did not stimulate chemokinesis (data not shown). This is in agreement with the observation that monocyte migration, in Boyden chamber experiments, was inhibited when U-II was added both in the upper and lower chambers and suggest that U-II-induced monocyte migration was essentially due to chemotaxis (data not shown). Our data thus suggest that the RhoA-Rho kinase signaling cascade plays a major role in the chemotaxis of monocytes induced by U-II. This signaling pathway has already been shown to be responsible for the chemotaxis of PBLs induced by the chemokine stromal cell-derived factor-1 (27). The fact that UT receptor is up-regulated by proinflammatory stimuli suggests that U-II could play a role in directing leukocyte migration to inflammatory sites. In addition, the potentiation of U-II activity by IL-1β could also be explained by a synergistic activation of RhoA-Rho kinase pathway. Indeed, we have shown that IL-1β stimulates RhoA-Rho kinase in human PBMC and that this pathway is involved in inflammatory responses (47).

In view of the present results, it is possible that U-II, which could be released from endothelial cells and vascular smooth muscle cells (23), contributes to the recruitment of monocyte expressing the UT receptor to inflamed tissues. This is particularly relevant to the atherosclerotic lesion formation in which monocyte/macrophage emigration from blood plays a central role (43). Several studies have shown that chemokine production, in particular by vascular endothelial and smooth muscle cells, is essential to this process (43, 48–50). This is well-established for MCP-1 since it has been reported that mice deficient for MCP-1 or its receptor CCR2 have an overall decrease in atherosclerotic lesion size associated with a reduced number of monocytes and macrophages in aortic walls (48, 49). Thus, blocking monocyte and macrophage recruitment in the vessel wall is considered a promising therapeutic approach against atherosclerosis development. The increased expression of U-II observed in endothelial and smooth muscle cells of aorta, carotid, and coronary arteries of patients with atherosclerosis might contribute to the attraction of monocyte/macrophage-expressing U-II receptors to vessel walls (22, 23). In addition to U-II, other vasoactive peptides could also participate in monocyte recruitment, either directly such as endothelin (51) or indirectly, such as angiotensin II, through stimulation of MCP-1 expression in arterial smooth muscle cells (52).

Our hypothesis for a role of U-II-mediated RhoA/Rho kinase-dependent monocyte recruitment in atherosclerotic lesions is in agreement with the previously described role of the RhoA/Rho kinase pathway in the development of atherosclerotic plaques (53). We have shown that inhibition of Rho kinase significantly reduces early atherosclerotic plaque development in the low density lipoprotein receptor-deficient mice. Inhibition of Rho kinase by Y-27632 should block most of the cellular processes induced by U-II including, vasoconstriction, smooth muscle cell proliferation, and also, as we show here, chemotaxis.

In conclusion, our data suggest that U-II could attract blood monocytes expressing the UT receptor to inflammatory sites and reinforce the idea of a possible role of U-II in the pathogenesis of atherosclerosis.

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Disclosures
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