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Bosentan, an endothelin receptor antagonist, ameliorates collagen-induced arthritis: the role of TNF-α in the induction of endothelin system genes

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Abstract

Objective Endothelins (ETs) are involved in several inflammatory events. The present study investigated the efficacy of bosentan, a dual ETA/ETB receptor antagonist, in collagen-induced arthritis (CIA) in mice.

Treatment CIA was induced in DBA/1J mice. Arthritic mice were treated with bosentan (100 mg/kg) once a day, starting from the day when arthritis was clinically detectable.

Methods CIA progression was assessed by measurements of visual clinical score, paw swelling and hypernociception. Histological changes, neutrophil infiltration and pro-inflammatory cytokines were evaluated in the joints. Gene expression in the lymph nodes of arthritic mice was evaluated by microarray technology. PreproET-1 mRNA expression in the lymph nodes of mice and in peripheral blood mononuclear cells (PBMCs) was evaluated by real-time PCR. The differences were evaluated by one-way ANOVA or Student’s t test.

Results Oral treatment with bosentan markedly ameliorated the clinical aspects of CIA (visual clinical score, paw swelling and hyperalgesia). Bosentan treatment also reduced joint damage, leukocyte infiltration and pro-inflammatory cytokine levels (IL-1β, TNFα and IL-17) in the joint tissues. Changes in gene expression in the lymph nodes of arthritic mice returned to the levels of the control mice after bosentan treatment. PreproET mRNA expression increased in PBMCs from rheumatoid arthritis (RA) patients but returned to basal level in PBMCs from patients under anti-TNF therapy. In-vitro treatment of PBMCs with TNFα upregulated ET system genes.

Conclusion These findings indicate that ET receptor antagonists, such as bosentan, might be useful in controlling RA. Moreover, it seems that ET mediation of arthritis is triggered by TNFα.

P. B. Donate and T. M. Cunha contributed equally to this study.

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Keywords  Rheumatoid arthritis · Collagen-induced arthritis · Bosentan · Inflammation · Microarray · Endothelins

Abbreviations
ET  Endothelin
RA  Rheumatoid arthritis
CIA  Collagen-induced arthritis
TNF  Tumor necrosis factor
IL  Interleukin
LT  Lymphotoxin
MTX  Methotrexate
CII  Collagen type 2
CFA  Complete Freund’s adjuvant
PBS  Phosphate-buffered saline
SAM  Significance analysis of microarrays
PBMCs  Peripheral blood mononuclear cells

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disorder of unknown cause. It is characterized by synovial leukocyte infiltration, pannus formation, degradation of cartilage and bone, and disabling pain [1, 2]. Increased levels of tumor necrosis factor α (TNF-α) and the interleukins IL-1β and IL-17 have been associated with variable degrees of bone and cartilage erosion, which causes a loss of function, disability, shortened life expectancy and considerable health care costs [3, 4].

Although no effective treatment is available, there have been major advances in the treatment of RA, including more aggressive use of disease-modifying antirheumatic drugs (DMARDs) and the development of immune therapies such as the anti-TNF agents [5, 6]. Despite these enormous advances, the large differences in patients’ responses, incidences of remission, and costs of treatments promotes a continuous search for the development of new drugs.

Endothelins (ETs) are a family of naturally occurring peptides [7] with growth-promoting, vasoactive, and nociceptive properties, and they affect the function of a number of tissues and systems [8]. There are 3 known, 21-amino-acid-long ETs in humans (ET-1, ET-2, and ET-3) that are produced as preproET and then cleaved by ET-converting enzymes to form big-ET and the active peptide [9].

These peptides have overlapping tissue distributions and are synthesized by vascular endothelial and smooth muscle cells and neural, renal, pulmonary and immune cells, such as macrophages and leukocytes [10]. Additionally, these peptides seem to exert an important influence by autocrine and paracrine actions mediated through G-protein-coupled specific ETA and ETB receptors [11].

ETs have pathophysiological roles in pulmonary hypertension, arterial hypertension, atherosclerosis, cerebral vasospasm and inflammatory processes [9, 12], in which they stimulate the formation of cytokines, such as interleukins and TNF [13]. In this context, high levels of ET-1 are detected in the synovial fluid of RA, osteoarthritis (OA), and gout patients. Plasma levels of ET-1 in active RA exceed the values in nonactive RA. Moreover, ET-1-like immunoreactivity in synovial fluid was found to be at levels several times higher than those in plasma and was found to be secreted from macrophage-like synoviocytes [14–16]. ETs may also act locally; ET-1-binding sites are localized in the synovial blood vessels, modulating synovial perfusion and exacerbating hypoxia in chronic arthritis [17]. Moreover, ET-1 has been shown to increase the net metalloproteases/tissue inhibitor of metalloproteases-1 balance and increase collagen degradation [18]. Recently, the participation of ETs in the development of arthritis and arthritic pain was demonstrated using the antigen-induced monoarthritis (AIA) model, with methylated bovine serum albumin as the antigen [19]. Taking into account the evidence mentioned above, we addressed the efficacy of the dual ET receptor antagonist bosentan in the collagen-induced arthritis (CIA) model, which is the animal model that best resembles human RA. Bosentan is the prototype of the sentan-class drugs and was first approved by the US Food and Drug Administration (FDA) for human use in pulmonary arterial hypertension. We also evaluated the impact of this treatment on differential gene expression profiles of draining inguinal lymph nodes. The modulation of ET system gene (preproET, ETA and ETB) expression was evaluated in peripheral blood mononuclear cells (PBMCs) from healthy donors and RA patients, and the impact of anti-TNF-α therapy in this process was evaluated.

Materials and methods

Animals

Male DBA/1J mice (12 weeks old; 18–22 g) were housed in temperature-controlled rooms (22–25°C) in the animal facility of the School of Medicine of Ribeirão Preto, University of São Paulo, São Paulo, Brazil, and received water and food ad libitum. The experimental protocols were approved by the local Ethical Committee on Animal Experimentation of the School of Medicine of Ribeirão Preto, University of São Paulo, São Paulo, Brazil (n244/2005).

Induction and assessment of CIA

Male DBA/1J mice received 200 μg bovine type II collagen (CII; Sigma) in complete Freund’s adjuvant (CFA) by
intradermal injection into the tail (day 0). CII [200 µg in phosphate-buffered saline (PBS)] was given again on day 21 by intraperitoneal injection [20]. Mice were monitored daily for signs of arthritis, for which severity scores were derived as follows: 0 = normal, 1 = erythema, 2 = erythema plus swelling, 3 = extension/loss function, and total score = sum of four limbs. Disease onset characterized by erythema and/or paw swelling was observed between days 25 and 35. The experimental control group corresponds to sham-immunized mice that received the same amount of CFA injection but without the administration of type II collagen. For the therapeutic approach, DBA/1J mice were treated with bosentan (Actelion Pharmaceuticals, Allschwil, Switzerland) (100 mg kg\(^{-1}\)) p.o. [21–23] once a day for a total of 11 days. The treatment began on the day that CIA was clinically detectable; thus, only mice that developed CIA were treated.

Measurement of mechanical hyperalgesia: electronic von Frey

The term hyperalgesia was used to define the decrease of nociceptive withdrawal threshold [24]. Mechanical hyperalgesia was tested in mice as previously reported [25]. The test consisted of evoking the hindpaw flexion reflex with a hand-held force transducer (electronic anesthesiometer; IITC Life Science, Woodland Hills, CA, USA) adapted with a 0.5-mm\(^2\) polypropylene tip. The investigator was trained to apply the tip perpendicular to the central area of the hindpaw with a gradual increase in pressure. The endpoint was characterized by removal of the paw followed by clear flinching movements. After paw withdrawal, the intensity of pressure was automatically recorded. The value for the response was obtained by averaging three measurements. The results are expressed by delta (Δ) withdrawal threshold (in g) calculated by subtracting zero-time mean measurements from time interval mean measurements.

Histological examination

For histological assessment, CIA mice treated with vehicle or bosentan were killed at the end of the experiments (11 days after CIA was clinically detected), and the paw joints were removed, fixed in 4% PFA buffer, and decalcified in EDTA. Sections (4 mm) were stained with H&E. The joint pathology was examined and scored. Scores are based on exudates, granulocyte infiltration, hyperplasia, mononuclear cell infiltration, periarticular mononuclear and granulocyte cell infiltration (each scoring 0–3), bone and cartilage destruction (scoring 0–4) and an additional score of 1 for fibrin deposition, resulting in a maximum possible score of 20. The sections were scanned with a Leica DM 4000B microscope, and the pictures were analyzed at a magnification of 500 µm with Leica LAS (Leica Application Suite) software.

Cytokine measurements

Eleven days after CIA was clinically detected, animals were killed by CO\(_2\) inhalation, and the surrounding joint tissue was removed and homogenized in 300 µL buffer containing protease inhibitors. IL-17, TNF-α and IL-1β levels were determined as described previously [26] by ELISA using paired antibodies (R&D Systems). The results are expressed as pg/joint for each cytokine. As a control, the concentration of each cytokine was determined in sham-immunized mice.

Myeloperoxidase activity assay

Neutrophil accumulation in the surrounding joint tissue of mice was evaluated by assaying myeloperoxidase (MPO) activity. Eleven days after CIA was clinically detected, animals were killed by CO\(_2\) inhalation, and the surrounding joint tissue was removed and homogenized with a tissue homogenizer (Power Gen 125, Fischer Scientific) in 0.2 mL pH 4.7 buffer (0.1 M NaCl, 0.02 M NaPO\(_4\), 0.015 M Na-EDTA). The suspensions were centrifuged at 3,000g for 15 min, the pellet was resuspended in lysis buffer (0.2% NaCl) and was further centrifuged for 15 min. The pellet cells were resuspended and homogenized in 0.5 mL H-TAB buffer (0.05 M NaPO\(_4\) buffer (pH 5.4) containing 0.5% dexamethasone) and centrifuged at 10,000g for 15 min, and the supernatants were used for determination of MPO activity. MPO activity in the resuspended pellet was assayed by measuring the change in absorbance at 450 nm using o-dianisidine dihydrochloride and 1% hydrogen peroxide. The results are reported as MPO units/mg of tissue. A unit of MPO activity was defined as that required to convert 1 µmol hydrogen peroxide to water in 1 min at 22°C.

Total RNA preparation

The DBA/1J mice were killed by CO\(_2\) inhalation, and the lymph nodes were surgically removed. To obtain sufficient mRNA for hybridization to the glass slides and the real-time PCR experiments, total inguinal lymph node RNA was pooled at each time point (n = 3 mice). Total RNA samples were prepared using Trizol® reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). For microarray and real-time PCR experiments, we used only undegraded and DNA-, protein- and phenol-free RNA preparations as evaluated by conventional agarose gel electrophoresis stained with ethidium bromide and ultraviolet spectrophotometry, respectively.
Human samples

Healthy donors and RA patients, from the Clinical Hospital of Ribeirão Preto–USP–SP, with active disease under treatment with methotrexate (MTX) or MTX plus anti-TNF (infliximab) were recruited, and they provided written informed consent. Peripheral blood samples from seven healthy volunteers and 14 RA patients were collected. All 14 RA patients recruited fulfilled the 1987 revised criteria of the American College of Rheumatology for the diagnosis of RA [27]. Seven of the patients were under treatment with methotrexate (MTX) alone, and seven of the patients were under treatment with MTX plus anti-TNF (infliximab) (clinical information is presented in Table 1). According to the Disease Activity Score 28 (DAS28, [28]), patients treated with MTX plus anti-TNF therapy exhibited lower levels of DAS28 (mean = 3.5) than those treated with MTX alone (DAS28: mean = 5.6). RA patients that received anti-TNF therapy presented a reduction of at least 1.6 in their DAS28 score, indicating the beneficial effect of anti-TNF therapy. The healthy donors did not present any clinical signs of disease upon anamnesis or clinical investigation.

For cell culture and RNA extraction, the PBMCs were separated on a Ficoll-Paque PLUS density gradient (GE Healthcare Life Sciences). For in-vitro experiments, 1 × 10⁶ PBMCs from six healthy donors and RA patients were cultured in 10% fetal bovine serum-supplemented RPMI 1640 medium at 37°C in a 5% CO₂ atmosphere in 96-well plates. PBMCs were incubated with 100 ng/mL TNF-α for 2 h. After stimulation, total mRNA was extracted from PBMCs using Trizol® reagent following the manufacturer’s instructions. The integrity of the total RNA samples was evaluated by denaturing agarose gel electrophoresis under standard conditions. The study was approved by the Human Ethics Committee of the Faculty of Medicine of Ribeirao Preto.

Gene expression analysis using microarrays

Gene expression in the lymph nodes was assessed using glass slide microarrays prepared on silane-coated UltraGAPS slides (#40015, Corning, New York, NY, USA). The arrays contained a total of 4,500 cDNA sequences that represented most murine tissues and organs. Sequences were obtained from the Soares thymus 2NbMT normalized library, which represents expressed sequence tag (EST) cDNA clones prepared from the thymus of a C57BL/6J 4-week-old male mouse, and is available at the IMAGE Consortium (http://image.hudsonalpha.org/).

The microarrays were prepared based on published protocols with PCR products from the cDNA clones [29] using a Generation III Array Spotter (Amersham Molecular Dynamics, Sunnyvale, CA, USA). A complete file that provides all of the genes and ESTs present on the microarrays used in this study is available on the MIAME database under accession code E-MEXP-2404 (http://www.ebi.ac.uk/microarray-as/ae/).

Complex cDNA probe preparation and hybridization

The cDNA complex probes derived from the total RNA obtained from the lymph nodes were prepared by reverse transcription using 10 µg of total RNA. The cDNA samples were monocolor labeled with Cy3 fluorochrome using the CyScribe post-labeling kit (GE Healthcare Life Sciences). Samples were hybridized for 15 h and then washed with an automatic slide processor system (ASP, Amersham Biosciences). Microarrays were scanned using a Generation III laser scanner (Amersham Biosciences).

As a reference for the hybridization procedure, we used equimolar quantities of cDNAs obtained from unrelated total RNA (mouse thymus total RNA). This approach allowed us to estimate the amount of target cDNA on each microarray spot.

Table 1 Clinical and demographic features of healthy donors and RA patients treated with MTX or MTX + anti-TNF (infliximab)

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>MTX only (n = 7)</th>
<th>MTX + infliximab (n = 7)</th>
<th>Healthy donors (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (years)</td>
<td>53.8 ± 4.6</td>
<td>47.2 ± 4.9</td>
<td>31.9 ± 2.7</td>
</tr>
<tr>
<td>Women n, (%)</td>
<td>5 (71.4%)</td>
<td>6 (85.7%)</td>
<td>4 (57.1%)</td>
</tr>
<tr>
<td>Caucasian n, (%)</td>
<td>7 (100%)</td>
<td>7 (100%)</td>
<td>7 (100%)</td>
</tr>
<tr>
<td>RF-positive n, (%)</td>
<td>6 (85.7%)</td>
<td>6 (85.7%)</td>
<td>0 (100%)</td>
</tr>
<tr>
<td>Anti-CCP positive n, (%)</td>
<td>4 (57.1%)</td>
<td>5 (71.4%)</td>
<td>0 (100%)</td>
</tr>
<tr>
<td>Mean disease duration (years)</td>
<td>3.5 ± 1.4</td>
<td>8.2 ± 2.4</td>
<td>None</td>
</tr>
<tr>
<td>MTX dose (mg/week)</td>
<td>11.2</td>
<td>15</td>
<td>None</td>
</tr>
<tr>
<td>MTX (months use, mean)</td>
<td>13 ± 1.9</td>
<td>22.6 ± 1.3</td>
<td>None</td>
</tr>
<tr>
<td>Infliximab dose (mg/kg/dose)</td>
<td>None</td>
<td>4</td>
<td>None</td>
</tr>
<tr>
<td>Infliximab use (&gt;6 months) n, (%)</td>
<td>None</td>
<td>7 (100%)</td>
<td>None</td>
</tr>
<tr>
<td>DAS28 (mean ± SEM)</td>
<td>5.6 ± 0.3</td>
<td>3.5 ± 0.5</td>
<td>None</td>
</tr>
</tbody>
</table>

RF rheumatoid factor, Anti-CCP anti-cyclic citrullinated peptide antibodies, MTX methotrexate, DAS Disease Activity Score
Microarray data analysis

Microarray images were quantified using Spotfinder software (http://www.tm4.org/spotfinder.html) and normalized using the R platform (http://www.r-project.org). Statistical analyses were performed using MeV software, version 3.1 (http://www.tm4.org/mev.html). Differentially expressed genes were identified using the significance analysis of microarrays (SAM) program [30], considering only those genes with an FDR (false discovery rate) ≤ 0.05.

To analyze the gene expression profiles from the SAM program data set, we used a hierarchical clustering method that grouped genes on the vertical axis and samples on the horizontal axis using similarity in their expression patterns. The similarities and dissimilarities in gene expression were presented as dendrograms, in which the pattern and length of the branches reflected the relatedness of the samples or genes, and as heat maps (http://rana.lbl.gov/EisenSoftware.htm) [31].

Real-time PCR

Real-time PCR was performed using a 7500 Real Time PCR system (Applied Biosystems). The cDNA was synthesized by oligo(dT) priming starting from 2.0 μg of lymph node total RNA as previously described, amplified using specific primers, and normalized to the amount of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (Gadph, ID: 008084). Reactions were performed in a final volume of 20 μL containing 10 pmol of each primer, 1× SYBR Green (Applied Biosystems) and 1 μL of first-strand cDNA. The specificity of the PCR products was assessed by melting curve analysis for all samples, and the products were also verified for correct size by agarose gel electrophoresis. Each sample was assayed in triplicate, and the mean Ct values were transformed into relative Amihe-like transcript quantities using the comparative Ct method (Applied Biosystems, user bulletin #2). The primers were identified using PRIMER3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and the cDNA sequences retrieved from GenBank (http://www.ncbi.nlm.nih.gov) for each gene. The following primers were used for mouse: Gadph, ID: 008084.2, sense 5′-GGGTGTGAACTACGAGAAT-3′, antisense 5′-CTTCCACAATGCGAAGTTT-3′; preproET, ID: 010104.2, sense 5′-CTGTCTCTTGACAGCTGCCT-3′, antisense 5′-CACCAAGCCTGTAGTATAC-3′; Mapk1, ID: 011949.3, sense 5′-CTCTCAACCTCTTGCTGAAAC-3′, antisense 5′-TGGAGCTCTGTACACGACGTG-3′; Ccr2, ID: 009915.1, sense 5′-GGGTGTGAACTACGAGAAT-3′, antisense 5′-CTTCCACAATGCGAAGTTT-3′; and Ltb, ID: 008518.1, sense 5′-TATCAGTGCTCTGGGCTGTG-3′, antisense 5′-TAGAGGTGAGGGCAAGATG-3′. Human primers were as follows: GAPDH, ID: 002046.3, sense 5′-CTGCACCACTAAGTGCTTA-3′, antisense 5′-CATGAGGCGAGGTCAGGTCTC-3′; PREPRO ET, ID: 001955.3, sense 5′-TGTTTCTTTGAGGTTCAGTCA-3′, antisense 5′-CTGTCTCTGGCAGCTTCTTG-3′; PRO-IL-1β, ID: 000002.11 sense 5′-GCAATGAGGATGACTTGGTTTGG-3′, antisense 5′-CAAGGTCCAGTCTGCTGGAA-3′; ETA, ID: 00004.11 sense 5′-CTGTCTGGAGGACAAAGTA-3′, antisense 5′-AGCAATCTGTTGGCGAGAT-3′; and ETB, ID: 00013.10 sense 5′-TCATCGGGAACCTCACACTT-3′, antisense 5′-GGATGTCAATGACGATGTG-3′.

Statistical analysis for real-time PCR and hyperalgesia

The results are presented as means ± SEM. The differences were evaluated by one-way ANOVA followed by Bonferroni’s t test (three or more groups) or Student’s t test (two groups). P < 0.05, P < 0.01, and P < 0.001 were considered statistically significant.

Results

Effects of bosentan treatment on collagen-induced arthritis

The increasing evidence of the role of ETs in the inflammatory process prompted us to evaluate the impact of bosentan treatment in a well-established model of arthritis, CIA. DBA/1J mice were immunized with type II collagen and therapeutically treated with bosentan for 11 days starting on the day that arthritis was clinically detectable. Oral treatment with bosentan ameliorated arthritis as determined by a reduction in the clinical score, the number of affected paws, and mechanical hyperalgesia observed during the course of the disease (Fig. 1a–c). A representative image of paws of vehicle- and bosentan-treated mice is shown in Fig. 1d. Infiltration of granulocytes and mononuclear cells into the inflamed joint and bone and cartilage destruction were lower in mice treated with bosentan than in the CIA vehicle-treated group (Fig. 2a). Bosentan-treated mice also presented significantly lower histological scores for inflammation and tissue destruction than the CIA vehicle-treated group (Fig. 2b; Table 2).

The reduction in the disease scores in bosentan-treated mice was also associated with a reduction in joint tissue levels of the pro-inflammatory cytokines IL-1β, TNF-α, and IL-17 (Fig. 3a–c). Bosentan-treated mice also presented a reduction in neutrophil accumulation in joint tissues compared with the CIA vehicle-treated group (Fig. 3d).
PreproET mRNA expression increase in lymph nodes of arthritic mice

ETs are produced as preproET, which is cleaved by ET-converting enzymes to form big-ET and the active peptide [9]. Therefore, we next analyzed the levels of the ET precursor preproET-1 by quantitative PCR in the inguinal lymph nodes of sham and immunized mice. As shown in Fig. 4, the levels of preproET-1 were higher in the lymph nodes of arthritic mice than in those of sham mice.

Bosentan treatment modulates gene expression in the lymph nodes

To elucidate the possible molecular mechanism associated with bosentan’s action and consequently the role of ETs in the CIA model, microarray technology was used to identify significant changes in gene expression during the treatment. The inguinal lymph node mRNA expression of sham and immunized mice was initially compared, and then we compared immunized mice before and after treatment with bosentan.

Although the expression pattern remained unchanged among the groups for a majority of the 4,500 sequences tested [presented $d(i) \approx d_E(i)]$, 508 genes were differentially expressed between sham and immunized mice, whereas 127 genes were found to be significantly modulated between immunized and bosentan-treated mice. With these data, clusters of repressed and induced genes were identified.

The Cluster-Tree View program was used to acquire hierarchical cluster analysis of results from the SAM program, and a comparison of the hybridization signatures showed variability among the three experimental groups. Based on heat map analysis, it was possible to distinguish the expression signature of the disease state and the treatment (Online Resource 1, 2). Genes that were differentially expressed in both heat maps were selected for further analysis according to their major biological processes. The genes were related to cell communication, cell cycle, cell death, response to stress and catabolic processes, among others. The genes involved with immune response are of particular importance, and a more careful analysis allowed a selection of those genes related to inflammatory processes, aside from being strictly related to CIA/RA. Among the genes analyzed, we found a group of important genes that exhibited an expression profile of particular interest. The Mapk1 (Mitogen-activated protein kinase 1), Ccr2...
Chemokine (C–C motif) receptor 2 (Ccr5) and Ltb (Lymphotoxin B) genes are involved in the arthritis process and are highly expressed during CIA development. On the other hand, during treatment with bosentan, their expressions were similar to control levels.

Real-time PCR confirmed the microarray data, showing increased transcription levels during disease in immunized mice and lower expression during bosentan treatment, with levels similar to control values (Fig. 5a–d).

PreproET mRNA is increased in RA-patient-derived PBMCs

The level of the ET precursor preproET was elevated in PBMCs from patients under treatment with MTX compared to healthy donors (Fig. 6a). On the other hand, PBMCs from patients receiving infliximab showed similar levels of preproET-1 compared with healthy donors (Fig. 6a). These results suggest that TNF-α could be involved in the upregulation of the ET system during RA. In an attempt to investigate this hypothesis, we performed in-vitro experiments using PBMCs from healthy donors and RA patients. PBMCs were cultured with TNF-α (100 ng/ml) for 2 h, followed by mRNA extraction. mRNA expression of preproET and ETA and ETB receptors increased in PBMCs from healthy donors and RA patients after TNF-α (Fig. 6b). However, this effect was more prominent in cells isolated from the RA patients (Fig. 6b). Interestingly, TNF-α also upregulated IL-1β mRNA expression in PBMCs from RA patients, but its expression was no different from cells from healthy donors.

Discussion

The pro-inflammatory properties of ETs were described immediately after their discovery, and they explain why these peptides contribute to the progression of a wide range of diseases that present inflammatory components, such as lupus erythematosus, systemic sclerosis and scleroderma [32, 33]. Our group and others have shown that ETs might participate in the pathophysiology of RA [14–17, 19]. Here, we extended these studies showing that bosentan, which is a dual ET receptor antagonist and was recently approved by the FDA for human use, ameliorated the
clinical and pathological inflammatory signals of CIA. Moreover, the bosentan treatment also prevented CIA-induced changes in the expression of important genes that encode key inflammatory mediators in the lymph nodes. In addition, the expression of ET precursor, preproET-1, was enhanced in the lymph nodes of CIA mice. From a clinical perspective, we also observed that the levels of ET precursors were elevated in PBMCs from RA patients who had undergone conventional treatment and were reduced by treatment with anti-TNF therapy. In vitro, TNF-α was able to enhance gene expression of the ET system (preproET-1, ETA and ETB) in PBMCs from RA patients compared with healthy donors.

Since their discovery, it has been clear that ETs are not merely vasoconstrictors but multifunctional peptides with cytokine-like activity, and they interfere with almost all aspects of cell function. Clinical studies have found elevated levels of ET-1 in synovial fluid and plasma of RA patients [14–16]. The strong association between these peptides and inflammation led us to investigate the efficacy of bosentan in CIA, which is a well-established experimental model for RA. CIA is characterized by acute and chronic inflammation of the joints and resembles most features of human RA, such as swelling, cartilage degradation, pain and loss of joint function [34]. Bosentan-treated mice presented a reduction in the following inflammatory parameters: edema, pain, joint movement, granulocyte and mononuclear cell infiltration into the joint tissue, bone and cartilage destruction, the production of pro-inflammatory cytokines and neutrophil migration. These results are in accordance with previous results that demonstrated that ET-1 mediates inflammatory events of zymosan-induced articular inflammation, including edema and neutrophil infiltration [35]. Moreover, ETs seem to be involved in pain in antigen-induced arthritis in mice [22, 36].

RA is a complex disease that results from an uncontrolled attack of the adaptive and innate immune system on joint structures. Although tissue and cells obtained from the

<table>
<thead>
<tr>
<th>#</th>
<th>Exudate</th>
<th>Granulocyte infiltration</th>
<th>Hyperplasia</th>
<th>Mononuclear infiltration</th>
<th>Periarticular mononuclear and granulocyte infiltration</th>
<th>Bone and cartilage destruction</th>
<th>Visible fibrin deposition</th>
<th>Total score</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIA #1</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>CIA #2</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>9</td>
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<td>CIA #3</td>
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<td>2</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>CIA #4</td>
<td>3</td>
<td>2</td>
<td>0</td>
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Mean ± SD 1.08 ± 0.90 1.9 ± 0.99 0.08 ± 0.28 1.66 ± 0.98 1.41 ± 0.79 1.41 ± 0.99 0.83 ± 0.38 8.5 ± 4.3

Bosentan #1 1 2 0 2 2 1 0 8
Bosentan #2 0 0 0 0 0 0 0 0
Bosentan #3 0 0 0 0 0 0 0 0
Bosentan #4 1 2 0 2 0 1 1 7
Bosentan #5 1 1 0 1 1 0 0 4
Bosentan #6 1 2 0 2 2 1 0 8
Bosentan #7 1 2 0 2 2 2 1 10
Bosentan #8 0 0 0 0 0 0 0 0
Bosentan #9 1 1 0 1 1 1 1 6
Bosentan #10 1 1 0 1 1 1 1 5
Bosentan #11 1 1 0 1 1 1 0 5
Bosentan #12 1 1 0 1 2 1 1 7

Mean ± SD 0.75 ± 0.45 1.08 ± 0.79 1.08 ± 0.79 1.0 ± 0.85 0.75 ± 0.62 0.33 ± 0.49 5.0 ± 3.4
inflamed joint might more accurately reflect the ongoing pathogenetic features of chronic diseases, lymph nodes and circulating immune cells are used to identify novel disease mediators, treatment responses and genetic disease variants [37, 38]. The use of large-scale analyses of gene expression patterns is increasing in many fields, including rheumatology. Array-based approaches allow the analysis of thousands of genes in a single experiment [39] and have been of special interest in the genetic study of heterogeneous autoimmune diseases such as RA. Therefore, in an attempt to elucidate the possible molecular mechanisms behind the role of ETs in CIA, the microarray technique was used to discover differences in lymph node gene profiles during CIA and bosentan treatment. Gene expression in the lymph nodes of arthritic mice was analyzed 11 days after bosentan treatment started because it was the time point when the effect of bosentan was most prominent. In our study, 4,500 sequences were analyzed by the SAM algorithm. A statistically significant set of 508 genes was found between sham and immunized mice, and a set of 127 genes was found between immunized mice treated with vehicle and those treated with bosentan. Moreover, a hierarchical cluster analysis of the results from SAM using the Cluster-Tree View program showed that the three experimental groups analyzed (sham, CIA vehicle- or bosentan-treated) had distinct profiles.

There were four main genes involved in the immune response whose expressions were increased during CIA and repressed by bosentan treatment. One gene was Mitogen-activated protein kinase 1 (Mapk1), a signaling molecule that regulates the production of cytokines and cytotoxic enzymes that have been implicated in RA pathogenesis. In agreement with our results, Mapk1 is induced through ETA and ETB receptors, and it mediates important peripheral functions of ETs, including DNA synthesis, mitogenesis, and the activation of vascular smooth muscle [40, 41]. Chemokine (C–C motif) receptor 2 (Ccr2) and chemokine (C–C motif) receptor 5 (Ccr5), which along
with their cognate ligands have been detected in the synovial fluid of patients with RA and in inflamed joints in CIA, were also affected by CIA and bosentan treatment [42–44]. Another affected gene was Lymphotoxin (LT) Beta, which is predominantly expressed in a variety of immune cells [45, 46] and is highly expressed in the RA synovial lymphoid follicles. In addition, treatment with LT-BetaR-Ig prevents the induction of murine CIA [47, 48]. It is important to mention that the expression of these genes described above was also evaluated by real-time PCR (Fig. 3), which confirmed the participation and modulation of these transcripts in the studied model, suggesting that they might have importance in the cascade of ET effects. Corroborating the participation of endogenous ETs in the modulation of these transcripts in peripheral lymph nodes, we detected an increased expression of preproET during CIA (Fig. 4). Therefore, it seems that ETs may have an immunomodulatory role by acting in the lymph nodes during experimental arthritis. This effect might contribute to the exacerbation of the immune response in RA. In agreement with this hypothesis, there is evidence that dendritic cells produce large amounts of

![Fig. 5 Confirmation of repressed gene expression in lymph nodes during bosentan treatment in CIA. Real-time PCR was used to confirm the mRNA levels of Mapk1 (a), Ltb (b), Ccr5 (c) and Ccr2 (d) in inguinal lymph nodes from sham, immunized and bosentan-treated DBA/1J mice. Lymph nodes were removed 11 days after CIA was clinically detected. Expression levels were normalized to Gapdh expression. The results are presented as mean ± SEM from one experiment with six mice per group. The differences between groups were evaluated by one-way ANOVA followed by Bonferroni’s t test. *P < 0.05 compared with sham-immunized mice; **P < 0.05 compared with CIA mice treated with vehicle.](image)

![Fig. 6 TNF-α induces the expression of ET system genes in PBMCs of healthy individuals and RA patients. Real-time PCR was used to detect the mRNA levels of (a) preproET in PBMCs from healthy individuals and RA patients and (b) IL-1β, preproET, and ETA and ETB receptors in PBMCs cultured with 100 ng/ml TNF-α or medium for 2 h. The expression levels were normalized to GAPDH expression. The results are expressed as mean ± SEM. The differences between responses were evaluated by one-way ANOVA followed by Bonferroni’s t test (three or more groups). *P < 0.05 and **P < 0.01 compared with healthy individuals and the MTX-treated patients group, respectively (a); *P < 0.05 and **P < 0.01 compared with control group (b).](image)
ET-1 and significantly increase the expression of ET receptors upon activation. Furthermore, selective blockade of the ETA receptor significantly reduced expression of the mature dendritic cell marker CD83, decreased the production of IL-12, downregulated the ability of dendritic cells to stimulate T cells, and promoted dendritic cell apoptosis [49].

Once the effectiveness of bosentan was demonstrated in the CIA model, suggesting the participation of the ET system in the pathophysiology of CIA, we then evaluated the expression of the ET precursor in immune cells from RA patients. Our results showed increased levels of preproET-1 in PBMCs from RA patients who were under MTX treatment compared with levels in cells from healthy donors. With a combination of MTX and anti-TNF (infliximab) therapy, preproET-1 gene expression level did not differ from the expression in healthy donors. These results suggest that upregulation of ET gene expression in PBMCs from RA patients seems to be controlled by pro-inflammatory cytokines, specifically TNF-α. There is a large body of evidence showing that TNF and other cytokines promote ET gene expression in a variety of cell types [50–52]. On the other hand, ETs can also contribute to the production of pro-inflammatory mediators such TNF, IL-1, IL-6, IL-8, monocyte chemotactic protein-1 and granulocyte/macrophage colony-stimulating factor [13, 52]. Aside from the possible immunomodulatory role of ETs in RA, we could not discount their local involvement in several inflammatory events in the joints. Indeed, as mentioned above, the levels of ET-1 were higher in RA patients than in osteoarthritic patients [15, 16]. Furthermore, an increased expression of preproET in zymosan- and antigen-induced articular inflammation was found in mice [22, 35]. Additionally, ETA and ETB receptors are functionally expressed in synovial tissue [23, 34]. Further supporting the involvement of TNF-α in the upregulation of ET system genes, we showed that TNF-α increased the expression of preproET-1, ETA and ETB in PBMCs from healthy donors and RA patients. TNF-α also increased the expression of IL-1β mRNA in PBMCs. Interestingly, the effect of TNF-α on the ET system genes was more prominent in cells from RA patients than in cells from healthy donors; however, this effect was not observed for IL-1β expression, suggesting a specific effect of TNF-α on the ET system. The intracellular mechanism by which TNF-α upregulates the gene expression of ETs in cells from RA patients was not addressed. Further studies are required to clarify the regulation of the TNF-α/ET axis, and a higher number of patients is necessary to achieve a definitive conclusion. Nevertheless, these results might provide an important novel mechanism by which anti-TNF-α therapy ameliorates inflammation via downregulation of ET system gene expression in leukocytes in RA.

Conclusions

The results from this investigation contribute to a better understanding of the mechanisms involved in CIA and the participation of ETs in CIA. These results could lead to future associated therapies for RA, including the use of ET receptor antagonists.

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