Etanercept improves endothelial function via pleiotropic effects in rat adjuvant-induced arthritis

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Abstract

Objectives. To determine the effect of etanercept on endothelial dysfunction and on traditional cardiovascular (CV) risk factors in the adjuvant-induced arthritis (AIA) rat model.

Methods. At the first signs of arthritis, etanercept (10 mg/kg/3 days, s.c.) or saline was administered for 3 weeks in AIA rats. Body weights and arthritis scores were monitored daily. Endothelial function was studied in aortic rings relaxed with acetylcholine (Ach) with or without inhibitors of nitric oxide synthase (NOS), cyclo-oxygenase (COX-2), arginase, endothelium-derived hyperpolarizing factor and superoxide anions (O$_2^•$) production. Aortic expression of endothelial nitric oxide synthase (eNOS), Ser1177-phospho-eNOS, COX-2, arginase-2, p22 phox and p47 phox was evaluated by western blotting analysis. Blood pressure, heart rate and blood levels of triglycerides, cholesterol and glucose were measured.

Results. Etanercept significantly reduced arthritis score (P < 0.001). It improved Ach-induced relaxation (P < 0.05) as a result of increased NOS activity, decreased COX-2/arginase activities and decreased O$_2^•$ production. These functional effects relied on increased eNOS expression and phosphorylation, and decreased COX-2, arginase-2 and p22 phox expressions. No correlation was found between arthritis score and Ach-induced relaxation. The treatment did not change triglycerides, cholesterol and glucose levels, but significantly increased systolic blood pressure and heart rate (P < 0.05).

Conclusion. Our data demonstrated that efficient dosage of etanercept on inflammatory symptoms improved endothelial function in AIA. This beneficial effect on endothelial function is disconnected from its impact on CV risk factors and relates to pleiotropic effects of etanercept on endothelial pathways. These results suggest that etanercept could be a good choice for patients with rheumatoid arthritis at high risk of CV events.

Key words: adjuvant-induced arthritis, endothelial dysfunction, etanercept, mechanisms

Rheumatology key messages

- Etanercept improves endothelial function in adjuvant-induced arthritis via pleiotropic effects on endothelial pathways.
- Positive effects of etanercept on endothelium are disconnected from its impact on cardiovascular risk factors.

Introduction

RA is the most common systemic autoimmune disease and is characterized by reduced life expectancy ranging between 3 and 18 years compared with the general population [1]. One of the leading causes of excess mortality in RA patients is cardiovascular (CV) disease due to accelerated atherogenesis, irrespective of classical CV risk factors [2]. Evidence from clinical studies shows that endothelial dysfunction (ED), the *sine qua non* condition...
for atherosclerosis appearance, is present in established RA [3] and is an important target for reducing CV diseases. Given that RA and atherosclerosis share common immunopathogenic features, it has been proposed that inflammatory mechanisms involved in the development of synovial lesions might contribute to ED [4]. This hypothesis is supported by in vivo experimental data showing that administration of the pro-inflammatory cytokine TNF-α depresses endothelium-dependent relaxation [5]. Surprisingly then, clinical studies on the effects of anti-TNF-α on ED in RA have provided conflicting results. Some have shown improvement of endothelial function [6–9], whereas some report no changes in ED despite efficient reduction of disease activity [10–15] and others demonstrate only a transient improvement of ED [16–18].

The major difficulties in addressing whether or not anti-TNF-α improves ED in clinical studies are that on the one hand, according to the international guidelines, TNF-α inhibitors are usually used in combination therapy with MTX, and on the other hand, RA patients take multiple medications, including CV drugs that can impact upon endothelial function. Thus, animal models of RA provide a unique opportunity to determine the impact of one given drug on endothelial function without the influence of other medications, with a direct assessment of vascular function of isolated vessels, and using rat cohorts with reproducible polyarthritis severity. Previous studies conducted on the widely used model of adjuvant-induced arthritis (AIA) in rats identified nitric oxide synthase (NOS) uncoupling, arginase-2 overactivation, endothelial function in the AIA model in rats. Endothelial function was studied on isolated aortic rings on day 33 after arthritis induction (a time at which ED was previously characterized [20]), and the mechanisms involved were dissected. The efficacy of etanercept on disease activity and joint damage was assessed by measurement of arthritis score and radiographic score, respectively. To assess whether the treatment modified CV risk factors, blood pressure, heart rate, glycaemia and lipid levels were also measured.

Methods

Animals

Six-week-old male Lewis rats (n = 60) were purchased from Janvier (Le Genest Saint Isle, France). Animals were kept under a 12:12 h light:dark cycle and allowed free access to food and water. The experimental procedures were approved by the local committee for ethics in animal experimentation no. 2012/001-CD of Franche-Comté University (Besançon, France) and complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (publication No. 85-23, revised 2011).

Induction and clinical evaluation of the arthritis model

Adjuvant arthritis was induced by a single intradermal injection at the base of the tail of 120 µl of 1 mg of heat-killed Mycobacterium butyricum (Difco, Detroit, MI, USA) suspended in 0.1 ml of mineral oil [Freund’s incomplete adjuvant (Difco, Detroit, MI, USA)]. The AIA model is characterized by rapid onset and progression of a robust and easily measurable polyarthritis (characterized by severe erythema and diffuse soft swelling with complete ankylosis and malformations in the paws), reduced locomotor activity, and is frequently associated with ear and tail inflammation, weight loss, anorexia and diarrhoea [21]. Rats were weighed and monitored for clinical signs of arthritis daily. The scoring system employed was as follows [22]: arthritis of one finger scored 0.1, weak and moderate arthritis of one big joint (ankle or wrist) scored 0.5, and intense arthritis of one big joint scored 1. Tarsus and ankle were considered as the same joint. The sum of the joint scores of the four limbs led to a maximum arthritis score of 6 for each rat.

Drug treatment

On the day of the first inflammatory symptoms (i.e. at day 11–12 post-immunization), AIA rats were randomized into two groups. One group received etanercept, at 10 mg/kg (s.c.) every 3 days for 3 weeks (Etanercept, n = 30). The dose of etanercept was chosen on the basis of previous data on the AIA model that showed a significant reduction in arthritis severity and radiographic lesion [23]. The other group received saline at 1 ml/kg (s.c.) for 3 weeks (Vehicle, n = 30).

Tissue collection, blood pressure and heart rate measurements

At 21 days after treatment initiation, rats were anaesthetized with pentobarbital (60 mg/kg, i.p.). Arterial systolic (SBP), diastolic (DBP), mean arterial blood pressure and heart rate were measured after cannulation of the left carotid artery and connection of the catheter to a pressure recorder system (Easy Graf, Gould, USA) under temperature control. Blood was withdrawn from the abdominal artery and centrifuged to obtain serum, divided into aliquots and stored at −80 °C until analysis. The thoracic aorta was removed and immediately used for the group of rats used for vascular reactivity studies (n = 15/group) or frozen in liquid nitrogen and stored at −80 °C until analysis for the group used for western blot (n = 15/group). Ankles were removed and placed in 4% formalin until assessment of radiographs.

Radiographical ex vivo analysis of joints of ankle and foot

Radiographs of hind paws were performed with a Block Matching Algorithm High Resolution Digital X-ray system (40 mV, 10 mA—D3A Medical Systems (Orleans, France). A score of 0–20 was determined for each paw using a
grading score used the scale: 0 (normal); 1 (slight); 2 (mild); 3 (moderate); and 4 (severe) abnormalities in the tissue for each of five characteristic features of AIA. Radiographs take into account: (i) the soft tissue swelling; (ii) the osteoporosis as measured by bone density; (iii) the loss of cartilage shown by narrowing of the joint spaces; (iv) the bone erosions; and (v) the heterotopic ossification defined as proliferation of new bone tissue. The maximum score for each rat is 40.

Vascular reactivity

At the end of the treatment period, the thoracic aorta was excised, cleaned of connective tissue and cut into rings of ~2 mm in length. The rings were suspended in Krebs solution (mol/l: NaCl 118, KCl 4.65, CaCl2 2.5, KH2PO4 1.18, NaHCO3 24.9, MgSO4 1.18, glucose 12, pH 7.4), maintained at 37 °C and continuously aerated with 95% O2, 5% CO2 for isometric tension recording in organ chambers, as previously described [25]. In some rings, the endothelium was mechanically removed. The completeness of endothelial denudation was confirmed by the absence of relaxation to the endothelium-dependent agonist acetylcholine (Ach, 10–6 mol/l). After a 90-min equilibration period under a resting tension of 2 g, to determine whether etanercept improved endothelial function, rings with intact endothelium were constricted with phenylephrine (PE, 10–6 mol/l), and endothelium-dependent relaxation to Ach (10–11 to 10–4 moles/l) was compared between the etanercept and the vehicle group. To investigate the contributions of NOS, tetrahydrobiopterin (the co-factor of NOS, BH4), arginine, O2−, COX-2 and EDHF, the rings were previously incubated for 1 h with the non-selective NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME, 10−4 mol/l), BH4 (10−7 mol/l), the arginase inhibitor Nω-hydroxy-nor-L-arginine (nor-NOHA, 10−4 mol/l), the superoxide dismutase mimetic Tempol (10−4 mol/l), the selective COX-2 inhibitor (NS-398, 10−5 mol/l) and the Ca2+−dependent K+ channels inhibitors apamin (10−7 mol/l) and charybdotoxin (10−7 mol/l), respectively. Endothelium-denuded rings were used to determine the vasocostrictive response to norepinephrine (NE, 10−11 to 10−4 mol/l) and the vasorelaxant response to the NO-donor sodium nitroprussiate (SNP, 10−11 to 10−4 mol/l) after pre-constriction with PE 10−6 mol/l.

Western blots

To investigate whether the effects of etanercept on endothelial function relied on changes in protein expression, the protein content of endothelial nitic oxide synthase (eNOS) and Phospho-Ser1177-eNOS (P-eNOS), an activated form of eNOS at serine 1177 that produces a sustained release of endothelial NO, arginase-2, COX-2, p22phox and p47phox (membrane-bound and cytosolic components, respectively, of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a major vascular enzyme responsible for O2− production in RA) [26], were assessed in thoracic aortas from vehicle-treated and etanercept-treated AIA rats. After homogenization in ice-cold lysis buffer, thoracic aortas were sonicated and centrifuged (12 000 g, 20 min, +4 °C) and the supernatants stored at −80 °C until protein measurement by the Lowry method. Total proteins were separated by SDS–PAGE on 12% (arginase-2, p22phox or p47phox) or 8% (COX-2, P-eNOS and eNOS) SDS–PAGE and transferred to polyvinyl difluoride (PVDF) membrane. After blocking unspecific binding with a 5% non-fat dry milk in Tris-buffered saline (TBS) buffer containing 0.1% Tween 20, membranes were incubated for 3 h at room temperature with anti-eNOS (mouse monoclonal 610297, BD Transduction Laboratories, 1/2500), anti-P-eNOS (mouse monoclonal 612393, BD Transduction Laboratories, 1/1000), anti–arginase-2 (rabbit polyclonal, Santa Cruz Biotechnology, 1/4000), anti–COX-2 (rabbit polyclonal aa 570–598, Cayman Chemical, 1/2500), anti-p22phox (mouse monoclonal sc-271968, Santa Cruz Biotechnology, 1/4000), anti-p47phox (mouse monoclonal sc-17845, Santa Cruz Biotechnology, 1/4000) or anti–β-actin (mouse monoclonal, A5441, Sigma-Aldrich, 1/5000) antibodies and then for 2 h with a horseradish peroxidase-conjugated anti–mouse IgGs (for eNOS, P-eNOS, p22phox, p47phox and β-actin, 115-035-166 Jackson ImmunoResearch) or anti–rabbit IgGs (for arginase-2 and COX-2, 111-035-144 Jackson ImmunoResearch) and visualized using enhanced chemiluminescence (ECL+, GE Healthcare). The band densities were determined by scanning densitometry, and values were expressed as arbitrary units.

Blood measurements

Total cholesterol and triglycerides were measured in serum (Vista, Siemens, USA) and glucose was measured in blood by using a glycometer (GlucoMen, Menarini diagnostics, Italy).

Data and statistical analysis

Values are presented as means (s.e.m.). Data were analysed using GraphPad Prism software (ver. 5.0). Contractile responses to NE were expressed as the percentage of the maximum response to KCl 100 mmol/l. Relaxant responses to SNP and Ach were expressed as the percentage of relaxation of the contractile response to PE 10−6 mol/l. Concentration–response curves to Ach, SNP and NE in vehicle-treated and etanercept-treated rats were compared by two-way analysis of variance for repeated measures. In each group (vehicle or etanercept), concentration–response curves to Ach with or without a specific inhibitor were compared by two-way analysis of variance for repeated measures. When necessary, to better understand the effect of inhibitors, the results were expressed as the area under the curve (AUC) (calculated from the individual concentration–response curves). Comparison between two values was assessed by an unpaired Student’s t test, or a Mann–Whitney test when the data were not normally distributed. The relationship between two parameters was determined by linear regression analysis, and Spearman’s correlation coefficient was calculated between these variables. P < 0.05 was considered statistically significant.
Results

Etanercept decreased clinical and radiological scores in AIA

Etanercept did not influence body weights as compared with the vehicle (Table 1). As expected, the treatment significantly decreased the mean arthritis scores (Fig. 1A). The clinical effect of etanercept was associated with a slight but significant decrease in radiological score (Fig. 1B). When detailing the different components of this score, it appeared that etanercept significantly reduced soft tissue swelling, loss of cartilage, and osteoporosis, but not bone erosion or new bone formation (supplementary Fig. S1, available at Rheumatology Online).

Table 1 Effect of etanercept on physiological and biological parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Vehicle</th>
<th>Etanercept</th>
</tr>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>218 (2)</td>
<td>224 (2)</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>114 (5)</td>
<td>125 (6)*</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>77 (3)</td>
<td>85 (5)</td>
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<tr>
<td>MAP (mm Hg)</td>
<td>89 (5)</td>
<td>103 (5)</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>256 (14)</td>
<td>294 (10)*</td>
</tr>
<tr>
<td>Total cholesterol (g/l)</td>
<td>0.99 (0.03)</td>
<td>0.88 (0.03)</td>
</tr>
<tr>
<td>Triglycerides (g/l)</td>
<td>0.47 (0.04)</td>
<td>0.45 (0.04)</td>
</tr>
<tr>
<td>Blood glucose (g/l)</td>
<td>1.10 (0.04)</td>
<td>1.13 (0.07)</td>
</tr>
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</table>

Values are expressed as means (s.e.m.) (n = 15 rats per group). All parameters were measured at day 33 post-immunization after 21 days of treatment (s.c.) with saline (vehicle) or etanercept (10 mg/kg/3 days). SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial blood pressure; bpm: beats per minute. *P < 0.05 different from vehicle.

Etanercept improved endothelial function in AIA

To determine whether etanercept modified endothelial function, the concentration–response curves to Ach were compared between etanercept- and vehicle-treated AIA rats. As shown in Fig. 2A, anti-TNFα treatment significantly improved Ach-associated vasorelaxation. To ascertain that this effect was not due to impaired response of vascular smooth muscle cells to vasoconstrictive stimulus or to the relaxant effect of NO, the effect of etanercept on NE-induced vasoconstriction and on SNP-induced vasodilation was determined on endothelium-denuded aortic rings. Our results demonstrated that etanercept-treated rats displayed similar constriction to NE (Fig. 2B) as well as similar relaxation to the NO-donor SNP (Fig. 2C). Of note, no correlation was found between the arthritis score and Ach-induced relaxation (AUC) (r = –0.322; P = 0.0817, all AIA rats, data not shown).

Etanercept improved the NOS/BH4/arginase balance in AIA

NO is the key endothelial-derived relaxing factor in the macrovasculature. Its production by eNOS depends, in part, on the competition between eNOS and arginase for their common substrate: L-arginine [26]. Incubation of rings with L-NAME significantly blunted Ach-associated relaxation in both the vehicle (Fig. 3A) and the etanercept group (Fig. 3B). However, the effect of L-NAME was greater in the etanercept group as compared with the vehicle group [% reduction of AUC: 87 (2) vs 77 (4) in vehicle, P = 0.042], indicating that NOS activity was enhanced by etanercept. This finding was confirmed by the higher expression of P-eNOS (the active form of eNOS) in the etanercept group (Fig. 3C) associated with a higher total eNOS expression (Fig. 3D). To assess the effect of treatment on arginase activity, aortic rings were incubated with nor-NOHA, an arginase inhibitor. In vehicle-treated rats

Fig. 1 Evolution of arthritis in vehicle- and etanercept-treated rats

(A) Arthritis scores were plotted over time after adjuvant-induced arthritis induction. (B) Radiographic scores were evaluated at the end of the treatment period. Values are the mean (s.e.m.) (n = 30/group). ***P < 0.001, *P < 0.05.
Fig. 2 Vascular reactivity to vasodilators and vasoconstrictive agents

Experiments were performed on thoracic aortic rings from vehicle- and etanercept-treated rats at the end of the treatment period (21 days after the onset of arthritis). Concentration–response curves of (A) Ach in endothelium-intact aortic rings preconstricted with PE 10⁻⁶ mol/l and (B) NE on endothelium-denuded aortic rings; (C) SNP on endothelium-denuded aortic rings preconstricted with PE 10⁻⁶ mol/l. Values are the mean (S.E.M.) (n = 10–15 aortic rings from 10–15 rats per group). **P < 0.01, by two-way ANOVA for repeated measures.

(Fig. 3E), nor-NOHA significantly improved Ach-induced vasorelaxation [AUC without inhibitor 304 (16) vs AUC with inhibitor 362 (20), P = 0.029], confirming the deleterious role of arginase in Ach-induced relaxation [27]. In the etanercept group, as a reflection of the normalization of arginase activity, nor-NOHA did not change the AUC of Ach-induced relaxation (Fig. 3F). Consistent with this, aortic arginase-2 expression was significantly reduced by etanercept (Fig. 3G). Additionally, since NOS activity also depends on the availability of its co-factor BH₄, we investigated the impact of etanercept on BH₄ deficiency. As previously reported in this model [28], incubation of aortic rings with BH₄ significantly improved Ach-induced relaxation in vehicle-treated AIA (Fig. 3H). In contrast, BH₄ did not improve the response to Ach in etanercept-treated rats (Fig. 3I). In summary, all the above indicated that the effect of etanercept on endothelial function was mediated by a modification of the NOS/BH₄/arginase balance in favour of increased NOS activity/expression and reduced BH₄ deficiency, along with decreased arginase activity/expression.

Etanercept blunted the COX-2 pathway but did not change EDHF production

Besides NO, EDHF and COX-derived products are endothelium-derived relaxing factors with a relevant contribution to normal endothelial function. Previous data on the AIA model demonstrated that impaired vascular EDHF production and enhanced COX-2 activity are both contributors to ED [27]. To assess the contribution of EDHF in Ach-induced relaxation, rings were incubated with apamin/chaerybdotoxin, two K⁺ channel inhibitors. We previously demonstrated that incubation of aortic rings from aged-matched, control non-AIA Lewis rats with apamin/chaerybdotoxin blunted the vasorelaxant response to Ach, as a reflection of the EDHF contribution to the relaxant effect of Ach in normal conditions [27]. In vehicle AIA, consistent with EDHF deficiency, apamin/chaerybdotoxin did not reduce, but actually slightly improved, Ach-induced relaxation (Fig. 4A). As shown in Fig. 4B, apamin/chaerybdotoxin did not change the response to Ach in etanercept-treated rats, thus indicating that etanercept did not improve EDHF production in AIA rats. As regards COX-2 activity, our results showed that NS-398, a COX-2 inhibitor, significantly improved Ach relaxation in the vehicle group (Fig. 4C). Conversely, the COX-2 inhibitor did not change the Ach response in the etanercept group (Fig. 4D), indicating that COX-2 activity was reduced by the treatment. Consistent with this, aortic COX-2 expression was significantly decreased by etanercept (Fig. 4E). Altogether, these data demonstrated that etanercept did not change EDHF production, but reduced the deleterious contribution of COX-2 to ED.

Etanercept decreased superoxide anions production and NADPH oxidase expression

The role of excessive O₂⁻ production and increased NADPH oxidase expression in AIA-associated ED has been previously demonstrated [28]. In the present study, the fact that the superoxide dismutase mimetic Tempol significantly improved Ach-induced vasodilation in the vehicle group confirmed this data (Fig. 5A). In etanercept-treated AIA rats, Tempol did not change the response to Ach, thus indicating that the O₂⁻ production decreased after treatment (Fig. 5B). Western blot analysis of the components of NADPH oxidase showed that the beneficial effect of etanercept on O₂⁻ production relied, at least in part, on decreased expression of p22phox (Fig. 5C), but not of p47phox (Fig. 5D).

Effect of etanercept on CV risk factors

Data are presented in Table 1. As compared with vehicle, etanercept induced a significant increase in SBP and heart rate (P < 0.05). Conversely, it did not change DBP, mean arterial blood pressure, blood glucose, total cholesterol or triglyceride levels.

Discussion

TNF-α is a pro-inflammatory cytokine secreted by activated macrophages and T cells and recognized by TNF-α receptors that are expressed on critical cellular effectors
Experiments were performed on thoracic aortic rings from vehicle- and etanercept-treated rats at the end of the treatment period (21 days after the onset of arthritis). Cumulative concentration–response curves of Ach were obtained after incubation or not with L-NAME at 10^{-4} mol/l (A and B), with the NOS cofactor BH4 at 10^{-7} mol/l (H and I) or with the arginase inhibitor nor-NOHA at 10^{-4} mol/l (E and F). Expression of eNOS (D), its phosphorylated form at Serine 1177, P-eNOS (C) and arginase-2 (G) were evaluated in aortas by western blotting. Values from the concentration–response curves are expressed as means (s.e.m.) (n=9–15 rings from 9–15 rats per group), compared by two-way ANOVA for repeated measures. Values from immunoblots are the mean (s.e.m.) (n=6 rats per group), compared by the Mann–Whitney test. *P < 0.05, ***P < 0.001.
of vascular function/dysfunction, including vascular endothelial and smooth muscle cells [29]. This cytokine was previously reported to contribute to ED in animal models of low-grade inflammation such as fructose-induced hypertension [30], ageing [31], oestrogen deficiency [32], chronic mild stress [33] and diabetes [34], as evidenced by the improvement achieved by etanercept of Ach-induced vasodilation in these models. A new finding provided by the present study is the positive impact of etanercept in ED associated with experimental arthritis, indicating that the cytokine also acts as a contributor to ED in situations associated with high-grade inflammation. This new data strongly suggests that lower CV events reported in RA patients treated with TNF-α inhibitors [35] are likely mediated by ED reduction and resonates with studies that show association between several polymorphisms of the TNF-α gene and predisposition to CV complications in RA patients [36, 37]. An intriguing result

Experiments were performed on thoracic aortic rings from vehicle- and etanercept-treated rats at the end of the treatment period (21 days after the onset of arthritis). Cumulative concentration–response curves of Ach were obtained after incubation or not with apamin/charybdotoxin at 10^{-7}/10^{-7} mol/l (A and B) or the COX-2 inhibitor NS-398 at 10^{-4} mol/l (C and D). COX-2 expression (E) was evaluated in aortas by western blotting. Values from the concentration–response curves are the mean (S.E.M.) (n = 8–15 rings from 8–15 rats per group), compared by two-way ANOVA for repeated measures. Data from immunoblots are the mean (S.E.M.) (n = 6 rats per group), compared by the Mann-Whitney test. *P < 0.05.
of our study was a lack of association between endothelial function and arthritis score in AIA rats. This data suggests that, even though TNF-α is likely involved in both vascular and joint detrimental processes in AIA, the pathomechanisms governing joint disease and vascular disease are probably distinct.

Classical CV risk factors, including hypertension, are minimally explaining the CV risk in RA [2, 38]. Besides, ED was reported to be present in RA patients independently of traditional risk factors [39]. Supporting this latter point are our results showing that etanercept did not modify triglycerides, total cholesterol or blood glucose levels, but resulted in a slight increase in SBP in AIA, as expected of traditional risk factors [39]. Supporting this latter result are our results showing that etanercept did not modify triglycerides, total cholesterol or blood glucose levels, but resulted in a slight increase in SBP in AIA, as expected of traditional risk factors [39].

The comprehensive analysis of the mechanisms underlying the effects of etanercept on endothelial function revealed both an improvement in NO production and a decrease in oxidative stress. This is consistent with studies showing an increased frequency of CV events and an increased risk of developing CV events in RA patients who carry eNOS [43] and methionine sulfoxide reductase gene polymorphisms [44], an oxidative stress-related enzyme. We showed that the improvement of endothelial NO production by etanercept was associated with an increase in eNOS phosphorylation at serine 1177 and in total eNOS expression, as previously reported in other animal models of ED [30–34]. The new finding of the present study is that increased NO output by the endothelium is also consequent upon a decrease in arginase-2 expression/activity, an enzyme that starves eNOS of its substrate L-arginine, and that was previously reported to be upregulated in vitro by TNF-α [45]. Besides a switch of the eNOS/arginase balance towards the NOS pathway, etanercept also reduced oxidative stress and (more precisely) the production of O2·−, a radical oxygen species that contributes greatly to ED in AIA [19]. Our results suggest that reduced O2·− production by etanercept may involve several mechanisms. A major mechanism is likely a decrease in the expression of p22phox, the membrane-bound subunit of NADPH oxidase. However, reduced arginase activity and the improvement of BH₄ availability may also be implicated. Indeed, under conditions of reduced substrate and/or cofactor availability, eNOS becomes uncoupled, that is, loses its ability to convert L-arginine to L-citrulline, but removes an electron from...
NADPH and donates it to molecular oxygen to yield $O_2^{-}$ instead of NO. An additional mechanism suggested by our study is a decrease in vascular COX-2 expression, since COX-2 is a source of oxygen radicals itself. Evidence that etanercept improves endothelial function through mechanisms other than increased NOS activity/expression might explain why the beneficial effects of DMARDs (including TNF-α inhibitors) on endothelial function were observed, despite unchanged plasma levels of dimethyl-L-arginine, an endogenous inhibitor of eNOS [46, 47].

In conclusion, despite the general consensus that the reduction of CV morbidity and mortality is of the utmost importance in RA, clinical data on the impact of TNF-α inhibitors on ED provided inconclusive results. This present study provides the first evidence of a favourable effect of etanercept on endothelial function in an animal model of RA, through pleiotropic effects on endothelial pathways, namely a switch of the eNOS/arginase balance towards the NOS pathway, a reduction of the NADPH oxidase/$O_2^{-}$ production pathway and a decrease in the COX-2 deleterious contribution. It is noteworthy that these pleiotropic effects of etanercept endorse the idea that etanercept interacts with a pivotal common endothelial effector (which is yet to be identified). The benefits of the TNF-α inhibitor on endothelial function occurred independently of its impact on CV risk factors. From a therapeutic point of view, this suggests that etanercept could be a good choice in RA patients at high risk of CV events. Further studies are warranted to determine whether the observed results are specific to etanercept or likely reflect a class effect of anti-TNF-α agents.

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Supplementary data

Supplementary data are available at Rheumatology Online.

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