EXTENDED REPORT

Increased inflammatory activity parallels increased basal nitric oxide production and blunted response to nitric oxide in vivo in rheumatoid arthritis

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Background: Endothelial dysfunction, defined as loss of bioactivity of NO in the vessel wall, is thought to precede atherosclerosis.

Objective: To determine whether endothelial dysfunction characterises patients with RA and whether these patients have increased inducible nitric oxide synthase (iNOS) dependent NO production in vivo.

Methods and results: Twenty patients with RA and 33 normal subjects received intrabrachial artery infusions of endothelium dependent (acetylcholine (ACh)) and independent (sodium nitroprusside (SNP)) vasodilators to determine arterial responsiveness to NO. Basal flow and its percentage decrease by NG-monomethyl-L-arginine (L-NMMA), an inhibitor of both iNOS and endothelium dependent NOS (eNOS), was used to determine the contribution of iNOS and eNOS dependent NO to basal flow. Both SNP ($p < 0.01$) and ACh ($p < 0.05$) increased blood flow significantly less in patients with RA than normal subjects. Serum concentrations of TNF α were, within the RA group, inversely correlated with blood flow responses to both SNP ($r = -0.67$, $p = 0.002$) and ACh ($r = -0.64$, $p < 0.005$). Basal flow was significantly increased in RA and correlated within this group with serum CRP ($r = 0.48$, $p < 0.05$), TNF α ($r = 0.61$, $p < 0.01$) concentrations, and ESR ($r = 0.68$, $p < 0.002$). L-NMMA decreased basal flow significantly more ($-34 \pm 2\%$) in the patients with RA than the normal subjects ($-24 \pm 3\%$, $p < 0.02$), suggesting in vivo of the blunted response to ACh, increased iNOS activity.

Conclusions: Patients with RA have a dual abnormality in NO dependent vascular function. Basal blood flow is increased in proportion to inflammatory activity and more inhibited by L-NMMA, suggesting increased iNOS activity, and responsiveness to NO is reduced.

Cardiovascular diseases have recently become the main cause of excessive mortality of patients with rheumatoid arthritis (RA).^{1–5} Because of some similarities between inflammation/autoimmune diseases and atherosclerosis, it has been suggested that inflammatory mediators may contribute to vascular dysfunction in patients with RA.⁶ In vitro, proinflammatory cytokines such as tumour necrosis factor α (TNF α) and interleukin 1 (IL1) induce endothelial activation, as measured by an increase in the expression and release of soluble E-selectin from endothelial cells.⁷ In human veins, local administration of TNF α and IL1 β increase basal nitric oxide (NO) dependent venodilatation but impair endothelium dependent venodilatation induced by bradykinin.⁸

Indirect measurements of NO production in patients with RA have suggested that the production of endogenous NO is increased owing to activation of the inducible form of nitric oxide synthase (iNOS). Serum concentrations of nitrite,^{9–10} nitrosoprotein¹¹ and 3-nitrotyrosine,¹² urinary nitrite excretion,¹³ and nitrate-creatinine ratios¹⁴ have been reported to be increased in RA. iNOS has been found to be overactive in circulating monocytes^{15–16} and ex vivo cultures of inflammatory synovium and cartilage.¹⁷ NO production is suppressed by NG-monomethyl-L-arginine (L-NMMA), which inhibits both endothelial NOS (eNOS) and iNOS.¹⁷ Interestingly, an increase in iNOS activity inhibits eNOS activity and responses to endothelium dependent vasodilators such as acetylcholine (ACh) in experimental models of sepsis,^{18–19} but whether this^{20–21} occurs in RA is unknown.

In this study we wished to determine whether inflammation in patients with RA is associated with alterations in NO production in vivo. In view of the above data, we suggested that although iNOS activity may be enhanced, eNOS may be

blunted. To distinguish between the two sources of NO, we determined the blood flow response to the endothelium dependent agonist ACh, which does not stimulate NO production by iNOS.¹⁹ The vasodilatory response to this agonist was thus used as a measure of endothelial function. The contribution of iNOS and eNOS to basal vascular tone was determined from the ability of an intrabrachial artery infusion of L-NMMA to decrease blood flow.²² Our results suggest that endogenous production of NO is enhanced in proportion to the degree of inflammation in patients with RA owing to enhanced iNOS activity while endothelium dependent vasodilatation is blunted.

SUBJECTS AND METHODS

Subjects

A total of 53 subjects were studied, 20 with RA and 33 normal subjects. Table 1 shows the clinical and biochemical characteristics of the subjects. The patients fulfilled the 1987 American College of Rheumatology (ACR) criteria for RA.²³ Fourteen patients were seropositive, and 11 had erosive disease. The patients were recruited from the outpatient clinic of the division of rheumatology by ML-R and the normal subjects by newspaper advertisement. None of the patients or normal subjects had hypertension and none of the normal subjects

Abbreviations: ACh, acetylcholine; ANOVA, analysis of variance; CRP, C reactive protein; eNOS, endothelial nitric oxide synthase; ESR, erythrocyte sedimentation rate; IL, interleukin; iNOS, inducible nitric oxide synthase; L-NMMA, NG-monomethyl-L-arginine; NO, nitric oxide; RA, rheumatoid arthritis; NSAIDs, non-steroidal anti-inflammatory drugs; SNP, sodium nitroprusside; TNF α , tumour necrosis factor α .

Table 1 Clinical and biochemical characteristics of the study groups. Data are shown as mean (SE)

	RA	Normal subjects
Sex (female/male)	17/3	27/6
Age (years)	53 (2)	54 (1)
Weight (kg)	69 (4)	74 (2)
Body mass index (kg/m ²)	26 (1)	26 (1)
Waist/hip	0.84 (0.02)	0.86 (0.02)
% Fat	32 (1)	34 (1)
S-IL6 (pg/ml)	12 (1)***	3 (1)
S-TNF α (pg/ml)	2.1 (0.3)***	1.2 (0.1)
ESR (mm/1st h)	41 (6)**	8 (1)
S-CRP (mg/l)	30 (6)**	4 (1)
S-LDL cholesterol (mmol/l)	3.16 (0.21)*	3.85 (0.15)
S-HDL cholesterol (mmol/l)	1.53 (0.08)	1.44 (0.08)
S-Triglycerides (mmol/l)	1.33 (0.13)	1.24 (0.10)
Current smokers	5/20 (25%)	12/33 (36%)
Number of tender joints	13 (2)	–
Number of swollen joints	11 (2)	–

*p<0.05, **p<0.01, ***p<0.001, for patients with RA v normal subjects.

RA, rheumatoid arthritis; IL6, interleukin 6; TNF α , tumour necrosis factor α ; ESR, erythrocyte sedimentation rate; CRP, C reactive protein; LDL, low density lipoprotein; HDL, high density lipoprotein.

used any drugs. None of the participants had a history of cardiovascular disease. Of the patients with RA, 10 were newly diagnosed and were studied before they had used any disease modifying drugs. The other 10 patients (mean (SD) duration of RA 16 (3) years) were treated with various disease modifying antirheumatic drugs, in eight patients combined with low dose (<10 mg daily) prednisone. Seven patients used non-steroidal anti-inflammatory drugs (NSAIDs). Before the endothelial function tests, the patients were instructed not to take acetylsalicylic acid or other NSAIDs for one week.

Design

Vascular function was measured in each subject after an overnight fast as detailed below. Before the vascular function study, venous blood samples were taken for measurement of serum lipids, IL6, TNF α , and C reactive protein (CRP) concentrations, and the erythrocyte sedimentation rate (ESR).

The purpose, nature, and potential risks of the studies were explained to the patients before their written informed consent was obtained. The experimental protocol was approved by the Ethics Committee, Department of Medicine, Helsinki University Central Hospital.

Vascular function

Vascular function was assessed in forearm resistance vessels by measuring forearm blood flow responses to intra-arterial infusions of endothelium dependent (ACh) and independent (sodium nitroprusside (SNP)) vasodilators and of L-NMMA, an arginine analogue which blocks generation of NO by both iNOS and eNOS,²⁴ as previously described in detail.²⁵ Because ACh stimulates NO production via eNOS but not iNOS,¹⁹ the blood flow response to ACh was used as a measure of endothelial function. The percentage decrease in blood flow below basal by infusion of L-NMMA reflects the contribution of NO produced both via iNOS and eNOS to basal flow.^{18, 24}

The study was begun after a 10–12 hour fast at 7.30 am. An indwelling cannula was inserted in an antecubital vein for blood sampling. A 27 G unmounted steel cannula (Coopers Needle Works, Birmingham, UK), connected to an epidural catheter (Portex, Hythe, Kent, UK), was inserted into the left brachial artery. All drugs were infused at a constant rate of 1 ml/min with infusion pumps (Braun AG, Mesungen, Germany). Subjects rested supine in a quiet environment for 30 minutes after needle placement before blood flow measurements were begun. Normal saline was first infused for 18

Figure 1 Blood flow responses (flow in experimental/control arm) to intrabrachial artery infusions of low (3 µg/min) and high (10 µg/min) doses of SNP and low (7.5 µg/min) and high (15 µg/min) doses of ACh in patients with RA (closed bars) and the normal subjects (open bars). *p<0.05, **p<0.01 for difference between groups by ANOVA.

minutes. Drugs were then infused in the following sequence: SNP (Nitropress, Abbott Labs, North Chigaco, IL) 3 (low dose) µg/min and 10 (high dose) µg/min, ACh (Miochol, OMJ Pharmaceuticals, San Germán, P.R.) 7.5 (low dose) and 15 (high dose) µg/min and L-NMMA (Clinalfa, Läufelfingen, Switzerland) 4 µmol/min. Each dose was infused for six minutes, and the infusion of each drug was separated by infusion of normal saline for 18 minutes, during which blood flow returned to basal values. Forearm blood flow was recorded for 10 seconds at 15 second intervals during the last three minutes of each drug and saline infusion period with a mercury-in-rubber strain gauge venous occlusion plethysmograph (EC 4 Strain Gauge Plethysmograph, Hokanson, Bellevue, WA), which was connected to a rapid cuff inflator (E 20, Hokanson), an analogue to digital converter (McLab/4e, AD Instruments Pty Ltd, Castle Hill, Australia) and a personal computer, as previously described.²⁵ Blood flow measurements were performed simultaneously in the infused (experimental) and control arm. Means of the final five measurements of each recording period were used for analysis. Blood pressure and heart rate were measured before and after the endothelial function test. Blood flow results during infusion of the vasodilators SNP and ACh are reported as a ratio of blood flow in the experimental arm divided by the blood flow in the control arm to correct for any differences in basal flow.²⁶

Other measurements

Serum TNF α and IL6 concentrations were measured using enzyme linked immunosorbent assay (ELISA) kits from R&D Systems (Minneapolis, Minnesota). Serum total and high density lipoprotein (HDL) cholesterol, and triglyceride concentrations were measured with respective enzymatic kits from Roche Diagnostics using an autoanalyser (Roche Diagnostics Hitachi 917, Hitachi Ltd, Tokyo, Japan). Whole body fat and fat-free mass were measured by a single frequency bioelectrical impedance device (model BIA-101A, Bio-Electrical Impedance Analyser System, Mt Clemens, MI).

Figure 2 Interrelationships between markers of inflammatory activity and basal flow (panels on the left) and responses to ACh or SNP (panels on the right) in patients with RA.

Statistical analyses

Group effects of blood flow responses (flow in experimental *v* control arm) were analysed by repeated measures analysis of variance (ANOVA) as described by Ludbrook *et al.*²⁷ Correlation analyses were calculated using Spearman's non-parametric rank and Pearson's correlation coefficient for non-normally and normally distributed data. The calculations were performed using the GraphPad Prism version 3.0 statistical program (GraphPad, San Diego, CA) or with Systat version 10.0 (SPSS, Evanston, IL). All data are shown as mean (SEM). A *p* value less than 0.05 was considered significant.

RESULTS

Physical and biochemical characteristics

Table 1 shows the physical and biochemical characteristics of the patients. The groups had similar age, weight, proportion of men and women, and body composition. The patients with RA had higher serum IL6, CRP concentrations, and a raised ESR compared with the normal subjects. The patients with RA had slightly lower low density lipoprotein (LDL) cholesterol concentrations than the normal subjects.

Endothelial function

During infusion of the endothelium independent vasodilator SNP, blood flow increased less ($p < 0.01$, ANOVA) during the low (4.1 (0.3) *v* 5.4 (0.4)) and high (5.4 (0.5) *v* 6.9 (0.5), flow in the experimental *v* control arm) doses in the patients with RA than in the normal subjects (fig 1). During infusion of the endothelium dependent vasodilator ACh, the blood response was also significantly ($p < 0.05$, ANOVA) blunted in the patients with RA compared with the normal subjects (fig 1). These defects in vascular function remained unchanged even if patients using corticosteroids were excluded (ACh low and

high dose: 3.0 (0.4) and 5.1 (0.5), $p < 0.05$ *v* normal subjects, SNP low and high dose: 3.8 (0.3) and 4.8 (0.4), $p < 0.05$ *v* normal subjects).

Serum TNF α ($r = -0.67$, $p < 0.002$) and CRP ($r = -0.48$, $p < 0.05$) but not IL6 or ESR were inversely correlated with the vasodilatory response to the low dose of SNP in the patients with RA. Serum TNF α ($r = -0.64$, $p < 0.005$) but not CRP, IL6, or ESR was inversely related to the blood flow response to the high dose of ACh. Figure 2 shows examples of these relationships within the RA group. In the normal subjects, serum TNF α but not CRP, IL6 or ESR, was inversely correlated with the blood flow response to the high ($r = -0.50$, $p < 0.01$) and low ($r = -0.36$, $p < 0.05$) dose of ACh. Serum TNF α was also inversely related to the blood flow response to the high dose of SNP ($r = -0.38$, $p < 0.05$).

Basal flow and effects of L-NMMA on basal flow

Basal blood flow was 40% higher in the patients with RA (2.5 (0.3) ml/dl.min) than the normal subjects (1.8 (0.1) ml/dl.min, $p < 0.05$). Both CRP ($r = 0.48$, $p < 0.05$), TNF α ($r = 0.61$, $p < 0.01$), and ESR ($r = 0.68$, $p < 0.002$), but not IL6, correlated significantly with basal blood flow in the patients with RA (fig 2). Systolic blood pressures were comparable in the patients with RA (137 (5) mm Hg) and normal subjects (135 (4) mm Hg), while diastolic blood pressure was slightly lower in the patients with RA (76 (2) mm Hg) than in the normal subjects (81 (1) mm Hg, $p = 0.064$). Consequently, peripheral vascular resistance (mean arterial pressure divided by blood flow) was lower in the patients with RA (47 (5) mm Hg/(ml/dl.min)) than in the normal subjects (66 (6) mm Hg/(ml/dl.min), $p < 0.05$).

During inhibition of NO synthesis (by both iNOS and eNOS) by infusion of L-NMMA, blood flow decreased

significantly more (−34 (2)%) in the patients with RA than in the normal subjects (−24 (3)%, $p < 0.02$).

DISCUSSION

We examined the integrity of NO dependent vasoregulation in patients with RA. We found that basal blood flow and its decrease by L-NMMA was increased compared with a group of healthy control subjects. In contrast, the vasodilatory responses to both SNP (an endothelium independent exogenous NO donor) and ACh (stimulates eNOS and endothelium dependent production of NO) were blunted in the patients with RA. The increase in basal flow was positively, and the responses to SNP and ACh inversely, correlated with inflammatory markers. Together these data suggest that iNOS activity was increased and the responsiveness to NO in the vessel wall impaired. Although correlations do not prove causality, the relationships of the vascular responses with the inflammatory markers suggest that inflammation might have contributed to the observed alterations.

In a clinical study of patients with active disease, it is unethical and impossible to withdraw drugs, which potentially could influence vascular responses, for prolonged periods. However, before the endothelial function tests the patients were instructed not to take acetylsalicylic acid or other NSAIDs for one week, and recent studies would suggest that neither naproxen²⁸ nor acetylsalicylic acid²⁹ alters vascular responses to the drugs infused in the present study. The rather close correlations between the circulating inflammatory markers and vascular responses further suggest that disease activity rather than the drugs was responsible for the alterations observed. Other evidence also supports the idea that inflammatory activity rather than drugs is responsible for the increase in cardiovascular complications in RA. A retrospective study of patients with RA who were followed up since diagnosis showed that high inflammatory activity predicted subsequent cardiovascular events.³⁰ In a recent prospective study, patients with RA had raised concentrations of markers of endothelial activation, including von Willebrand factor, D dimer, and PAI-1 antigen. All these markers correlated with the ESR,³¹ a finding resembling that found in the present study (fig 2). PAI-1 antigen and D dimer concentrations also correlated with cumulative disease activity.³¹ Active use of disease modifying antirheumatic drugs, especially methotrexate, has been shown to decrease cardiovascular mortality particularly.³²

Although there is no “gold standard” for assessing endothelial function, impaired vasodilatory responses to ACh or shear stress are considered early abnormalities in vascular function preceding atherosclerosis.³³ Vasodilatory responses to ACh are blunted in patients with cardiovascular risk factors^{34–35} and predict cardiovascular events.^{36–37} Because ACh does not increase iNOS production,¹⁹ a blunted vasodilatory response suggests impaired synthesis of NO by eNOS or accelerated NO destruction. Because the vasodilatory response to SNP was also blunted, the latter possibility appears the correct one. Although accelerated destruction would also apply to iNOS derived NO, its rate of production was sufficient to result in a net iNOS dependent overproduction of NO.

The finding of increased basal flow and the fraction of basal flow which is inhibited by L-NMMA in vivo is consistent with several reports of increased NO production by various indirect measurements in vitro.^{10–12 14–17 38–41} We found that TNF α , CRP, and ESR correlated with basal flow, in keeping with a previous study demonstrating a significant positive relationship between CRP and the number of iNOS positive mononuclear cells in synovial fluid samples.³⁸ The serum concentration of TNF α has been shown to correlate with enhanced mitochondrial radical production in patients with RA.⁴² The activity of iNOS of freshly isolated blood mononuclear cells has also been shown to correlate significantly with disease activity.¹⁶

The findings in the patients with RA are reminiscent of those in sepsis, during which NO production by iNOS is markedly increased while the response to ACh, which stimulates eNOS,¹⁹ is blunted.^{18 19} Attenuation of the endothelial vasodilatory response to ACh can be restored by specific inhibitors of iNOS such as L-NG-(1-iminoethyl)-lysine.¹⁸

We considered the possible mechanisms underlying the blunted vasodilatory responses to ACh and SNP. We found that serum concentrations of TNF α were inversely correlated with responses to both agents. Local administration of TNF α and IL1 β but not IL6 alone attenuates bradykinin and arachidonic acid induced dilatation in human veins precontracted with norepinephrine, implying that both TNF α and IL1 β can acutely cause endothelial dysfunction.⁸ One possible mechanism underlying the effect of TNF α is reduction of the half life of the mRNA encoding eNOS.⁴³ TNF α is thus one potential cause of endothelial dysfunction in patients with RA. However, this would not explain the blunted response to the exogenous NO donor SNP. Another possibility, which appears to characterise at least experimental arthritis, is that excessive NO production by iNOS results in formation of excessive amounts of superoxide, and peroxynitrate from superoxide and nitric oxide.⁴⁴ The latter reaction could reduce the amount of NO reaching vascular smooth muscle cells during stimulation of endogenous NO production from endothelial cells by ACh and that liberated from SNP. Superoxide production from multiple cellular sources is increased in RA, correlated with serum concentrations of TNF α ,⁴² and thought to perpetuate the chronic inflammatory state.⁴⁴ Levels of nitrotyrosine, a marker of peroxynitrate formation, are increased in the synovial fluid of patients with RA.¹² Superoxide has been shown to increase TNF α production in human monocytes in a dose dependent fashion⁴⁵—that is, superoxide formation may precede increases in TNF α . Regardless of the sequence of events, an increase in superoxide could limit the ability of endogenous and exogenous NO to induce vasodilatation.

To conclude, the present data demonstrate blunted vasodilatory response to NO in patients with RA in the forearm vascular bed. Given that a blunted blood flow response, measured as in this study, has been shown to predict future cardiovascular disease,^{36 37 46} the observed vascular dysfunction might be an early sign of atherosclerosis in patients with RA.

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