Increased serum N^G-hydroxy-L-arginine in patients with rheumatoid arthritis and systemic lupus erythematosus as an index of an increased nitric oxide synthase activity

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Abstract

Objectives—To determine the feasibility of monitoring the serum concentration of N^G-hydroxy-L-arginine (L-NHA) as an index of an increased nitric oxide (NO) synthase activity in patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) compared with nitrate (NO₃⁻), the major circulating metabolite of NO whose concentration is influenced by dietary intake.

Methods—The serum concentrations of L-NHA, L-arginine (L-Arg), and NO₃⁻ were determined in 33 patients with RA, 25 patients with SLE and, 29 healthy subjects.

Results—Serum L-NHA was significantly increased in RA patients with high disease activity (287% of control, $p < 0.01$), but not with low disease activity (115%), as well as in patients with SLE (173%, $p < 0.01$). In contrast, serum NO₃⁻ did not differ significantly between either group of patients and the respective control group.

Conclusion—NO synthase activity or expression, or both, is upregulated in RA patients with high disease activity and in patients with SLE. Serum L-NHA seems to be a more specific and reliable index of an increased activity of this enzyme in patients with acute or chronic inflammatory diseases than NO₃⁻.

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There is now substantial evidence suggesting a role for nitric oxide (NO) in the pathogenesis of rheumatoid arthritis (RA). Increased production of nitrite (NO₂⁻) and nitrate (NO₃⁻), as an index of NO formation, has been reported in several animal studies of adjuvant induced arthritis, an experimental immunopathy that is believed to share many features with human RA.^{1,2} Moreover, suppression of NO generation by in vivo administration of NO synthase inhibitors, such as N^G-monomethyl-L-arginine (L-NMA), attenuates or inhibits the development of the disease, whereas supplementation with the NO precursor L-arginine (L-Arg) results in an exacerbation of the inflammatory process.^{3,4} Increased concentrations of NO₂⁻ or NO₃⁻, or both,⁵⁻⁷ and nitrotyrosine⁸ have also been detected in the serum and synovial fluid^{5,6,8} or urine⁷ of patients with RA. In this context, however, it is

important to note that the single determination of the concentration of NO₂⁻ does not provide a reliable index of endogenous formation of NO because of the rapid conversion into NO₃⁻, the predominant circulating metabolite of NO in the body. Moreover, the concentration of both compounds is strongly influenced by dietary intake and hence derived to a large extent from NO synthase independent sources.⁹

In endotoxin treated rats, the NO synthase metabolite N^G-hydroxy-L-arginine (L-NHA) is released from NO producing cells and accumulates in the circulating blood,¹⁰ presumably because of a competition of the amino acid with the circulating L-Arg for the y⁺ amino acid transport system in vascular cells, hence limiting its metabolism.¹¹ As there is no source other than NO synthase known to produce L-NHA in the body, we have proposed that monitoring the serum concentration of this amino acid may represent a specific and thus more reliable index of an increased NO synthase activity than serum NO₂⁻ or NO₃⁻, or both, in patients with acute or chronic inflammatory diseases.

To substantiate this hypothesis, we have determined the concentration of L-NHA and L-Arg by high performance liquid chromatography (HPLC) analysis in serum samples from patients with RA and systemic lupus erythematosus (SLE) as well as in healthy subjects. In addition, we have determined the concentration of NO₃⁻ in these serum samples by using the nitrate reductase assay.

Methods

PATIENTS

Serum samples were obtained from 33 patients with RA (19 women, 14 men) and 25 female patients with SLE that fulfilled at least four of the seven American College of Rheumatology 1987 criteria for the classification of rheumatic diseases. Exclusion criteria were inflammatory diseases other than RA or SLE. In addition, blood samples were obtained from 29 healthy subjects (15 women, 14 men). All patients were attending a rheumatology outpatient department. The group of RA patients was divided into two subsets, one comprising patients with RA of low (RA-) and one group with high inflammatory activity of the disease (RA+), the latter defined by involvement (swellings or tenderness, or both) of four or more joints as well as C reactive protein (CRP)

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> 15 mg/l or an erythrocyte sedimentation rate (ESR) > 30 mm/1st h, or both. Of the SLE patients, 23 displayed moderate disease activity and two were graded as mild cases.¹² Some of the patients received non-steroidal anti-inflammatory drugs, whereas the majority received second line antirheumatic drugs including corticosteroids, methotrexate, and azathioprine.

HPLC ANALYSIS

Blood samples were centrifuged at $1300 \times g$ for seven minutes and serum was stored at -20°C . Before HPLC analysis, 1.5 ml serum was mixed with 1.5 ml 500 mM borate buffer (pH 4.1) and 1 pmol L-[³H]lysine (Amersham, specific activity 2.92 TBq/mmol) and passed through a cation exchange cartridge (Merck LiChrolut SCX 200 mg). The cartridge was subsequently washed with double distilled water and 10 mM sodium acetate (pH 4.5), and the adsorbed basic amino acids were eluted with 1 ml of 200 mM sodium acetate (pH 8.5). Reproducibility of the extraction procedure was monitored by determination of the recovery of L-[³H]lysine by β liquid scintillation counting. An aliquot of the eluate (40 μl) was then subjected to pre-column derivatisation with *o*-phthalaldehyde (Sigma, 10 μl) followed by HPLC/fluorescence detection analysis.^{10,11} The HPLC column (Merck LiChrospher 100, RP-18 endcapped, 125×40 (id) mm) was isocratically eluted with 10 mM KH_2PO_4 , pH 5.85/acetonitrile/methanol/tetrahydrofuran 80:9.5:9.5:1 (v/v/v/v) at a flow rate of 1 ml/min. L-NHA and L-Arg were eluted from the column with retention times of 13.1 and 14.5 minutes, respectively. The amount of each amino acid in the sample was calculated on the basis of the integrated peak area relative to those of the authentic standards (50 pmol) and the overall recovery, which had been individually determined for each amino acid before. Interassay and intra-assay variability was below 10%. The serum concentration of NO_3^- was assessed using the nitrate reductase method.¹³

STATISTICAL ANALYSIS

Unless indicated otherwise (RA+ group), data analysis was performed by two sided Student's *t* test for unpaired data.

Results

Table 1 summarises the demographic and relevant clinical data of the patients. In the group of patients with RA, the Ritchie articular

Figure 1 Serum concentration of N^ω-hydroxy-L-arginine (L-NHA) in the groups of patients with RA of high inflammatory activity (RA+, n=12) and low inflammatory activity (RA-, n=21), in the group of patients with systemic lupus erythematosus (SLE, n=25), and in the respective control groups (Con). The figure depicts the individual concentrations of each patient or control subject, as determined by HPLC analysis, and the means (SD). Only in the RA+ group did the L-NHA concentrations not follow a Gaussian distribution, so that the non-parametric Mann-Whitney U test instead of the Student's *t* test was used for statistical analysis.

index¹⁴ for assessment of joint tenderness was 27.4 (2.1) (mean (SEM), range 15-46) for the RA+ subset and 8.3 (0.7) (range 0-12) in the RA- group. L-NHA and L-Arg were detected in the serum samples from both healthy subjects and patients. Their presence was confirmed indirectly by co-elution with the authentic standards on two additional HPLC columns (Merck LiChrospher 60 RP-select B and HPLC Technology UltraTechsphere 5-ODS) and, in addition, by gas chromatography mass spectrometry analysis (EI mode) of their pentafluoropropionic anhydride methyl ester derivatives (not shown).

The average concentration of L-NHA in serum samples of patients with low inflammatory activity of RA was not significantly increased compared with the age and sex matched RA- control group (table 1, fig 1). Serum concentrations of L-Arg were also not significantly different between the two groups, while that of NO_3^- was significantly lower (table 1). In patients with high inflammatory activity of RA, however, serum L-NHA values were significantly higher than in the RA+ control group (table 1, fig 1), while serum NO_3^- concentrations were not significantly different.

In the group of patients with SLE, which comprised only women, the serum concentra-

Table 1 Serum concentrations of N^ω-hydroxy-L-arginine (L-NHA), L-arginine (L-Arg), demographic and laboratory parameters of patients included in the study

Group	L-NHA (μM)	L-Arg (μM)	NO_3^- (μM)	ESR (mm/1st h)	CRP (mg/l)	Age (y)	Number (f/m)
Control (RA+)	7.0 (1.2)	101.4 (17)	27.6 (3.8)	—	—	44.8 (2.1)	8/4
Patients (RA+)	20.1 (3.3)†	109.7 (5.5)	33.8 (5.1)**	40.7(7.7)*	16.0(3.2)**	54.1 (4.0)	8/4
Control (RA-)	7.3 (0.9)	94.4 (11.3)	28.6 (3.1)**	—	—	40.0 (1.9)*	11/10
Patients (RA-)	8.5 (1.6)	92.6 (5.6)	17.0 (1.6)	17.7(3.1)	9.2(1.7)	53.3 (2.6)	11/10
Control (SLE)	9.3 (1.5)	98.5 (13.8)	22.5 (2.6)	—	—	37.3 (2.7)	15/0
Patients (SLE)	15.9 (1.9)‡	95.9 (5.5)	21.3 (2.4)	14.8(2.0)	ND	35.5 (2.5)	25/0
Normal range	—	—	—	3-8 (m) 6-11 (f)	1-9	—	—

Values are the means (SEM). ESR = erythrocyte sedimentation rate; CRP = C reactive protein; RA+ = rheumatoid arthritis with high inflammatory activity; RA- = rheumatoid arthritis with low inflammatory activity; SLE = systemic lupus erythematosus; ND, not determined. * $p < 0.05$, ** $p < 0.01$ v patients (RA-). † $p < 0.01$ (Mann-Whitney U test) v control (RA+). ‡ $p < 0.01$ v control (SLE).

tion of L-NHA also showed a significant increase compared with the SLE control group (table 1, fig 1). NO_3^- concentrations, on the other hand, were virtually identical (table 1).

Discussion

In this study we present evidence that the serum concentration of L-NHA, an index of an increased NO synthase activity or expression, or both, is significantly raised in patients with RA of high inflammatory activity compared with concentrations found in the serum of healthy subjects or patients with RA of low inflammatory activity. This finding is compatible with earlier observations of an increased concentration of nitrotyrosine in the serum of patients with active RA, but not in the serum of patients without severe inflammation of the joints.⁸ An increased concentration of NO_2^- or NO_3^- , or both, has also been demonstrated in the serum^{5,6} and urine⁷ of patients with RA.

In the group of patients with SLE, L-NHA concentrations were also significantly increased compared with the age matched female control group, suggesting that an increase in NO synthase activity is also associated with the pathogenesis of SLE in humans, as previously predicted from a SLE-like autoimmune disease model in mice.¹⁵

It is important to note that we cannot ascertain to what extent our results were influenced by the antirheumatic medication of the patients at the time of blood sampling. The serum concentrations of L-Arg, however, were virtually identical in all groups of patients compared with the respective control group, thus excluding the possibility that the increase in serum L-NHA was based on renal insufficiency in SLE or the use of non-steroidal anti-inflammatory drugs, or both.

Serum L-NHA and NO_3^- were significantly increased only in patients with RA of high inflammatory activity when compared with the respective control group or RA- patients, mainly because the serum concentration of NO_3^- in the group of patients with RA of low inflammatory activity was significantly lower even than that in the group of healthy subjects. The serum concentration of L-NHA therefore seems to be indeed a sensitive and more reliable parameter to monitor an upregulation of NO synthase activity in the body than that of NO_2^- or NO_3^- alone or the sum of NO_2^- and NO_3^- , which thus far has been determined in patients with rheumatic diseases.

In summary, our findings suggest that the serum concentration of L-NHA is a useful and

reliable index of increased NO synthase activity or expression, or both, in patients with RA and SLE and possibly also in other patients with acute or chronic inflammatory diseases.

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