Short-term effect of anti-TNF-α therapy on nitric oxide production in patients with severe rheumatoid arthritis

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Abstract

Objective

TNF-α increases expression of inducible nitric oxide synthase (iNOS) in macrophages and vascular endothelial cells. Under normal conditions, iNOS activity is very low. However, iNOS activity is stimulated during inflammation by cytokines such as TNF-α and the amount of NO produced by iNOS may be a 1,000-fold greater than that produced by endothelial NOS. Since functional iNOS gene polymorphisms have been associated with susceptibility to rheumatoid arthritis (RA), drugs blocking TNF-α might decrease production of cytotoxic concentrations of NO leading to beneficial effect on RA or its complications. In the present study we investigated whether the infusion of the anti-TNF-α-infliximab may yield a short-term effect altering circulating NO oxidation products in patients with severe RA.

Methods

We investigated 33 RA patients on periodical treatment with infliximab. Serum levels of nitrates, nitrites and NOx (nitrites+nitrates) were determined immediately prior to and after infliximab infusion. Correlation with clinical variables, laboratory markers of inflammation, metabolic syndrome features, adipokines and adhesion molecules was also assessed.

Results

Upon infliximab administration, serum NOx concentrations (μM) decreased significantly ([mean±SD: 15.0±8.8; median: 11.9; interquartile range: 9.2-18.5] before infliximab-time 0 (baseline) and [12.9±6.3; 10.9; 7.8-17.2] after infliximab infusion-time 120 minutes; p=0.03). It was also the case for nitrates (9.8± 8.3; 7.6; 5.5-10.2] before infliximab and [7.5±4.0; 6.6; 5.2-10.0] after infliximab infusion; p=0.008). There was a positive correlation between basal levels of nitrites and leptin concentration prior to infliximab administration. However, no significant correlations between NO oxidation products and clinical or other laboratory variables were found.

Conclusions

Our results show, for the first time, a short-term effect of anti-TNF-α therapy on the levels of nitric oxide production.

Key words

Rheumatoid arthritis, inflammation, nitric oxide, anti-TNF-α antibody-infliximab, cardiovascular morbidity.

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Introduction

A number of potential mechanisms mediated by TNF-α blockers may explain the reduction of high cardiovascular morbidity observed in rheumatoid arthritis (RA) (1). Improvement of insulin resistance in patients who started anti-TNF-α therapy (2), and rapid increase in insulin sensitivity (3) and ghrelin (4) and reduction of resistin serum levels (5) following anti-TNF-α-monoclonal antibody-infliximab infusion have recently been observed in RA. Furthermore, infliximab infusion was associated to rapid improvement of flow-mediated endothelial-dependent vasodilatation (6) and decrease of biomarkers of endothelial dysfunction (7).

Nitric oxide (NO) is the product of conversion of L-arginine to L-citrulline by NO synthases (NOS). Besides neuronal NOS, there are two forms of NOS; endothelial (eNOS), expressed constitutively on the endothelial cells lining the vasculature and inducible NOS (iNOS), expressed only in response to certain inflammatory stimuli (8, 9). TNF-α increases expression of iNOS in macrophages, vascular endothelial and smooth muscle cells. Under normal conditions, iNOS activity is very low. However, iNOS activity is stimulated during inflammation by cytokines such as TNF-α and the amount of NO produced by iNOS may be a 1,000-fold greater than that produced by eNOS (8).

Since functional iNOS gene polymorphisms have been associated with susceptibility to RA (9), drugs blocking TNF-α might decrease production of cytotoxic concentrations of NO leading to beneficial effect on RA or its complications.

Direct *in vivo* measurement of NO is very difficult because of the extremely low levels and its short half-life. However, it can be measured its more stable oxidation products nitrate and nitrite in plasma. When combined measurements of nitrate/nitrite are made, this is described as NOx. NO is oxidized almost exclusively to nitrite, whereas in whole blood in the presence of oxygenated hemoglobin, nitrite is rapidly further oxidized to nitrate. Plasma nitrate is normally much higher than nitrite and almost identical to NOx (8).

To assess whether infliximab administration yields an immediate effect on circulating concentrations of NO oxidation products, we studied the short-term effect of the administration of infliximab on 33 consecutive nondiabetic RA patients on periodical treatment with this TNF-α blocker because of severe disease (4, 5, 7). Moreover, since the presence of inflammation is directly implicated in the development of the metabolic syndrome observed in patients with RA (10), in this study we also investigated whether circulating concentrations of NO oxidation products may correlate with biomarkers of inflammation and metabolic syndrome.

Patients and methods

Patients

We investigated 33 consecutive patients that met the 1987 American College of Rheumatology criteria for RA (11) and that were recruited from Hospital Xeral-Calde, Lugo, Northwest Spain. Information on the characteristics of this Caucasian population was reported elsewhere (12, 13). They formed part of an ongoing study on CV disease in RA (3-7, 14-16).

Each of the RA patients had been switched from traditional disease modifying antirheumatic drugs (DMARDs) to anti-TNF-α -infliximab treatment because of severe and active disease (Disease Activity Score-28 [DAS28] >5.1) (7, 17). In all patients, treatment with a DMARD had been initiated when a diagnosis of RA was made. Prior to anti-TNF-α therapy, patients were required to have been treated with at least two DMARDs including chloroquine, sulphasalazine, gold, methotrexate (at least 15 mg/week), leflunomide, and cyclosporine A (3 mg/kg/day). Infliximab therapy (initial dose of 3 mg/kg) was administered intravenously at 0, 2, 6 weeks and subsequently every 8 weeks. However, in some patients, because of disease severity, the dose was increased to 5 mg/kg and, if deemed necessary, the interval between infliximab infusions was shortened to 6 weeks. At the time of the present study the median duration of infliximab therapy in this series of RA patients was 2 years.

All patients had received treatment

with both non-steroidal antiinflammatory agents and low doses of prednisone (generally 5 mg bid) immediately after disease diagnosis. At the time of the study, each patient was on infliximab 3 or 5 mg/kg given at 6 or 8 weekly intervals (a range of treatment duration, 1-4.5 years), oral methotrexate 15-25 mg weekly with or without chloroquine 250 mg daily, prednisone 2.5-7.5 mg daily and a non-steroidal antiinflammatory agent (naproxen 500-1000 mg or diclofenac 50-100 mg daily). The blood pressure was below 140/90 mmHg in each patient at the time of the study. However, 7 were taking antihypertensive agents (enalapril [n=3]; losartan [n=3]; enalapril and hydrochlorothiazide [n=1]). Four patients were using a statin (simvastatin 20-40 mg daily). Patients with diabetes were excluded. For ethical reasons, patients included in the present study were not randomized to a placebo group. The same procedure has been found acceptable and followed in a recent study on the effect of infliximab therapy on lipid profiles in patients with RA (18). The local institutional committee approved anti-TNF-α therapy and each patient gave informed consent to participate in the study. Neither this study nor previous studies on RA patients receiving periodical treatment with infliximab (3-5, 7, 14, 15) were supported by any pharmaceutical drug company.

Study protocol

As previously reported (3, 5, 7), in each patient a DAS28 (17) was recorded by the same rheumatologist (MAG-G) prior to infliximab infusion (the same day). In all cases, the drug was given at 8am as an intravenous infusion in a saline solution.

Blood samples were taken immediately before and after the intravenous administration of infliximab. This anti-TNF-α monoclonal antibody was given intravenously over a period of 120 minutes (4, 5, 7). The first sample test was considered time 0. The second sample test after the end of the infliximab administration was considered time 120 minutes. None of the patients received any nutrient before and during infusion (all of them were fasting patients).

All measurements were made in the fasting state. Blood samples were taken at 0800 hours for determination of the erythrocyte sedimentation rate-ESR-(Westergren), CRP (latex immuno-turbidimetry, Nephelometer Analyzer II, Dade Behring, Marburg, Germany), lipids (enzymatic colorimetry), plasma glucose and serum insulin (DPC, Dipesa, Los Angeles, CA, USA). We measured serum concentrations of NOx, nitrates and nitrites, immediately prior to (at time 0) and immediately after infliximab infusion (just at the time that the infusion of the drug ended-at time 120 minutes) using Griess reaction method (Cayman Chemical Company, Ann Arbor, MI, USA); sensitivity 2.5 μM; intra-assay and interassay variations 3.4% and 2.7%. Insulin resistance was estimated by the homeostasis model assessment of insulin resistance (HOMA-IR) using the formula = (insulin (μU/ml) x glucose (mmol/l)÷22.5 (3). Also, as previously reported (7), soluble (s) circulating levels of adhesion molecules, intercellular cell adhesion molecule-1 (ICAM-1), ICAM-3, vascular cell adhesion molecule-1 (VCAM-1), E-selectin and P-selectin, and serum ghrelin (4), resistin (5), adiponectin (14) and leptin (19) were measured prior to infliximab infusion.

Subsequently, final blood sampling was performed for determination of NOx, nitrate and nitrite, insulin, glucose, adiponectin, resistin, leptin, ghrelin and adhesion molecules concentrations immediately after the end of infliximab infusion, which was administered over 120 minutes.

Statistical analyses

Results were expressed as mean± standard deviation (SD), median and interquartile (IQ) range, or number (n) (%). The associations between baseline characteristics and NOx, nitrate and nitrite concentrations were assessed by estimating the Spearman correlation coefficient, as NOx, nitrate and nitrite concentrations were not normally distributed. Also, correlation between changes in serum levels of NOx, nitrates and nitrites at time 120 (immediately after the end of infliximab infusion) with changes in other laboratory data following infusion of this drug were analyzed by the Spearman correlation coefficient.

Changes in NOx, nitrate and nitrite concentrations upon infliximab therapy (just prior to infusion at time 0 and immediately after the end of infliximab infusion at time 120 minutes) were evaluated using the Wilcoxon signed-rank test. Statistical significance was accepted at $p<0.05$.

Results

Descriptive data

The baseline-recorded variables in this series of 33 RA patients on periodical treatment with infliximab are shown in Table I. Despite clinical improvement, as reflected by a reduction in the DAS28 score compared to that found prior to the onset of anti-TNF-α-therapy (see above), all except one patient still had active disease (DAS28 >2.6) (20).

Correlations between the basal recorded characteristics and levels of NOx, nitrites and nitrates

No correlations between NOx, nitrite and nitrate concentrations obtained before infliximab administration and the age at the time of the study or at the onset of the disease and disease duration were found. In addition, there were no statistically significant correlations between tender and swollen joints, DAS28, the mean ESR and CRP from disease diagnosis, the ESR, CRP and platelet count at the time of the study or the cumulative prednisone dose and baseline NO oxidation product levels (Table II).

Relationships of basal NOx, nitrate and nitrite concentrations with metabolic syndrome features

There was no correlation between basal (prior to infliximab infusion) levels of NOx, nitrates and nitrites and body mass index of RA patients. It was also the case when correlation between basal concentrations of NO oxidation products and HOMA-IR, basal insulin or glucose was assessed (Table II). Although a positive correlation between basal levels of nitrites and leptin concentrations prior to infliximab administration was found, basal levels of NOx, nitrites and nitrates were not

Table I. Baseline characteristics in 33 RA patients on treatment with anti-TNF-α-monoclonal antibody-infliximab. Results are expressed as mean± standard deviation; median (interquartile range).

Age, years	
At disease onset	43.3 ± 12.0; 42 (37-57)
At the time of the study	55.3 ± 12.8; 55 (46-65)
Women, n. (%)	25 (76)
Disease duration, years	12.3 ± 7.5; 11 (5-16)
Time from the onset of RA to the beginning of infliximab therapy, years	10.0 ± 7.3; 9 (4-15)
Rheumatoid factor positive, n. (%)	30 (91)
Disease activity	
DAS28	4.4 ± 1.1; 4.4 (3.6-5.1)
Swollen joint count, n.	4.8 ± 4.0; 3 (2-7)
Tender joint count, n.	4.1 ± 3.7; 3 (1-6)
VAS patient disease activity	41.2 ± 17.0; 40 (30-50)
CRP at the time of the study, mg/l	14.2 ± 16.0; 5.5 (4.0-23.4)
Mean CRP from disease diagnosis, mg/l	20.9 ± 12.6; 16 (12-28)
ESR at the time of the study, mm/hr	30.2 ± 19.4; 28 (16-39)
Mean ESR from disease diagnosis, mm/hr	36.6 ± 20.8; 31 (23-45)
Platelet count at the time of the study, $x10^9$/l	289.9 ± 81.6; 270 (245-327)
Cumulative prednisone dose, gr	28.72 ± 18.86; 27.45 (10.60-48.25)
Duration of treatment with infliximab (years)	2.5 ± 1.2; 2.0 (1.5-3.5)
Metabolic syndrome features	
Body mass index, kg/m^2	25.4 ± 4.4; 24.0 (22.6-28.9)
Hypertension, n. (%)	7 (21)
Systolic blood pressure, mmHg	120.2 ± 10.9; 120 (115-130)
Diastolic blood pressure, mmHg	73.3 ± 7.1; 75 (70-80)
Glucose, mmol/l	4.85 ± 0.78; 4.88 (4.27-5.28)
Insulin, pmol/l	107.6 ± 69.5; 84.7 (59.7-131.2)
HOMA-IR,μU.mmol/ml.l	3.4 ± 2.3; 2.9 (1.9-4.2)
Total cholesterol, mmol/l	4.97 ± 0.80; 5.12 (4.42-5.49)
HDL cholesterol, mmol/l	1.64 ± 0.31; 1.63 (1.45-1.86)
LDL cholesterol, mmol/l	2.70 ± 0.51; 2.74 (2.33-3.13)
Triglycerides, mmol/l	1.22 ± 0.50; 1.15 (0.98-1.56)
Adiponectin, ng/ml	25790 ± 28122; 15705 (11240-26180)
Resistin, ng/ml	21.9 ± 9.9; 18.8 (15.0-26.8)
Leptin, ng/ml	16.2 ± 15.5; 10.9 (5.3-19.4)
sICAM-1, ng/ml	349.8 ± 103.2; 345.0 (288.4-395.6)
sICAM-3, ng/ml	58.4 ± 15.1; 53.9 (48.6-64.2)
sVCAM-1, ng/ml	1098 ± 370; 1013 (882-1254)
sE-selectin, ng/ml	53.0 ± 27.9; 42.4 (33.5-67.4)
sP-selectin, ng/ml	291 ± 356; 221 (141-316)
ghrelin, pg/ml	896.1 ± 314.8; 861.2 (700.5-879.9)
NOx, μM	15.0 ± 8.8; 11.9 (9.2-18.5)
Nitrates, μM	9.8 ± 8.3; 7.6 (5.5-10.2)
Nitrites, μM	5.3 ± 4.2; 3.9 (1.9-9.0)

DAS: disease activity score; VAS: visual analog scale; BMI: body mass index; HOMA-IR: homeostasis model assessment of insulin resistance; HDL: high-density lipoprotein; LDL: low-density lipoprotein; ICAM-1: intercellular cell adhesion molecule-1; ICAM-3: intercellular cell adhesion molecule-3; VCAM-1: vascular cell adhesion molecule-1. NOx: Nitrites+nitrates.

significantly correlated with basal levels of total cholesterol, HDL and LDL cholesterol, triglycerides, adiponectin, resistin, ghrelin and adhesion molecules (Table II).

Changes in concentrations of NOx, nitrites and nitrates upon infliximab therapy.

Upon infliximab administration, serum NOx concentrations (μM) decreased significantly ([mean±SD: 15.0±8.8; median: 11.9; interquartile range: 9.2-18.5] before infliximab-time 0 (baseline) and [12.9±6.3; 10.9; 7.8-17.2] after infliximab infusion-time 120 minutes; p=0.03). It was also the case for nitrates as a significant reduction in nitrate levels following infliximab infusion was also seen ([mean±SD: 9.8±8.3; median: 7.6; interquartile range: 5.5-10.2] before infliximab-time 0 (baseline) and [7.5±4.0; 6.6; 5.2-10.0] after infliximab infusion-time 120 minutes; p=0.008) (Fig. 1). However, no significant change in nitrite concentration was seen following infliximab infusion ([mean±SD: 5.3±4.2; median: 3.9; interquartile range: 1.9-9.0] before infliximab-time 0 (baseline) and [5.4±4.3; 4.3; 1.9-8.3] after infliximab infusion-time 120 minutes; p=0.98).

Correlation between changes in levels of NO oxidation products at time 120 (immediately after the end of infliximab infusion) with changes in other laboratory data following infliximab infusion

As shown in Table III, the change in NOx concentration following infliximab infusion showed a significant correlation with the change in the levels of nitrates that decreased moderately simultaneously upon infusion of the drug. However, changes in the levels of NO oxidation products following infliximab infusion did not show significant correlations with changes in the levels of adiponectin, resistin, ghrelin, leptin, adhesion molecules or HOMA-IR after the infusion of the drug.

Discussion

NO can be both cytotoxic and anti-cytotoxic, depending on co-signaling, cellular/tissue context, the level of oxidative stress and the NOS isozymes involved. NO can be protective in endothelial cells. However, TNF-α induces production of cytotoxic concentrations of NO by stimulating *de novo* synthesis of iNOS (8, 21). In this study, we have found that TNF-α blockade in patients with severe RA results in decreased circulating NO concentrations. The short-term decrease of NOx and nitrates in serum after infliximab infusion shown in the present report offers a possible explanation for the improvement of endothelial function determined by flow mediated endothelial dependent vasodilatation in RA following the use of the anti-TNF-α monoclonal antibody-infliximab, which was for first time measured using the flow mediated vasodilatation of the brachial artery and published by the authors from Zürich a few years ago (22).

Table II. Association between baseline patient characteristics and levels of NO oxidation products in 33 patients with rheumatoid arthritis.*

Variable	NOx	Nitrates	Nitrites
Age at disease onset	-0.129 (0.48)	0.012 (0.95)	-0.100 (0.58)
Age at study	-0.145 (0.28)	-0.015 (0.94)	-0.158 (0.35)
Disease duration	-0.134 (0.46)	-0.125 (0.50)	-0.005 (0.98
DAS 28	-0.151 (0.40)	-0.124 (0.50)	0.054 (0.77)
Swollen joint count	-0.109 (0.55)	-0.050 (0.78)	-0.023 (0.90)
Tender joint count	-0.149 (0.41)	-0.152 (0.41)	0.10 (0.95)
VAS patient disease activity	-0.216 (0.23)	-0.195 (0.29)	-0.042 (0.82)
CRP at the time of the study	0.058 (0.75)	-0.080 (0.67)	0.265 (0.14)
Mean CRP from disease diagnosis	0.000 (1.00)	-0.090 (0.64)	0.193 (0.31)
ESR at the time of the study	0.044 (0.81)	0.108 (0.56)	0.149 (0.41)
Mean ESR from disease diagnosis	0.075 (0.70)	0.126 (0.51)	0.134 (0.48)
Platelet count at the time of the study	-0.125 (0.49)	-0.172 (0.35)	0.111 (0.54)
Body mass index	0.221 (0.22)	-0.026 (0.89)	0.338 (0.055)
Glucose	-0.009 (0.96)	-0.249 (0.18)	0.184 (0.32)
Insulin	-0.021 (0.91)	-0.142 (0.45)	0.135 (0.47)
HOMA-IR	-0.134 (0.51)	-0.189 (0.35)	0.099 (0.63)
Triglycerides	-0.115 (0.52)	-0.168 (0.36)	0.208 (0.25)
Total cholesterol	-0.150 (0.41)	-0.124 (0.50)	0.049 (0.79)
HDL cholesterol	-0.110 (0.54)	0.164 (0.369)	-0.179 (0.32)
LDL cholesterol	-0.278 (0.12)	-0.333 (0.06)	0.068 (0.71)
Systolic AP	-0.092 (0.61)	-0.256 (0.16)	0.108 (0.55)
Diastolic AP	-0.015 (0.94)	-0.189 (0.30)	0.340 (0.06)
Cumulative prednisone dose	0.532 (0.86)	0.013 (0.94)	0.218 (0.22)
Time from onset to infliximab therapy	-0.127 (0.48)	-0.134 (0.47)	0.035 (0.85)
Adiponectin	0.059 (0.75)	0.294 (0.10)	-0.117 (0.52)
Resistin	0.098 (0.59)	-0.025 (0.89)	0.147 (0.43)
Leptin	0.312 (0.08)	0.137 (0.46)	0.392 (0.02)
Ghrelin	-0.024 (0.90)	0.178 (0.33)	-0.235 (0.19)
sICAM-3	0.185 (0.30)	0.186 (0.31)	0.059 (0.08)
sVCAM-1	-0.011 (0.95)	0.074 (0.69)	0.02 (0.93)
sE-selectin	0.214 (0.23)	-0.055 (0.76)	0.350 (0.05)
sP-selectin	-0.144 (0.42)	0.063 (0.74)	-0.279 (0.12)

*Data are expressed as r (p).

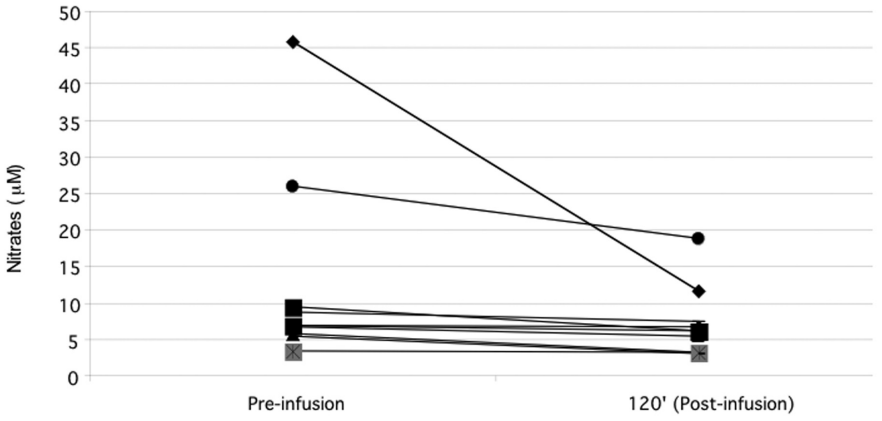

Fig. 1. Change in the serum concentration of Nitrates pre-infusion (at time 0 – immediately before) and post-infusion (at 120' – immediately after the end of the intravenous infliximab therapy). The figure shows 10 out of the 33 randomly selected patients disclosing the decrease of serum nitrate levels following infliximab infusion (after the end of the inflixmab administration at time 120 minutes) compared to the levels obtained immediately prior to the intravenous administration of the drug (considered as time 0).

Cytokines are potent upregulators of cellular adhesion molecule expression on endothelial cells. TNF-α mediates endothelial dysfunction via diminished expression of eNOS and cyclo-oxygenase-1. Also, TNF-α impedes degradation of asymmetric dimethylarginine, the endogenous inhibitor of NOS (23). Moreover, using human umbilical vein endothelial cells in culture, TNF-α has been found to have a direct cytotoxic effect on endothelial cells, causing cell apoptosis (24). Even more importantly in view of our present results, TNF-α also induces production of cytotoxic concentrations of NO by stimulating *de novo* synthesis of the iNOS. This, in turn, has been found to result in further generation of $O2^-$ by macrophages (25) and acts in regulating the nuclear transcription factor, NFκB (21), required for transcription of many cytokines (26) and vascular adhesion molecules (27).

TNF-α produces a myriad atherogenic effects and anti-TNF-α therapy may improve endothelial function via a number of anti-inflammatory mechanisms. Certainly, although the potential mechanisms underlying the rapid but transient improvement of endothelial function following infliximab infusion (6) require further biochemical investigation, it is clear that infliximab binds TNF-α interfering in the cytokine cascade and this effect might provide a pharmacokinetic and dynamic explanation for the vascular improvement (6, 22) and the reduction of cardiovascular mortality mediated by this drug (28).

We have previously described a strong positive correlation (r=0.665, $p<0.001$) between body mass index in RA patients undergoing infliximab therapy because of severe disease and serum levels of leptin in these patients prior to the infusion of infliximab (19). In the present study we have also found a positive correlation between basal levels of nitrites and leptin concentration determined before infliximab infusion. Interestingly, studies on rats have demonstrated that as the body weight of the rats increased, the concentration of both leptin and nitrites and nitrates linearly increased in plasma (29). However, the rate of increase of nitrites-nitrates was 75,000-fold greater than that of leptin in these animals (29). These results suggested a potential role of leptin in the mechanisms of NOS activation (29). On the other hand, more recent studies have shown that leptin promotes iNOS up-regulation (30). Leptin increased the synthesis of iNOS protein and inhibits the contractile response induced by angiotensin II in vascular

Table III. Correlation between the changes in the levels of NO oxidation products with the changes in the other laboratory data at time 120 (immediately after the end of the intravenous infusion of infliximab).*

Changes in the Variable	Changes in NOx	Changes in Nitrates	Changes in Nitrites
NOx	1 (-)	0.428 (0.02)	0.688 (<0.001)
Nitrates	0.428 (0.02)	1 (-)	-0.297 (0.11)
Nitrites	0.688 (<0.001)	-0.297 (0.11)	1 (-)
Resistin	-0.023 (0.90)	0.042 (0.82)	-0.215 (0.23)
Adiponectin	-0.277 (0.12)	-0.379 (0.05)	0.062 (0.73)
Leptin	0.113 (0.53)	0.238 (0.20)	-0.145 (0.42)
Ghrelin	0.078 (0.67)	0.231 (0.21)	-0.085 (0.64)
sE-selectin	-0.027 (0.88)	0.075 (0.69)	-0.008 (0.96)
sP-selectin	-0.056 (0.76)	-0.150 (0.42)	-0.034 (0.85)
sVCAM-1	0.204 (0.25)	-0.046 (0.80)	0.179 (0.32)
sICAM-1	-0.091 (0.61)	-0.063 (0.74)	-0.050 (0.78)
sICAM-3	0.323 (0.06)	-0.007 (0.97)	0.294 (0.10)
HOMA-IR	-0.222 (0.27)	-0.226 (0.28)	-0.020 (0.92)

*Data are expressed as r (p).

smooth muscle cells of the aorta (30). This endothelium-independent depressor action of leptin is mediated by an increase of NO bioavailability in vascular smooth muscle cells that requires the up-regulation of iNOS (30).

There are, however, number of potential limitations of this study that we would like to discuss. First, the decrease in the concentration of nitrates observed immediately after infliximab infusion was significant but moderate. Second, it is known that the normal concentration of these oxidation products have a wide range, so, the differences found in this study should be interpreted with caution. Third, the increase in nitrates is most probably the result of an increased oxidation by hemoglobin in peripheral blood. Moreover, our series of RA patients were under treatment with other drugs (including steroids and other anti-inflammatory agents) that certainly modify the metabolism of NO. Finally, although the main purpose of our study was to establish if TNF-α blockade by the infusion of infliximab was able to yield an immediate decrease in the levels of NO oxidation products and, because of that, determinations were performed immediately before and after the infusion of the drug, it is possible that further determinations performed several weeks after the administration of the drug might yield a more important reduction in the concentration of nitrates. In this regard, Reddy *et al*. described a statistically significant decrease in the median values for serum nitrite after 4 weeks of leflunomide therapy (31), a drug used for the management of patients with RA that has been shown to cause cell specific inhibition of inducible iNOS activation in animal models.

In conclusion, our results show, for first time, the immediate effect of anti-TNF-α therapy on the levels of nitric oxide production**.**

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