Induction of Inducible Nitric Oxide Synthase, Argininosuccinate Synthase, and GTP Cyclohydrolase I in Arthritic Joints of Human Tumor Necrosis Factor-α Transgenic Mice

MIKA HUKKANEN, LOUISE A.M. PLATTS, SYLVA HARALAMBOUS, MARI AINOLA, YRJÖ T. KONTTINEN, GEORGE KOLLIAS, and JULIA M. POLAK

ABSTRACT. ***Objective.*** Transgenic mice that express human tumor necrosis factor-α (Tg197 h-TNF-α) develop polyarthritis at 3 to 4 weeks of age leading to severe joint destruction at 8 to 10 weeks of age. Studies have suggested that inducible nitric oxide synthase (iNOS) activity can modulate the progression of arthritis. We investigated the induction of iNOS together with argininosuccinate synthase (AS) and GTP cyclohydrolase I (GTPCH), 2 of the rate-limiting enzymes for high output NO generation, in the Tg197 h-TNF-α transgenic model of arthritis.

Methods. We used 4 and 8-week-old Tg197 h-TNF-α transgenic mice and wild-type CBA × C57B1/6 control mice to investigate the expression of iNOS with respect to that of AS, GTPCH, and 3-nitrotyrosine by quantitative RT-PCR and immunocytochemistry. Urinary NO metabolites were analyzed using a chemiluminescence assay.

Results. Inducible NOS, AS, and GTPCH mRNA was found in all study groups; however, only iNOS mRNA showed a clear increase in 4-week-old Tg197 h-TNF-α transgenics in comparison to age matched wild-type controls. Abundant iNOS protein expression was found in macrophages and vascular smooth muscle cells in hyperplastic synovium and pannus. AS expression was found in vascular endothelium and fibroblasts of the inflammatory synovium and pannus. GTPCH immunoreactivity was mostly restricted to macrophages in inflammatory synovium. Localization of 3-nitrotyrosine overlapped with that of iNOS, indicating formation of reactive nitrogen species. Consistent with the high output NO generation, there was a 5-fold increase in urinary NO metabolites in 8-week-old Tg197 h-TNF-α transgenic mice.

Conclusion. We characterized the Tg197 h-TNF-α transgenic model of inflammatory arthritis in terms of high output NO-generating pathway, and showed that both AS and GTPCH are intimately associated with inflammatory arthritis. The concomitant induction of AS and GTPCH with that of iNOS suggests that they may be important modulators of arthritis, and that they may represent novel targets for modulation of disease activity. (J Rheumatol 2003;30:652–9)

Key Indexing Terms:
ARGININOSUCCINATE SYNTHASE GTP CYCLOHYDROLASE NITRIC OXIDE
3-NITROTYROSINE TUMOR NECROSIS FACTOR-α

From the Institute of Biomedicine/Anatomy, Biomedicum Helsinki, University of Helsinki; ORTON Research Institute, the Orthopaedic Hospital of the Invalid Foundation, Helsinki; Department of Medicine/Invärtes Medicin, Helsinki University Central Hospital, Helsinki, Finland; Tissue Engineering Centre, Imperial College School of Medicine, Chelsea and Westminster Hospital, London, UK; Laboratory of Molecular Genetics, Hellenic Pasteur Institute, Athens, Greece; and Institute of Immunology, Biomedical Sciences Center "Alexander Fleming," Varkiza, Greece.

Supported by grants from the Academy of Finland, the University of Helsinki, clinical EVO research grants (from the Invalid Foundation and TYH 0056; TYH 0215; TYH 0341; TYH 8307), and the Group of Excellence scheme by the Academy of Finland, the National Technology Agency, Ministry of Education, and University of Helsinki, Finland.

M. Hukkanen, PhD, Associate Professor and Academy Fellow, Institute of Biomedicine/Anatomy, Biomedicum Helsinki, University of Helsinki, and ORTON Research Institute, Orthopaedic Hospital of the Invalid Foundation; L.A.M. Platts, PhD, Research Scientist; J.M. Polak, MD, DSc, FRCP, FRCPath, Professor, Tissue Engineering Centre, Imperial College School of Medicine, Chelsea and Westminster Hospital; S. Haralambous, PhD, Senior Scientist, Laboratory of Molecular Genetics, Hellenic Pasteur Institute; M. Ainola, MSc, Research Scientist, Institute of Biomedicine/Anatomy, Biomedicum Helsinki, University of Helsinki; Y.T. Konttinen, MD, PhD, Professor, Department of Medicine/Invärtes Medicin, Helsinki University Central Hospital, Helsinki, and ORTON Research Institute, Orthopaedic Hospital of the Invalid Foundation; G. Kollias, PhD, Professor, Laboratory of Molecular Genetics, Hellenic Pasteur Institute, Institute of Immunology, Biomedical Sciences Center "Alexander Fleming."

Address reprint requests to Dr. M. Hukkanen, Biomedicum Helsinki, Institute of Biomedicine/Anatomy, PO Box 63 (Haartmaninkatu 8), FIN-00014 University of Helsinki, Helsinki, Finland.
E-mail: mika.hukkanen@helsinki.fi

Submitted February 28, 2002; revision accepted September 10, 2002.

Persistent nitric oxide (NO) production by the cytokine inducible NO synthase (iNOS) has been suggested as one of the contributing factors for the development of inflammatory lesions and joint destruction/deformations seen in rheumatoid arthritis (RA)[1,2] and several animal models of

the disease. In most model systems, induction of iNOS synthesis and high output NO production precede the onset of the clinical hallmarks of the disease such as erythema and joint swelling. NO production has been shown to persist through the chronic phase of the disease, suggesting that NO may be involved not only in the initiation but also in the maintenance of inflammatory arthritides[3]. The animal models that have been characterized in terms of iNOS-driven high output NO production include the streptococcal cell-wall fragment model[4], MRL/lpr mice[5], adjuvant induced arthritis[6], and collagen II induced arthritis[7]. Many reports agree that inhibition of NOS activity can be prophylactic against polyarthritis. However, the use of various animal models of RA and the availability of an array of pharmacological compounds that inhibit NOS enzyme activity have raised much controversy. In these rodent models, the increase in NO typically precedes the onset of the symptoms of inflammatory arthritis. Prophylactic administration of L-NMA or L-NAME (nonselective inhibitors of NOS activity) have been shown to suppress the disease onset[4-6], whereas aminoguanidine (which exhibits selectivity towards iNOS activity) has been shown to have little effect in preventing the development of arthritis[8,9]. L-NIL (a selective inhibitor of iNOS activity) has indeed been shown to aggravate the inflammation associated with the streptococcal cell-wall fragment model[10]. Further, a recent report shows that in homozygous iNOS gene-deficient mice with antigen induced arthritis, the clinical scores of joint inflammation were higher in iNOS mutants compared with their wild-type counterparts[11].

Despite these conflicting reports on the various inhibitors of NOS activity and models of arthritis, NO metabolite concentrations in serum and synovial fluid have been shown to correlate well with the acute flares of the disease[12-14]. Prednisolone, glucosamine, methotrexate, and anti-tumor necrosis factor (TNF-α) therapy have all been reported to reduce endogenous NO synthesis in correlation with amelioration of the disease[13,15-17]. These studies together with those of Sakurai, *et al* and McInnes, *et al*[1,2] have suggested that intraarticular NO production may be a contributory factor for the induction and maintenance of RA.

NO reacts with superoxide at near diffusion-limited rate to form peroxynitrite, a potent oxidant capable of damaging cellular proteins, lipids, and DNA bases[18-21]. NO and peroxynitrite have well documented effects on induction of cyclooxygenase-2 synthesis and activity[22-23]. It follows that interest should also be directed to other complementary pathways that are required for the maintenance of high output NO generation by the activity of iNOS. All 3 recognized NOS isoforms require continuous *de novo* production of L-arginine as a substrate and 5,6,7,8-tetrahydrobiopterin (BH4) as a cofactor for NO generation[24,25]. One of the synthetic pathways for the endogenous L-arginine production is its recycling from L-citrulline. This reaction requires the activity of 2 urea cycle enzymes, namely, argininosuccinate synthase (AS) and argininosuccinate lyase (AL). The synthesis of iNOS and AS mRNA follow a similar time course after immunostimulation with lipopolysaccharide or cytokines such as TNF-α, interleukin 1 (IL-1), and/or interferon-γ (IFN-γ)[26,27]. Additionally, NOS enzyme activity is dependent on the synthesis and availability of BH4 that involves GTP cyclohydrolase I (GTPCH), the first and rate-limiting enzyme in the GTP–biopterin pathway. GTPCH has been shown to be induced in several cell types in parallel with iNOS and AS mRNA as a result of TNF-α, IL-1, and/or IFN-γ stimulation[27,28].

We hypothesized that spontaneous overexpression of TNF-α, as seen in Tg197 h-TNF-α transgenic mice, who characteristically develop chronic polyarthritis with 100% phenotypic penetrance of the disease, leads to increased synthesis and coinduction of iNOS, AS, and GTPCH, together with peroxynitrite induced oxidative damage evidenced by 3-nitrotyrosine (3-NT). For the study, we used 4 and 8-week-old Tg197 h-TNF-α transgenic mice and age matched nontransgenic CBA × C57B1/6 wild-type controls.

MATERIALS AND METHODS

Tg197 human-TNF-α transgenic mice. Transgenic mice carrying and expressing human-TNF-α gene constructs with modified 3' region were developed[29]. The 3' region of the human TNF-α gene containing its 3' untranslated and 3' flanking sequences was replaced with that of the human ß-globin gene[29]. Microinjection of the construct into mouse zygotes resulted in founder transgenic mice, some of which (designated Tg197) developed swelling of the ankle joints and exhibited impaired movement. A male Tg197 (treated with an anti-h-TNF-α antibody) was mated with an F1 female and the litter was grown in a specific pathogen-free environment. The transgenic line of arthritic Tg197 h-TNF-α mice was maintained by breeding into F1 female (CBA × C57B1/6) mice. These Tg197 h-TNF-α mice develop 100% phenotypic penetrance of disease progeny with progressive polyarthritis and impairment of hind leg movement. Human TNF-α expression has been described in the arthritic joints of Tg197 mice[30]. For this study, Tg197 h-TNF-α transgenics were bred and killed at 4 and 8 weeks of age to represent the acute and advanced/chronic stages of inflammatory arthritis.

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated using TRIzol reagent (GibcoBRL, Life Technologies, Paisley, Scotland) from frozen ankle joints of 4 and 8-week-old CBA × C57B1/6 wild-type controls and Tg 197 h-TNF-α mice (n = 6 for all 4 groups). The amount of the RNA was measured spectrophotometrically and the quality was determined with ethidium bromide stained 1% agarose gel under ultraviolet light. Five micrograms of the RNA was DNase treated (Promega, Madison, WI, USA), and 2.5 µg of that was transferred to cDNA with SuperScript Preamplification System using oligo(dT)$_{12-18}$ for priming and RNase H for removal of mRNA according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). cDNA synthesis without enzyme and without sample were used for negative controls.

Quantitative PCR amplification was performed from 0.25 µg cDNA in LightCycler™ SYBR Green I PCR mix by LightCycler™ PCR machine (Roche Molecular Biochemicals, Mannheim, Germany). PCR amplification was performed using 0.25 µM of target-specific primers for ß-actin (accession X03672): sense 5'-CTTCTTTGCAGCTCCTTCGT-3' and antisense 5'-GTGCCAGATCTTCTCCATGT-3', producing a 310 bp band; for iNOS (accession U43428): sense 5'-TCCCAAGTACGAGTGGTTCC-3'

and antisense 5'-TGGTCACATTCTGCTTCTGG-3', producing a 299 bp band; for AS (accession XM-123777): sense 5'-TGGAATGAAGTCCC-GAGGTA-3' and antisense 5'-ACGTTCATGCTCACCAGCTC-3', producing a 315 bp band; and for GTPCH (accession L09737): sense 5'-GGCTGCTTACTCGTCCATTC-3' and antisense 5'-AGCCAATATG-GACCCTTCCT-3', producing a 251 bp band. For primers, corresponding sequences were searched from NCBI Entrez search system and sequence-similarity search was done using the NCBI blastn program. The identity of the PCR product was verified by a melting curve analysis. Serial dilutions of cloned PCR fragments in plasmid DNA were used to determine the copy number of the amplicon per 1000 ß-actin mRNA copies. The amplified PCR fragments were cloned with a TOPO TA cloning kit (Invitrogen) into a pCRII-TOPO vector. Plasmid DNA was isolated with a High Pure Plasmid Isolation Kit (Roche). The concentration of the plasmids was analyzed spectrophotometrically. The plasmid used for quantitative PCR was sequenced using fluorescein labeled dye terminator kits supplied by ABI (Applied Biosystems, Foster City, CA, USA) and analyzed on an automatic 373A sequencer (Applied Biosystems). The acquired sequence was verified with NCBI blastn program. Each individual sample was amplified at least 2 times in all genes. Statistical significance was evaluated by 2 tailed unpaired Student t test between the age matched wild-type and Tg 197 h-TNF-α transgenic groups. A p value < 0.05 was regarded as statistically significant and the data are expressed as mean ± SEM.

Immunocytochemistry and antisera. The hind limbs from 4 and 8-week-old CBA × C57B1/6 wild-type controls (n = 7 for both groups) and Tg 197 h-TNF-α transgenics (n = 9 for both groups) were dissected from the pelvis and immersion fixed in 10% (v/v) formalin in 0.1 M phosphate buffered 0.9% saline overnight (PBS, pH 7.4), and thoroughly washed in PBS prior to decalcification and embedding in Paraplast (BDH Laboratory Supplies, Dagenham, UK). Limbs were oriented so that longitudinal sagittal sections could be cut through the ankle joints. Paraffin sections of 3 µm were cut, captured onto 3,3'aminopropyltriethoxy-silane coated slides, and dewaxed. Endogenous peroxidase activity was exhausted by incubation in methanol with 0.3% hydrogen peroxide for 20 min at room temperature. The sections were washed in PBS for 3 × 5 min and incubated with normal sera as appropriate for the specific primary antibody for 30 min. Sections were then incubated with primary antibodies (details below) overnight at 4°C, washed and incubated with biotinylated goat anti-rabbit or rat anti-mouse antibodies as necessary for 30 min, washed and incubated with a peroxidase labeled avidin-biotin complex (ABC) for 1 h (ABC Elite kit, Vector Laboratories, Peterborough, UK). The immunoreactivity was visualized using a solution of 3,3'diaminobenzidine (DAB) as chromogen with 0.2% hydrogen peroxide in PBS, to provide a brown reaction product, counterstained, dehydrated, cleared, and mounted. Polyclonal antibodies raised against amino acid residues 961–1144 of mouse macrophage iNOS were obtained from Transduction Laboratories (Lexington, KY, USA). Polyclonal antibodies raised against bacterially expressed and enzymatically competent AS and GTPCH were raised by Prof. S.S. Gross, Cornell University, Ithaca, NY, USA[31,32]. Polyclonal antibodies raised against 3-NT were kindly provided by Prof. J.S. Beckman[33]. Rat anti-mouse macrophage (Pharmingen, San Diego, CA, USA) and rat anti-mouse granulocyte (clone 7/4, Serotec, Oxford, UK) antibodies were used in some cases to confirm immunoreactive cell types expressing iNOS, 3-NT, AS, or GTPCH in the arthritic joints of Tg197 h-TNF-α transgenics.

Measurement of NO metabolites NO_2–/NO_3– by chemiluminescence assay. Overnight urine samples were collected from 8-week-old CBA × C57B1/6 wild-type controls (n = 5) and Tg197 h-TNF-α transgenics (n = 5) and stored at 70°C until analysed for the NO metabolites NO_2–/NO_3–. Triplicate 50 µl samples were injected into the purge vessel and urinary NO_2–/NO_3– was converted to NO in a reducing mixture of 0.1 M VCl_3 in 1.0 M HCl at 87°C. NO was detected by the chemiluminescence reaction with ozone generated from pure oxygen using a Sievers NOA 270B NO analyzer (Sievers Instruments, Boulder, CO, USA). Comparisons were made against standard curves generated by known concentrations of sodium nitrate. Statistical significance was evaluated by 2 tailed unpaired Student t test. A p value < 0.05 was regarded as statistically significant and the data are expressed as mean ± SEM.

RESULTS

Nontransgenic CBA × C57B1/6 wild-type control mice showed intact synovial intima and normal articular structure with no evidence of inflammation (Figure 1A). In contrast,

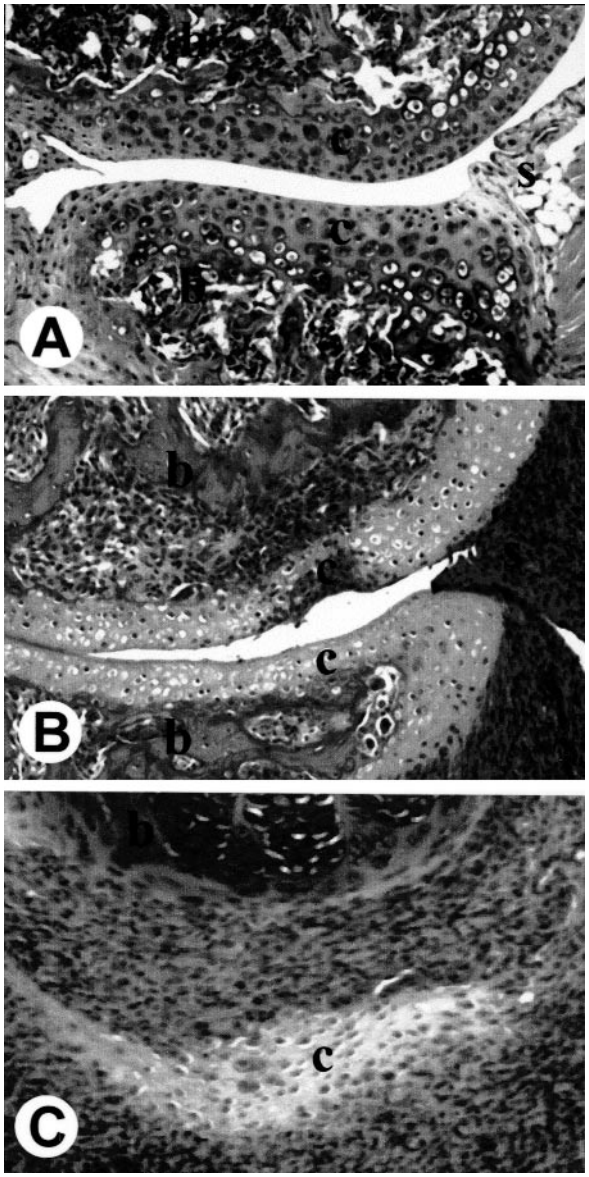

Figure 1. Photomicrographs of 4-week-old CBA × C57B1/6 wild-type control and 4 and 8-week-old Tg197 h-TNF-α transgenic mice showing progressive articular destruction. (A) Histology of normal noninflammatory 4-week-old wild-type ankle joint for comparative purposes. (B) In 4-week-old Tg197 h-TNF-α transgenics there was evidence of cartilage surface erosion, synovial hyperplasia and pannus growth, narrowing of joint cavity, and infiltration of subchondral trabeculae by inflammatory cells and fibrotic tissue growth with reactive bone formation. (C) In more severely affected 8-week-old Tg197 h-TNF-α transgenics, the articular cartilage was almost completely eroded, with extensive invasion and integration by pannus tissue leading to almost complete loss of the articular architecture. b: subchondral bone; c: cartilage; s: synovium. Original magnification ×50.

4 and 8-week-old Tg197 h-TNF-α transgenics had a progressive arthritis characterized by synovial hyperplasia, granulomatous lesions, and extensive erosions of cartilage and subchondral bone (Figures 1B, 1C). In 4-week-old Tg197 h-TNF-α transgenics, the distal tibial epiphysis and associated calcaneous bone were affected with discrete erosions of cartilage and underlying bone, even in the absence of pannus tissue overlying the cartilage (Figure 1B). In contrast, 8-week-old Tg197 h-TNF-α transgenics had massive joint destruction. Synovium was intensely hyperplastic and infiltrated by numerous macrophages and polymorphonuclear cells with lymphocytes dispersed among areas of granulomatous lesions. An extensive pannus formation was evident, with destruction of the underlying cartilage and subchondral bone (Figure 1C).

The copy numbers of iNOS, AS, and GTPCH mRNA from 4 and 8-week-old Tg197 h-TNF-α transgenics are shown in Table 1. Although the copy numbers of all the 3 enzyme mRNA studied seemed to increase in accord with disease progression, only iNOS mRNA showed a statistically significant difference between 4-week-old Tg197 h-TNF-α transgenics and age matched CBA × C57B1/6 wild-type controls (2.00 ± 0.894 vs 18.00 ± 3.759 iNOS mRNA copies/1000 ß-actin mRNA copies; p = 0.0020). However, it was of interest that both AS and GTPCH mRNA copy numbers were substantially higher than those of iNOS in all wild-type and transgenic groups studied (Table 1).

The cellular distribution of the molecules investigated was similar in 4 and 8-week-old Tg197 h-TNF-α transgenics, despite clear differences in the degree of tissue damage. Therefore, the results are discussed as Tg197 h-TNF-α transgenics compared with CBA × C57B1/6 wild-type controls. In wild-type controls, iNOS expression was limited to a few bone marrow cells. In Tg197 h-TNF-α transgenics, many of the infiltrating macrophage-like and some of the polymorphonuclear cells in hyperplastic synovium and pannus were heavily immunoreactive for iNOS (Figures 2A, 2B). Most of these cells were identified as macrophages and granulocytes using mouse-specific antibodies. Many of the macrophages, granulocytes, and lymphocytes infiltrated into the synovial fluid were immunoreactive for iNOS (Figure 2A). Inducible NOS was also found in vascular smooth muscle of the subintimal synovial stroma, together with 3-NT immunoreactivity. Many of the articular chondrocytes and macrophages within both subchondral and deep bone erosions showed intense immunoreactivity for iNOS (Figure 3A). Localization of 3-NT mostly overlapped with that of iNOS, but was also observed in the extracellular matrix, especially in granulomatous lesions surrounded and sometimes infiltrated by macrophages expressing iNOS (Figures 2B, 2C). No immunoreactivity for any of the antigens investigated was seen in control sections, where the primary antibody had been omitted or replaced with an irrelevant antibody of the same IgG class (Figure 3B).

In the CBA × C57B1/6 wild-type controls, AS immunoreactivity was observed in the vascular endothelium, but not in the synovial intima/subintima or cells in the deeper stroma. There was widespread induction of AS in Tg197 h-TNF-α transgenics with arthritis, most notably in fibroblast-like cells in hyperplastic synovium and pannus and macrophage-like cells at sites of subchondral bone erosions (Figures 4A, 4B), with only a partial overlapping with that of iNOS. Intense GTPCH immunoreactivity was evident in macrophages in the intima/subintima and deeper stroma in Tg197 h-TNF-α transgenics, but could not be found in the synovium from the CBA × C57B1/6 wild-type controls (Figures 5A, 5B, 5C).

As the severity of arthritis was seen to progress with age, NO metabolite NO_2–/NO_3– levels were analyzed (as a confirmation of iNOS enzyme activity and NO generation) from overnight urine samples. A significant increase in urinary NO_2–/NO_3– was found in 8-week-old Tg197 h-TNF-α transgenics compared with CBA × C57B1/6 wild-type controls (588.0 ± 194.9 and 114.2 ± 12.6 µM, respectively; p = 0.0415).

DISCUSSION

The development of the Tg197 h-TNF-α transgenic mouse line, which constitutively overexpress intact human-TNF-α protein, has provided a predictive genetic animal model of spontaneous RA[28]. Thus the Tg197 h-TNF-α transgenic line provides a model for investigations of modulators or copromoters of RA. In the Tg197 h-TNF-α transgenic model, TNF-α upregulates IL-1 synthesis with subsequent exacerbation of synovial inflammation, cartilage, and bone erosion, together with IL-1 mediated maintenance of inflammation. Administration of neutralizing antibodies to either TNF-α or IL-1 receptors has been shown to have

Table 1. Results of quantitative reverse transcriptase-polymerase chain reaction analyses.

Group	iNOS	AS	GTPCH
Wild-type, 4 weeks	2.00 (0.894)	128.5 (43.37)	518.8 (148.0)
Tg 197, 4 weeks	18.00 (3.759)*	190.3 (27.51)	514.8 (103.1)
Wild-type, 8 weeks	2.17 (0.401)	59.0 (8.521)	578.8 (194.1)
Tg 197, 8 weeks	25.33 (15.21)	309.2 (135.5)	899.3 (283.0)

Results are normalized and given per 1000 ß-actin copies (mean ± SEM). * p = 0.002.

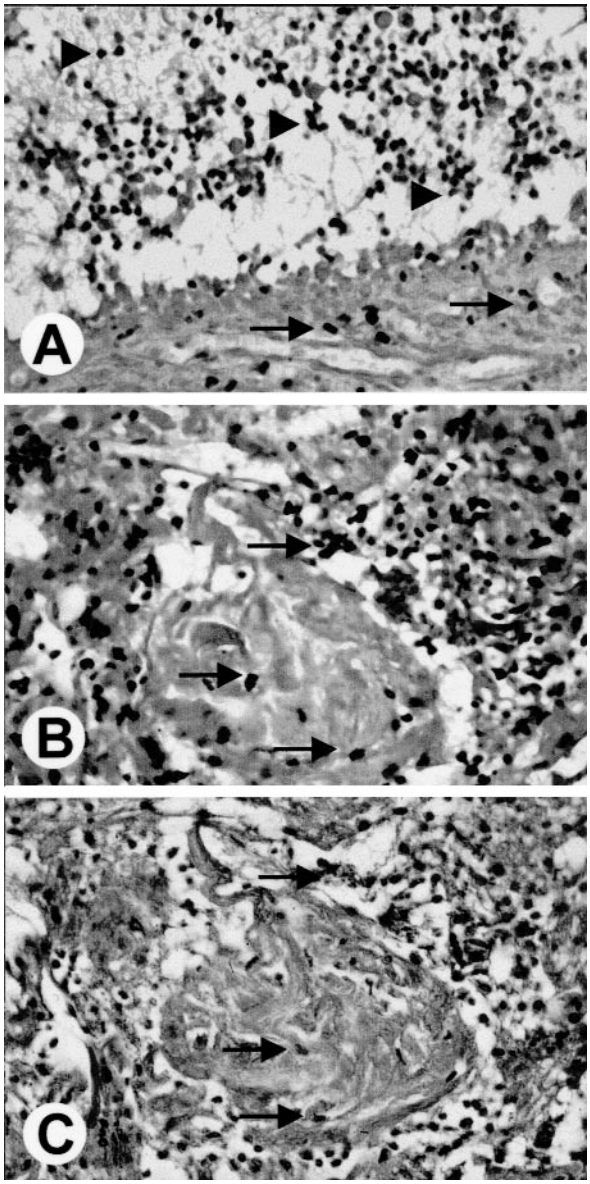

Figure 2. Localization of iNOS and 3-NT in 8-week-old Tg197 h-TNF-α transgenic mice. (A) Many infiltrating macrophages in hyperplastic synovium (arrows) and macrophages, granulocytes, and lymphocytes within the synovial fluid/exudate (arrowheads) were heavily immunoreactive for iNOS (A and B). (B) Fibrin deposition was evident within granulomatous synovial stroma infiltrated predominantly with macrophages that were also immunoreactive for iNOS (arrows). (C) Peroxynitrite induced oxidative damage and nitrosylation of tyrosine groups was detected with antibodies to 3-NT. 3-NT immunoreactivity was extensively localized to the granulomatous extracellular matrix surrounded and infiltrated by iNOS-expressing macrophage-like cells. 3-NT was also localized to some inflammatory cells that appeared to have a similar distribution to those that were characterized by iNOS immunoreactivity (arrows). Original magnification ×100.

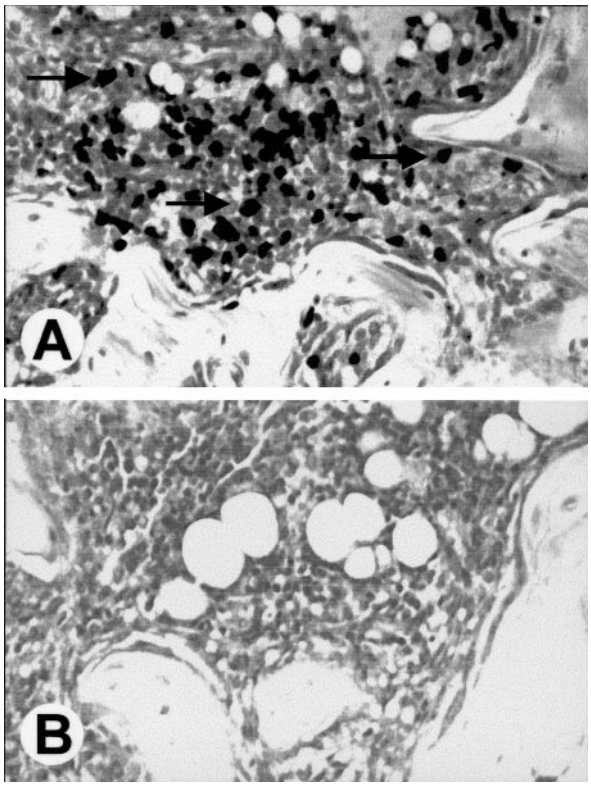

Figure 3. Inducible NOS localization in 8-week-old Tg197 h-TNF-α transgenic bone erosions. (A) Immunoreactivity for iNOS was localized to macrophage-like cells (arrows) within the calcaneous bone erosions. (B) The immunoreactivity was absent when antiserum for iNOS had been replaced with an irrelevant antibody with the same IgG class. Original magnification ×100.

prophylactic effects on the development of the clinical and histopathological hallmarks of RA[29,34,35].

Our study provides evidence that the L-arginine/NO pathway, including AS and GTPCH, the 2 rate-limiting enzymes for high output NO generation, is induced in the inflamed joints of Tg197 h-TNF-α transgenics. We observed that iNOS is found most abundantly *in situ* in macrophages that are involved in destruction of the articular architecture. These cells produced such quantities of NO that peroxynitrite was formed, as shown by the widespread localization of 3-NT. The high urinary NO_2–/NO_3– concentrations provided further evidence of activation of the L-arginine/NO pathway. In 8-week-old Tg197 h-TNF-α transgenics urinary NO metabolites NO_2–/NO_3– were increased 5-fold compared with the nontransgenic wild-type controls. This was further substantiated by the fact that iNOS mRNA increased significantly during the acute phase of the disease. We did not detect clear differences in iNOS, AS, or GTPCH expression and/or their distribution between 4 and 8-week-old Tg197 h-TNF-α transgenics, despite the progressive nature of the disease. Although iNOS is often only transiently induced during the acute flares of inflammation, the constitutive overexpression of h-TNF-α in Tg197 h-TNF-α transgenic joints seems to be sufficient to maintain the activation of iNOS protein for prolonged periods of time and throughout the pathogenesis of joint

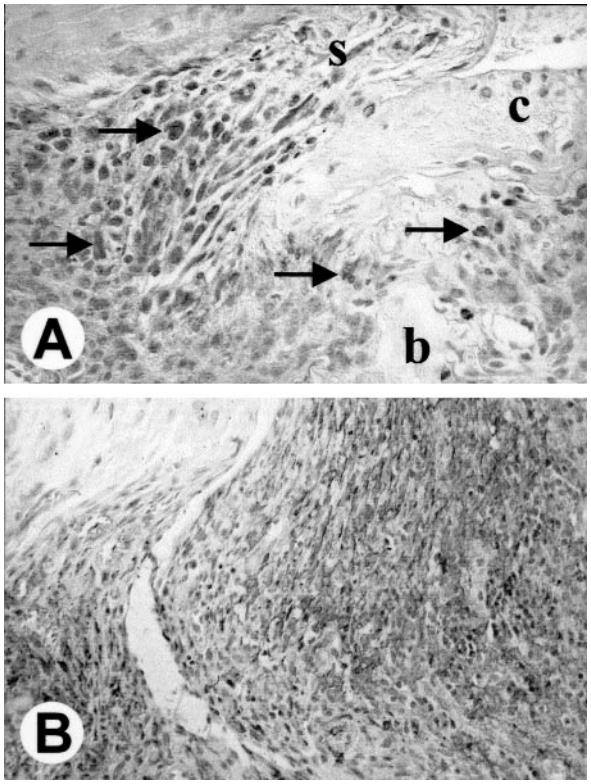

Figure 4. AS immunoreactivity in 4 and 8-week-old Tg197 h-TNF-α transgenics. (A) In 4-week-old Tg197 h-TNF-α transgenics, AS-immunoreactive fibroblast-like cells were evident in the inflammatory synovium and macrophage-like cells in pannus and at sites of subchondral bone erosions (arrows). (B) In 8-week-old Tg197 h-TNF-α transgenics, AS expression was evident in numerous stromal fibroblast-like cells in synovium and pannus. Original magnification (A) ×100 and (B) ×75.

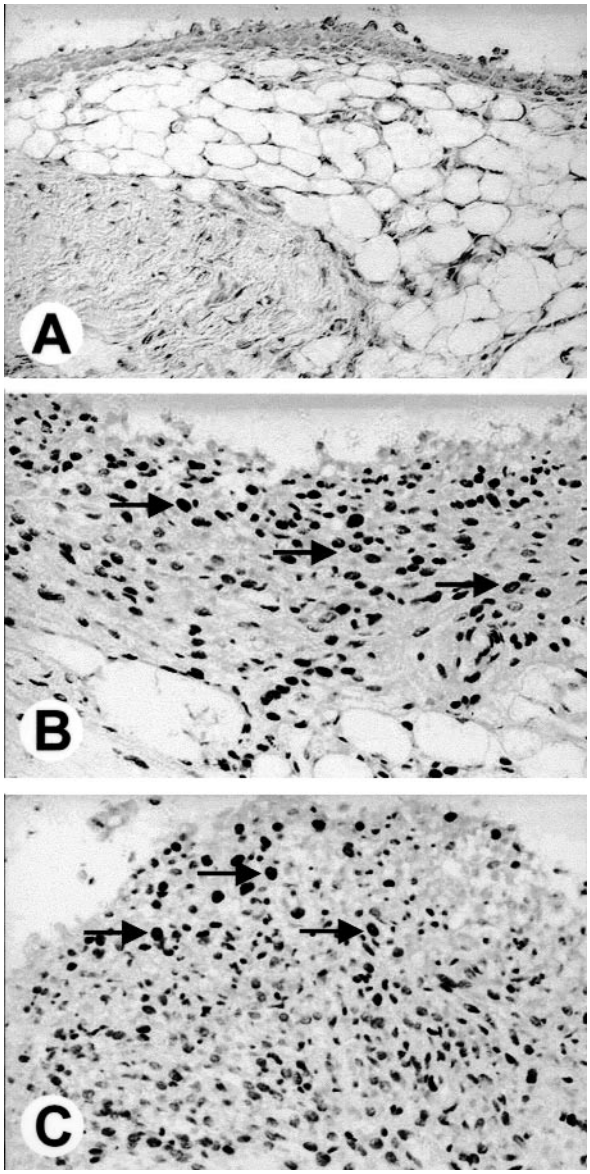

Figure 5. GTP cyclohydrolase immunoreactivity in 4-week-old CBA × C57B1/6 wild-type control and 4 and 8-week-old Tg197 h-TNF-α transgenic synovium. (A) Immunoreactivity for GTP cyclohydrolase was not found in the wild-type control synovium. In contrast, GTP cyclohydrolase immunoreactivity was widespread within the macrophage-like cells (arrows) in the subintimal and deeper stroma and pannus from 4-week-old (B) and 8-week-old (C) Tg197 h-TNF-α transgenic mice. Original magnification ×100.

destruction. Perhaps more important, coinduction of AS and GTPCH protein was also maintained from acute (4 weeks) to chronic phase (8 weeks) of the disease. This implies that the increased synthesis of L-arginine and BH4 were both maintained and able to provide the essential substrate (L-arginine) and cofactor (BH4) for prolonged activity of iNOS with subsequent high output NO generation, as evidenced by 3-NT and urinary NO metabolites.

We observed widespread iNOS expression in the inflamed joints of Tg197 h-TNF-α transgenics with spontaneous arthritis. Immunoreactivity for 3-NT was shown to overlap and in part exceed that of iNOS. Previous studies have described 3-NT in serum and synovial fluid in RA, whereas none was detected in healthy controls or osteoarthritis[19]. In a recent study by Mapp and co-workers, 3-NT was immunolocalized to vascular smooth muscle cells and CD 68+ macrophages in human RA synovium[21]. Detection of 3-NT has commonly been taken as evidence of NO mediated peroxynitrite generation[18-20,33]. Peroxynitrite is a potent and highly reactive oxidant species that can suppress cellular respiration and cause damage to cell membrane lipids and DNA bases, contributing to cell injury and death. Peroxynitrite forms in a reaction between NO and superoxide and its decomposition produces the hydroxyl and nitronium ions that contribute to nitration of aromatic amino acids including tyrosine[18]. Peroxynitrite may have other proinflammatory actions as well. One of the proinflammatory actions of TNF-α is through NO mediated induction of cyclooxygenase-2 transcription and transla-

tion[22]. Peroxynitrite can mediate induction of cyclooxygenase activity by serving as a substrate for the peroxidase activity of the enzyme[36]. Taken together, the colocalization of 3-NT in the inflammatory joints of Tg197 h-TNF-α transgenics with iNOS positive macrophages provides a strong indication that peroxynitrite mediated oxidation and nitration occurs as a result of h-TNF-α mediated iNOS synthesis and NO generation.

In the arthritic joints of Tg197 h-TNF-α transgenics, AS and GTPCH protein were coinduced together with iNOS. AS and GTPCH mRNA copy numbers seemed to increase in accord with disease progression, although they did not reach significance concentrations due to high intraassay variability. This may have been due to the severity of inflammation with subsequent mRNA cleavage and degradation. However, the results of the quantitative RT-PCR assay showed that both AS and GTPCH mRNA were much higher than iNOS mRNA copy numbers in all groups studied. This may imply that the synthesis of AS and GTPCH, 2 of the rate-limiting enzymes for high output NO generation, is more strictly regulated at the posttranscriptional than the transcriptional level *in vivo*. AS and GTPCH have not been previously studied in inflammatory arthritides. AS and argininosuccinate lyase (AL) together confer the ability of cells to regenerate the NOS substrate L-arginine from L-citrulline (a coproduct of NOS activity)[31,37]. In inflammation and during periods of high output NO generation, the availability of free L-arginine is rate-limiting to NO synthesis. While AL mRNA and protein synthesis are constitutive or only weakly regulated, AS mRNA and protein synthesis, at least *in vitro*, are thought to be highly regulated and induced by immunostimulants such as TNF-α, IL-1, and IFN-γ[31,37]. In this study, AS was localized in CBA × C57B1/6 wild-type controls in the vascular endothelium. In contrast, in Tg197 h-TNF-α transgenic arthritic joints AS was induced in fibroblasts in hyperplastic synovium and pannus and some macrophage-like cells at sites of subchondral bone erosions. An interesting finding was that iNOS was mostly localized to macrophage-like cells, while AS expression was most common in fibroblasts, with only discrete macrophage-like cells being positive. Therefore, there was only partial overlapping of iNOS and AS, implying possible paracrine interactions between macrophages that synthesize iNOS and their neighboring fibroblasts that synthesize AS. Nevertheless, these findings imply that the arginine-citrulline cycle is strongly induced in the Tg197 h-TNF-α transgenics with spontaneous arthritis, and suggest that the induced AS synthesis and its activity may provide the source of L-arginine for high output NO generation. We also found that GTPCH is induced concurrently with iNOS and AS in Tg197 h-TNF-α transgenics, and predominantly in macrophage-like cells scattered throughout the synovial intima/subintima and stroma. GTPCH is the first and rate-limiting enzyme in the *de novo* 3 step sequential synthesis of BH4 from GTP. The other enzymes in the GTP-biopterin pathway are less regulated 6-pyruvoyl tetrahydrobiopterin synthase and sepiapterin reductase that catalyze the reaction from dihydroneopterin triphosphate to BH4, an essential cofactor for NOS activity and NO generation[25]. The hyperphenylalaninemic hph-1 mutant mouse displays 90% deficiency in GTPCH activity that results in a concomitant loss of the activity of the endothelial isoform of NOS, which under physiological conditions usually produces only small amounts of NO in comparison to iNOS[38,39]. It follows that GTPCH must be upregulated in parallel with iNOS for high output iNOS activity, and studies have indeed shown an identical time-course for GTPCH and iNOS mRNA, protein, and activity after immunostimulation with TNF-α, IL-1, and/or IFN-γ[28]. In addition, increased urinary and synovial fluid neopterin concentrations have been reported by many authors, and several studies suggest that neopterin levels may reflect the clinical severity of RA[40]. These findings strongly suggest that coinduction of GTPCH with iNOS and AS is essential for high output NO generation and that the GTP-biopterin pathway is induced in arthritis, at least in the Tg197 h-TNF-α transgenic model of RA.

We characterized the Tg197 h-TNF-α transgenic mouse model of RA in terms of the high output NO-generating pathway including AS and GTPCH, the 2 rate-limiting enzymes necessary for maximal NO production. The concomitant induction of AS and GTPCH with iNOS suggests that they are important modulators or copromoters of inflammatory arthritis, and that they may represent novel targets for modulation of disease activity.

ACKNOWLEDGMENT

We thank Prof. S.S. Gross, Department of Pharmacology, Cornell University Medical College, Ithaca, New York, for the generous gift of AS and GTPCH antibodies, and Prof. J.S. Beckman, Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon, USA, for the generous gift of 3-NT antibodies.

REFERENCES

1. Sakurai H, Kohsaka H, Liu MF, et al. Nitric oxide production and inducible nitric oxide synthase expression in inflammatory arthritides. J Clin Invest 1995;96:2357-63.
2. McInnes IB, Leung BP, Field M, et al. Production of nitric oxide in the synovial membrane of rheumatoid and osteoarthritis patients. J Exp Med 1996;184:1519-24.
3. Evans CH, Stefanovic-Racic M. Effects of nitric oxide synthase inhibitors in experimental models of arthritis. In: Hukkanen MVJ, Polak JM, Hughes SPF, editors. Nitric oxide in bone and joint disease. Cambridge: Cambridge University Press; 1998:61-9.
4. McCartney-Francis N, Allen JB, Mizel DE, et al. Suppression of arthritis by an inhibitor of nitric oxide synthase. J Exp Med 1993;178:749-54.
5. Weinberg JB, Granger DL, Pisetsky DS, et al. The role of nitric oxide in the pathogenesis of spontaneous murine autoimmune disease: increased nitric oxide production and nitric oxide synthase expression in MRL-lpr/lpr mice, and reduction of spontaneous glomerulonephritis and arthritis by orally administered NG-monomethyl-L-arginine. J Exp Med 1994;179:651-60.

6. Stefanovic-Racic M, Meyers K, Meschter C, Coffey JW, Hoffman RA, Evans CH. N-monomethyl arginine, an inhibitor of nitric oxide synthase, suppresses the development of adjuvant arthritis in rats. Arthritis Rheum 1994;37:1062-9.
7. Cannon GW, Openshaw SJ, Hibbs JB Jr, Hoidal JR, Huecksteadt TP, Griffiths MM. Nitric oxide production during adjuvant-induced and collagen-induced arthritis. Arthritis Rheum 1996;39:1677-84.
8. Connor JR, Manning PT, Settle SL, et al. Suppression of adjuvant-induced arthritis by selective inhibition of inducible nitric oxide synthase. Eur J Pharmacol 1995;273:15-24.
9. Stefanovic-Racic M, Meyers K, Meschter C, Coffey JW, Hoffman RA, Evans CH. Comparison of the nitric oxide synthase inhibitors methylarginine and aminoguanidine as prophylactic and therapeutic agents in rat adjuvant arthritis. J Rheumatol 1995;22:1922-8.
10. McCartney-Francis NL, Song X, Mizel DE, Wahl SM. Selective inhibition of inducible nitric oxide synthase exacerbates erosive joint disease. J Immunol 2001;166:2734-40.
11. Veihelmann A, Landes J, Hofbauer A, et al. Exacerbation of antigen-induced arthritis in inducible nitric oxide synthase-deficient mice. Arthritis Rheum 2001;44:1420-7.
12. Farrell AJ, Blake DR, Palmer RM, Moncada S. Increased concentrations of nitrite in synovial fluid and serum samples suggest increased nitric oxide synthesis in rheumatic diseases. Ann Rheum Dis 1992;51:1219-22.
13. Stichtenoth DO, Fauler J, Zeidler H, Frolich JC. Urinary nitrate excretion is increased in patients with rheumatoid arthritis and reduced by prednisolone. Ann Rheum Dis 1995;54:820-4.
14. Ueki Y, Miyake S, Tominaga Y, Eguchi K. Increased nitric oxide levels in patients with rheumatoid arthritis. J Rheumatol 1996;23:230-6.
15. Di Rosa M, Radomski M, Carnuccio R, Moncada S. Glucocorticoids inhibit the induction of nitric oxide synthase in macrophages. Biochem Biophys Res Commun 1990;172:1246-52.
16. Perkins DJ, St. Clair EW, Misukonis MA, Weinberg JB. Reduction of NOS2 overexpression in rheumatoid arthritis patients treated with anti-tumor necrosis factor alpha monoclonal antibody (cA2). Arthritis Rheum 1998;41:2205-10.
17. Meininger CJ, Kelly KA, Li H, Haynes TE, Wu G. Glucosamine inhibits inducible nitric oxide synthesis. Biochem Biophys Res Commun 2000;279:234-9.
18. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA 1990;87:1620-4.
19. Kaur H, Halliwell B. Evidence for nitric oxide-mediated oxidative damage in chronic inflammation. Nitrotyrosine in serum and synovial fluid from rheumatoid patients. FEBS Letts 1994; 350:9-12.
20. Halliwell B, Zhao K, Whiteman M. Nitric oxide and peroxynitrite. The ugly, the uglier and the not so good: a personal view of recent controversies. Free Radic Res 1999;31:651-69.
21. Mapp PI, Klocke R, Walsh DA, et al. Localization of 3-nitrotyrosine to rheumatoid and normal synovium. Arthritis Rheum 2001;44:1534-39.
22. Hughes FJ, Buttery LD, Hukkanen M, O'Donnell A, Maclouf J, Polak JM. Cytokine-induced prostaglandin E2 synthesis and cyclooxygenase-2 activity are regulated both by a nitric oxide-dependent and -independent mechanism in rat osteoblasts in vitro. J Biol Chem 1999;274:1776-82.
23. Honda S, Migita K, Hirai Y, et al. Induction of COX-2 expression by nitric oxide in rheumatoid synovial cells. Biochem Biophys Res Commun 2000;268:928-31.
24. Nathan C, Xie QW. Regulation of biosynthesis of nitric oxide. J Biol Chem 1994;269:13725-8.
25. Xie L, Smith JA, Gross SS. GTP cyclohydrolase I inhibition by the prototypic inhibitor 2,4-diamino-6-hydroxypyrimidine. J Biol Chem 1998;273:21091-8.
26. Hattori Y, Campbell EB, Gross SS. Argininosuccinate synthetase mRNA and activity are induced by immunostimulants in vascular smooth muscle. Role in the regeneration or arginine for nitric oxide synthesis. J Biol Chem 1994;269:9405-8.
27. Nussler AK, Billiar TR, Liu ZZ, Morris SM Jr. Coinduction of nitric oxide synthase and argininosuccinate synthetase in a murine macrophage cell line. Implications for regulation of nitric oxide production. J Biol Chem 1994;269:1257-61.
28. Hattori Y, Gross SS. GTP cyclohydrolase I mRNA is induced by LPS in vascular smooth muscle: characterization, sequence and relationship to nitric oxide synthase. Biochem Biophys Res Commun 1993;195:435-41.
29. Keffer J, Probert L, Cazlaris H, et al. Transgenic mice expressing human tumor necrosis factor: a predictive genetic model of arthritis. EMBO J 1991;10:4025-31.
30. Douni E, Akassoglou K, Alexopoulou L, et al. Transgenic and knockout analyses of the role of TNF in immune regulation and disease pathogenesis. J Inflamm 1995-96;47:27-38.
31. Xie L, Gross SS. Argininosuccinate synthetase overexpression in vascular smooth muscle cells potentiates immunostimulant-induced NO production. J Biol Chem 1997;272:16624-30.
32. Hwang O, Baker H, Gross S, Joh TH. Localization of GTP cyclohydrolase in monoaminergic but not nitric oxide-producing cells. Synapse 1998;28:140-53.
33. Haddad IY, Pataki G, Hu P, Galliani C, Beckman JS, Matalon S. Quantitation of nitrotyrosine levels in lung sections of patients and animals with acute lung injury. J Clin Invest 1994;94:2407-13.
34. Kollias G, Douni E, Kassiotis G, Kontoyiannis D. On the role of tumor necrosis factor and receptors in models of multiorgan failure, rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease. Immunol Rev 1999;169:175-94.
35. Van den Berg WB, Joosten LA, Kollias G, van de Loo FA. Role of tumor necrosis factor α in experimental arthritis: separate activity of interleukin 1ß in chronicity and cartilage destruction. Ann Rheum Dis 1999;58 Suppl I:140-8.
36. Landino LM, Crews BC, Timmons MD, Morrow JD, Marnett LJ. Peroxynitrite, the coupling product of nitric oxide and superoxide, activates prostaglandin biosynthesis. Proc Natl Acad Sci USA 1996;93:15069-74.
37. Xie L, Hattori Y, Tume N, Gross SS. The preferred source of arginine for high-output nitric oxide synthesis in blood vessels. Semin Perinatol 2000;24:42-5.
38. Gutlich M, Ziegler I, Witter K, et al. Molecular characterization of HPH-1: a mouse mutant deficient in GTP cyclohydrolase I activity. Biochem Biophys Res Commun 1994;203:1675-81.
39. Cosentino F, Barker JE, Brand MP, et al. Reactive oxygen species mediate endothelium-dependent relaxations in tetrahydrobiopterin-deficient mice. Arterioscler Thromb Vasc Biol 2001;21:496-502.
40. Altindag ZZ, Sahin G, Inanici F, Hascelik Z. Urinary neopterin excretion and dihydropteridine reductase activity in rheumatoid arthritis. Rheumatol Int 1998;18:107-11.