Elevated Serum Nitric Oxide Levels in Patients with Inflammatory Arthritis Associated with Co-expression of Inducible Nitric Oxide Synthase and Protein Kinase C-η in Peripheral Blood Monocyte-Derived Macrophages

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ABSTRACT. ***Objective.*** To quantify circulating nitric oxide (NO) levels and inducible NO synthase (*iNOS*) expression in peripheral blood monocyte-derived macrophages (PB-MDM) from patients with inflammatory arthritis (IA) as a measure of disease activity, and to determine if there is a correlation between expression of *iNOS* and protein kinase C-η (*PKC-η*).

Methods. PB-MDM were isolated from whole blood of 20 patients with IA (14 rheumatoid arthritis and 6 peripheral spondyloarthropathies). Thirteen patients with osteoarthritis (OA) and 9 healthy individuals were controls. Serum NO levels were measured by indirect determination of nitrite and nitrate. Expression of *PKC-η* and *iNOS* was investigated by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.

Results. Serum NO (189.9 ± 49.7 µM) was significantly higher ($p < 0.0028$) in IA patients than in controls (131.1 ± 18.5 µM) or patients with OA (126.9 ± 37.1 µM). IA patients with severe inflammation had highest levels of NO, while those with mild inflammation had normal levels of NO. RT-PCR showed that PB-MDM from IA patients with active disease co-expressed *iNOS* and *PKC-η*. This was observed in 15 out of 16 cases. All other groups with normal plasma NO expressed neither gene.

Conclusion. Our findings show that elevated plasma NO levels were only present in IA patients with severe disease activity. We show for the first time a positive correlation between *PKC-η* and *iNOS* expression in arthritis, supporting our earlier *in vitro* findings that *PKC-η* expression was essential for lipopolysaccharide-mediated *iNOS* induction and NO production in human monocytes. *PKC-η* may be important for the development of IA-induced *iNOS* positive phenotype in human PB-MDM. (J Rheumatol 2003;30:2529–34)

Key Indexing Terms:
NITRIC OXIDE INDUCIBLE NITRIC OXIDE SYNTHASE INFLAMMATORY ARTHRITIS
PROTEIN KINASE C-η REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION
PERIPHERAL BLOOD MONOCYTE-DERIVED MACROPHAGES

Rheumatoid arthritis (RA) is a prototype autoimmune disease characterized by inflammation of the joints. The principal pathological features of joint inflammation include invasion of leukocytes, their proliferation in the synovium, and accumulation of peripheral mononuclear cells in the synovial fluids. It is thought that infiltration of these cells in their activated stage results in overproduction of cytokines such as tumor necrosis factor (TNF)-α and interleukin 1 (IL-1) as well as a number of other inflammatory mediators including nitric oxide (NO), matrix-metalloproteases, and prostaglandin E_2 [1]. Dysregulation of these molecules eventually leads to damage to the joints.

NO is produced by members of the NO synthase (NOS)

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family, which consists of endothelial, neuronal, and inducible isoforms. When synthesized by the inducible NOS (*iNOS*), NO is crucial to host defense against a whole range of microbes including fungi, protozoa, intracellular bacteria, and certain viruses[2]. *iNOS* expression in monocytes may be induced by endotoxin or cytokines as a result of infection or diseases. Failure to regulate NO production can result in tissue injury. Several studies have shown that NO is associated with pathogenesis of systemic sclerosis, RA, and AIDS-associated dementia[3-5]. Disease models in animals have also shown that HN^G-monomethyl L-arginine monoacetate (L-NMMA), which inhibits all 3 NOS enzymes, could ameliorate adjuvant[6] or streptococcus-induced arthritis[7].

In vitro induction of *iNOS* expression has proven very difficult in human monocytes and cell lines. It is possible that the lack of key signaling molecules required for induction of *iNOS* and production of NO explains this phenomenon. In an earlier study, we found that protein kinase C-η (*PKC-η*) expression was completely deficient in human monocytic cells and that *iNOS* could not be induced by lipopolysaccharide (LPS) or cytokines. Subsequent transfections of human monocytic Mono Mac 6 cell line with *PKC-η* resulted in *iNOS* induction and NO production by these cells following LPS stimulation. These data suggest that *PKC-η* expression may be required prior to the induction of the *iNOS* gene in human monocytic cells. We tested this hypothesis in peripheral blood monocytes from patients with inflammatory arthritis (IA) to see whether we could establish an *in vivo* correlation between these 2 genes.

MATERIALS AND METHODS

Subjects. A total of 33 patients who fulfilled the American College of Rheumatology criteria[8] and 9 healthy volunteers (age 22 to 48 yrs) were recruited for the study. The patients were placed in 3 different subgroups based on their medical diagnosis: osteoarthritis (OA, n = 13, age 47 to 81 yrs), RA (n = 14, age 36 to 76 yrs), and peripheral spondyloarthropathies (SpA, n = 6, age 24 to 63 yrs). While patients with OA were considered as having noninflammatory arthritis, those with RA, psoriatic arthritis (PsA), or ankylosing spondylitis (AS) were considered as having inflammatory arthritis. The healthy volunteers and OA patients were the controls for our study. Depending on the swollen/tender joint count at the time of study, patients with RA (rheumatoid factor, RF, positive) or PsA (RF negative) were further subdivided into either a severely (minimum 5 active joints) or mildly inflamed (less than 5 active joints) group. Of the 6 patients with peripheral SpA, who were serologically negative for RF, 3 had AS and 3 had PsA. The project was approved by the university's human investigation committee, and written consent obtained from all patients and healthy individuals. Disease status of the patients was withheld from the laboratory investigators until all experimental assessments were complete.

Isolation of peripheral blood monocytes. Peripheral blood mononuclear cells (PBMC) were prepared from heparinized venous blood samples by density gradient centrifugation with Ficoll-Paque as per manufacturer's protocol (Pharmacia Biotech Inc., Piscataway, NJ, USA). After extensive washing, the PBMC were resuspended in RPMI-1640 media supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 µg/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, and 1 mM MEM amino acids (Invitrogen Life Technologies, New York, NY, USA), and cultured for 2 h at 37°C in a humidified incubator (95% air and 5% CO_2). Nonadherent cells were discarded; the adherent monocytes were washed twice with PBS, and allowed to differentiate to macrophages for 5 days.

RNA extraction of monocytes and reverse transcriptase-polymerase chain reaction (RT-PCR). RNA was extracted from the differentiated cells using TRIzol Reagent (Invitrogen) as per protocol. Aliquots of 1.5–2.0 µg of the total RNA was used (per reaction) for cDNA synthesis by SuperScript H-RT (Invitrogen). In some experiments, RT with ^{32}P incorporation was performed to ensure validity of the experiment. The resulting cDNA (2 µl) was used as a template for amplification by PCR.

The *iNOS* primers were CGG TTC TAC TCC ATC AGC TC (sense) and TGC CAG AAA CTG CGG AAG GG (antisense), while those for *GAPDH* were TCA CCA GGG CTG CTT TTA AC (sense) and GGA GGC ATT GCT GAT GAT CT (antisense). The primers for *PKC-η*[9] were AAC GAG GAG TTT TGC GCT AA (sense) and TGG TAA AAT GTT TGA AGA TCC G (antisense). After an initial denaturation for 5 min at 95°C, the templates (*GAPDH* and *iNOS*) were subject to a 30-cycle amplification of 1 min denaturation at 94°C, 1 min annealing at 60°C, and 30 s elongation at 72°C. The cycling parameters for *PKC-η* were 1 min denaturation at 94°C, 2 min annealing at 58°C, and 1 min elongation at 72°C for 35 cycles. A 10 min final extension at 72°C was applied in all 3 cases. The expected sizes for *iNOS*, *GAPDH*, and *PKC-η* were 264, 400, and 259 base pairs, respectively. A mock reaction, which contained all the PCR reagents but no template, was prepared in parallel to eliminate false positive amplification. Samples that had all reagents minus the RT served as another type of negative control. Nine microliters of PCR products was separated on a 1.5% agarose (Sigma) gel containing 0.5 µg/ml EtBr (Sigma). The gel image was photographed and scanned.

Nitrate and nitrite determination from biological fluids. Plasma samples (50 µl) were diluted 4-fold with sterile distilled water. Then 90 U/l *E. coli* nitrate reductase and 44 µM NADPH/FAD (Boehringer Mannheim, USA) were then added, and the samples incubated for 24 h at room temperature. Excess NADPH was eliminated with a mixture containing 480 U/l of L-glutamic dehydrogenase, 4 mM α-ketoglutaric acid, and 84 mM $(NH_4)_2SO_4$ (Sigma). The samples were further incubated for 20 min at room temperature, deproteinated with 15 g/l $ZnSO_4$, vortexed, and clarified by centrifugation for 5 min at 10,000 g. In a 96-well microtiter plate, 100 µl of the supernatants was well mixed with 100 µl of the Griess reagent [50 µl of 1% (w/v) sulfanilamide in 5% H_3PO_4 plus 50 µl of 1% (w/v) N-(1-naphthyl)ethylenediamine in water], incubated in the dark for 15 min, and measured for optical density at 590 nm with a microplate reader (Model 3550, Bio-Rad, Hercules, CA, USA). Purified sodium nitrite (Sigma) was used to construct a standard curve from which the unknowns were to be extrapolated. Samples lacking either sulfanilamide or N-(1-naphthyl)ethylenediamine of the Griess reagent were negative controls.

Data and statistical analysis. Data were analyzed by Graph Pad Prism and the results were expressed as mean ± SD. Where appropriate, statistical analyses (unpaired Student's t tests) were carried out using the INSTAT software.

RESULTS

Elevated serum NO associated with active disease status in RA patients. NO accumulation in the serum (Figure 1) was assessed by indirect measurements of its stable products nitrite and nitrate in patients with inflammatory arthritis (RA, n = 14 and SpA, n = 6), OA (n = 13), or healthy individuals (n = 9). For the purpose of this study, disease activity of patients within the seropositive RA group was determined by clinical assessments of the number of tender and swollen joints manifested at the time of study. Patients classified in the severe inflammatory group (Figure 1, severe) had between 5 and 12 active joints, while those in the mild inflammatory group had less than 5 (Figure 1, mild).

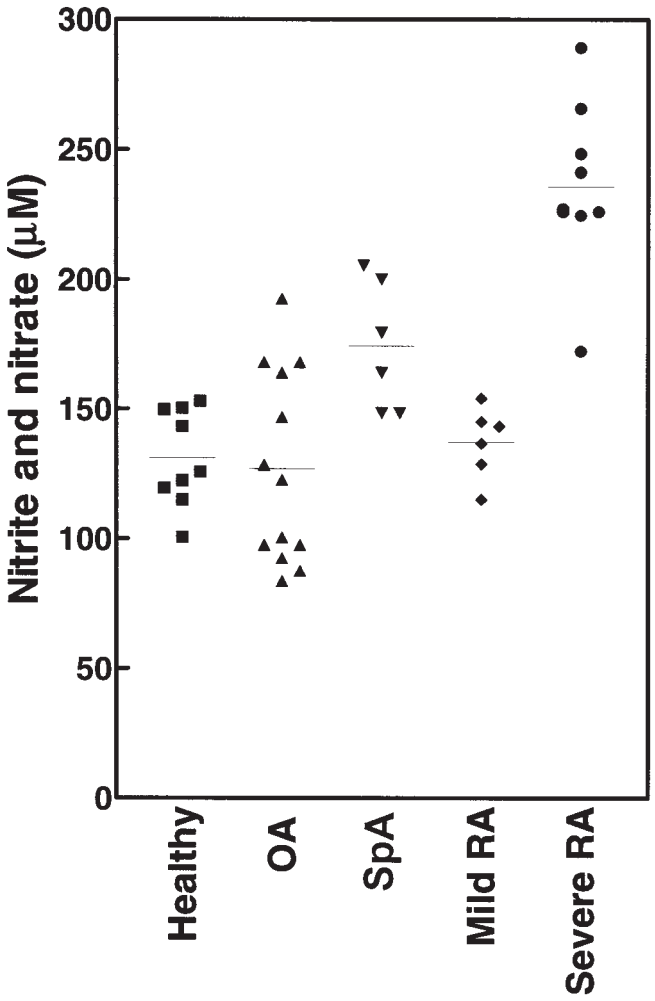

Figure 1. Elevated serum NO in patients with inflammatory arthritis. NO was measured from 50 µl of fresh plasma from healthy volunteers or patients with RA, peripheral SpA, or OA by indirect assessments of nitrite. Nitrate was first reduced by nitrate reductase to nitrite, which was then measured by Griess assay. Based on clinical assessments of the swollen and tender joint count, the patients within the RA group were further categorized as having mild RA (< 5 active joints) or severe RA (5–12 active joints). Each symbol represents a patient and the mean is indicated.

Generally, patients with inflammatory arthritis had significantly higher levels of circulating NO than patients with OA or healthy volunteers. Within the seropositive RA group, elevated levels of plasma NO were directly correlated with degree of inflammation; patients with severe inflammation (236.9 ± 37.1 µM) had markedly higher NO levels in the circulation than patients with mild inflammation (137.1 ± 13.8 µM). The latter group had levels of NO comparable to those seen in healthy individuals (131.1 ± 18.5 µM) and OA patients (126.9 ± 37.1 µM).

Coexpression of iNOS and PKC-η associated with elevated circulating NO. Our findings from an earlier study[9a] showed that human mononuclear phagocytes (from acute leukemic Mono Mac 6 cell line) transfected with *PKC-η* were capable of expressing *iNOS* and releasing NO into the culture media following endotoxin stimulation. Thus, we wished to examine whether these *in vitro* findings would be applicable in clinical situations. With this aim, we used arthritis as a model of choice. In Figure 1, we showed that NO levels in the circulation were elevated in RA patients with active disease, and that the absolute levels were correlated with progression of the condition. We next wanted to investigate if the upregulation of NO in the serum of these patients might be attributed to expression of the *iNOS* gene in human monocytes. Using gene-specific primers, *iNOS* expression in PB-MDM was examined by RT-PCR. As shown in a representative RT-PCR experiment (Figure 2A), RA and SpA patients with active disease strongly expressed *iNOS* mRNA, but the healthy individuals or those with noninflammatory OA did not. Also, PB-MDM from patients with inflammatory arthritis with normal circulating NO did not express *iNOS*.

We showed previously that *PKC-η* was essential for the *in vitro* induction of *iNOS* by LPS in human monocytic cells. It was of interest to see if *PKC-η* would also be expressed in these patients, and whether its expression profile could be correlated with that of *iNOS*. We again carried out the investigation by RT-PCR. Figure 2B represents data from one such experiment. *PKC-η* was found to be highly expressed in RA patients with active disease. In sharp contrast, healthy volunteers and patients with OA who lacked *iNOS* were also negative for *PKC-η*. Taken together, we observed (1) a statistically significant ($p < 0.0021$) upregulation of circulating NO compared to that in the control groups (healthy or OA) or in patients with mildly inflammatory RA (Figure 1); and (2) a constitutive coexpression of *PKC-η* and *iNOS* in PB-MDM only in patients with inflammatory arthritis with severe disease.

Interestingly, one anomaly was observed in Patient 23 (Table 1). Clinical assessments of the number of tender and swollen joints had shown that this particular patient had severe inflammation with more than 10 active joints. The patient's plasma NO (227 µM) was notably elevated compared to that of the controls. In accord with this finding, RT-PCR analyses showed the expression of *iNOS* in PB-MDM. However, *PKC-η* appeared to be completely absent from the patient's PB-MDM.

DISCUSSION

NO, TNF-α, and IL-1 are major pleiotropic inflammatory mediators overproduced in arthritis-affected joints[10]. Highly active free radical NO can act in many different ways on different molecules to exert its toxic effects, leading to eventual joint destruction. At the onset of the disease, NO is found to be mostly concentrated in the inflamed synovium as a result of *iNOS* expression in activated chondrocytes and synovial macrophages present in the local environment. However, as the chronic condition progresses, NO may be

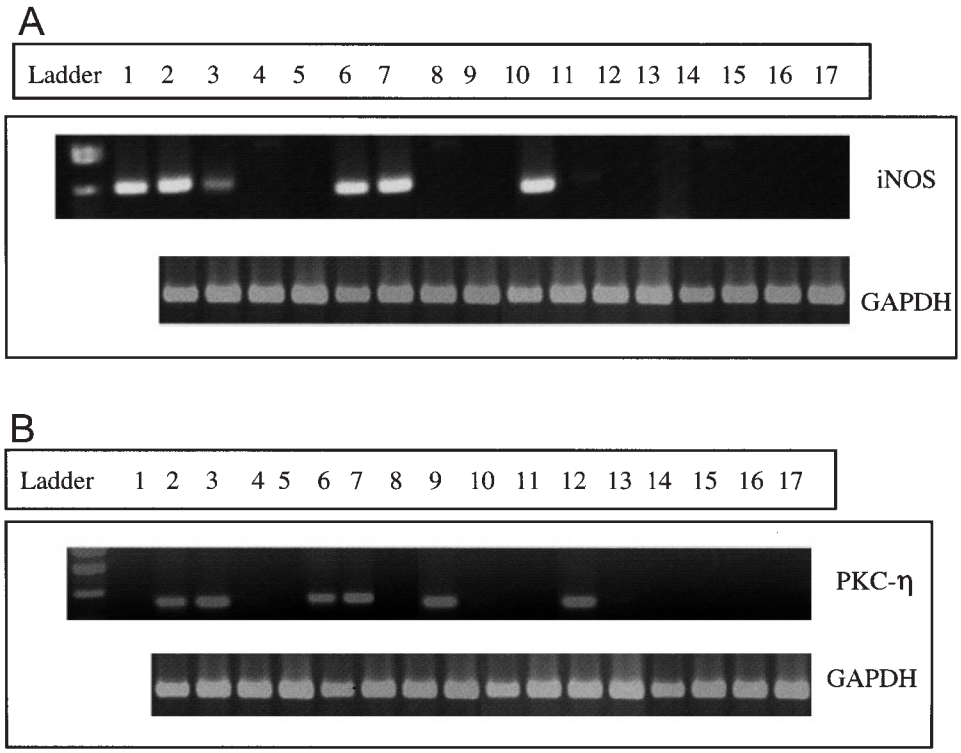

Figure 2. Investigation of *iNOS* and *PKC-η* in PB-MDM from arthritides by RT-PCR. PB-MDM from healthy individuals and patients with IA were isolated, cultured, and assessed for *iNOS* (A) or *PKC-η* (B) expression by RT-PCR using gene-specific primers. GAPDH amplified from the same samples was an internal control. 0.5 µg of 100 bp DNA ladder was loaded as markers to the well furthest to the left of the gel. PCR products (9 µl) were separated on a 1% agarose gel, visualized by a UV light source, and photographed. Except for the control lanes, all others are labeled by patient ID number (diagnosis, disease activity). A: Lane 1: pBABE (plasmid contains full-length human *iNOS* cDNA, positive control); Lane 2: Patient 42 (PsA, severe); Lane 3: Patient 39 (AS, inflammation); Lane 4: Patient 38 (AS, clinically quiescent); Lane 5: Patient 37 (AS, clinically quiescent); Lane 6: Patient 29 (RA, severe); Lane 7: Patient 28 (RA, severe); Lane 8, Patient 26 (RA, mild); Lane 9, Patient 24 (RA, mild); Lane 10, Patient 23 (RA, severe); Lane 11: Patient 22 (OA, no inflammation); Lane 12: Patient 18 (OA, no inflammation); Lane 13: Patient 14 (OA, no inflammation); Lane 14: Patient 11 (OA, no inflammation); Lane 15: Patient 4 (healthy); Lane 16: Patient 2 (healthy); and Lane 17: Patient 1 (healthy). B: Lane 1: no-template PCR (negative control); Lane 2: Patient 42 (PsA, severe); Lane 3: Patient 39 (AS, inflammation); Lane 4: Patient 38 (AS, clinically quiescent); Lane 5: Patient 37 (AS, clinically quiescent); Lane 6: Patient 29 (RA, severe); Lane 7: Patient 28 (RA, severe); Lane 8: Patient 26 (RA, mild); Lane 9: Patient 25 (RA, severe); Lane 10: Patient 24 (RA, mild); Lane 11: Patient 23 (RA, severe); Lane 12: pks1.*PKC-η* (plasmid encoding full-length *PKC-η* cDNA, positive control); Lane 13: Patient 22 (OA, no inflammation); Lane 14: Patient 18 (OA, no inflammation); Lane 15: Patient 14 (OA, no inflammation); Lane 16: Patient 4 (healthy); and Lane 17: Patient 2 (healthy).

found circulating in the bloodstream. Here, we have shown that inflammatory arthritis patients with severe inflammation had upregulated serum NO not seen in controls or patients with OA (Figure 1). These elevations in NO levels appear to correlate with monocyte expression of the *iNOS* gene as its mRNA accumulation was also increased in these same patients (Figure 2 and Table 1). Although upregulation of NO in sera of patients with RA has been reported[11], our findings are novel in 2 aspects. First, we were able to show that the elevated NO levels were only observed in patients with severe disease as defined by clinical assessments of the number of inflamed joints. RA patients with mild inflammation had comparable levels of NO to those found in controls or in patients with noninflammatory OA. Second, to our knowledge, this is the first time that a positive correlation between *PKC-η* and *iNOS* expression by PBM has been reported in arthritic patients. Specifically, the 2 genes were either both expressed or not expressed at all. This observation was found in all patients except Patient 23. In this patient with active RA, NO levels in the circulation were elevated, and her PB-MDM did accordingly express *iNOS*. However, in contrast to other inflammatory arthritis patients with severe disease, who were positive for the 2 genes in their PB-MDM, this patient did not appear to express *PKC-η* in these cells. Presently, we have no explanation for this observation.

Table 1. Elevated plasma NO is correlated with co-expression of *iNOS* and *PKC-η* in peripheral blood monocyte-derived macrophages from patients with RA.

Patient (ID#)	Age, yrs	Sex	Diagnosis	Activity	Steroids	DMARD	NO, µM	*PKC-η*	*iNOS*
1 (001)	26	F	Healthy	NA	N	N	143.2	N	N
2 (002)	42	F	Healthy	NA	N	N	150.4	N	N
3 (046)	22	F	Healthy	NA	N	N	153.0	ND	ND
4 (047)	25	F	Healthy	NA	N	N	149.8	N	N
5 (048)	32	F	Healthy	NA	N	N	122.4	ND	ND
6 (057)	40	F	Healthy	NA	N	N	119.6	N	N
7 (058)	44	F	Healthy	NA	N	N	125.6	N	N
8 (061)	48	F	Healthy	NA	N	N	100.5	N	N
9 (062)	43	F	Healthy	NA	N	N	115.0	N	N
10 (006)	54	F	OA	No inflammation	N	N	146.8	N	N
11 (007)	76	M	OA	No inflammation	N	N	168.0	N	N
12 (009)	58	F	OA	No inflammation	N	N	168.0	N	N
13 (020)	81	F	OA	No inflammation	N	N	192.4	ND	ND
14 (025)	49	F	OA	No inflammation	N	N	97.6	N	N
15 (027)	56	M	OA	No inflammation	N	N	83.6	N	N
16 (028)	60	M	OA	No inflammation	N	N	164.0	ND	ND
17 (029)	61	F	OA	No inflammation	N	N	97.6	N	N
18 (032)	47	F	OA	No inflammation	N	N	122.8	N	N
19 (034)	48	F	OA	No inflammation	N	N	87.6	N	N
20 (035)	55	F	OA	No inflammation	N	N	128.4	N	N
21 (037)	50	M	OA	No inflammation	N	N	92.4	N	N
22 (038)	63	M	OA	No inflammation	N	MTX	100.4	N	N
23 (004)	57	F	RA	Severe	Prednisone	MTX	227.2	N	Y
24 (005)	39	F	RA	Mild	Prednisone	MTX	154.0	N	N
25 (017)	36	M	RA	Severe	N	N	289.2	Y	Y
26 (019)	44	M	RA	Mild	N	MTX	143.2	N	N
27 (021)	76	F	RA	Severe	Prednisone	N	172.2	ND	ND
28 (023)	54	M	RA	Severe	N	MTX	248.4	Y	Y
29 (030)	46	F	RA	Severe	N	MTX	226.2	Y	Y
30 (031)	51	F	RA	Mild	Prednisone	MTX	136.6	ND	ND
31 (053)	50	F	RA	Severe	N	MTX	265.7	Y	Y
32 (054)	54	F	RA	Severe	Prednisone	N	241.3	Y	Y
33 (055)	60	M	RA	Mild	N	MTX	115.0	N	N
34 (056)	52	F	RA	Severe	Prednisone	MTX	224.7	Y	Y
35 (059)	59	F	RA	Mild	Prednisone	MTX	145.0	N	N
36 (060)	75	F	RA	Mild	Prednisone	MTX	128.6	N	N
37 (011)	60	M	SpA (AS)	Clinically quiet	Prednisone	N	148.4	N	N
38 (012)	24	M	SpA (AS)	Clinically quiet	N	MTX	148.4	N	N
39 (013)	36	M	SpA (AS)	No inflammation	Prednisone	N	205.6	Y	Y
40 (018)	37	M	SpA (PsA)	Mild	N	MTX	164.0	ND	ND
41 (022)	33	F	SpA (PsA)	Mild	N	MTX	179.3	ND	ND
42 (026)	63	F	SpA (PsA)	Severe	Prednisone	N	200.0	Y	Y

ND: not done.

Although TNF-α and IL-1 have long been considered attractive targets for pharmacologic intervention and gene therapy for inflammatory diseases like arthritis, *iNOS* has also begun to gain attention as yet another candidate for the development of novel treatments for the disorders. As for *iNOS* inhibitors, work with L-NMMA in animal models showed reduced paw swelling, indicating attenuation of inflammation[12]. However, as NO does have other physiological roles (endothelium-relaxing factor and neurotransmitter) besides that of an inflammatory mediator, such therapeutic approach may have severe side effects, as L-NMMA suppresses all 3 NO synthases. Obviously, the ultimate goal would be to suppress the overproduction of NO in arthritis-affected joints and peripheral blood cells without compromising other important functions of NO.

Considering that PKC plays an essential role in the signal transduction of various cellular processes including gene expression, several reports have proposed a role of PKC inhibitors (benzylidene derivative and staurosporine) as possible antirheumatic agents in that they led to attenuation of IL-1α or PMA-induced IL-1ß production by synovial cells from patients with RA[13]. In addition, PKC inhibitor gold sodium thiomalate (GSTM), employed as a therapeutic agent for RA for many years, has been shown by Hashimoto

and colleagues[14] to suppress mitogen-induced T-cell proliferation. Similarly, in an experimental model of adjuvant-induced arthritis, PKC inhibitors debromohymenialdisine (a catalytic site inhibitor)[15] and Ro 32-0432[16] have been shown to alleviate rat paw swelling via suppression of the proliferation of autoreactive lymphocytes.

We have shown a strong association between *PKC-η* and *iNOS* in inflammatory arthritis patients with severe disease. These findings may pave the way for future investigation of *PKC-η* as a potentially therapeutic target for the treatment of inflammatory conditions such as RA where overproduction of NO and/or persistent expression of *iNOS* by peripheral blood monocytes has been shown to contribute to disease pathogenesis[10,17].

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