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Inflammation Research

Widespread expression of inducible NOS and citrulline in lupus nephritis tissues

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Abstract. *Objective:* Examine the presence of functional inducible nitric oxide synthase (iNOS) in lupus nephritis lesions.
Methods: Seventeen kidney biopsies from patients with lupus nephritis and an equal number of normal control kidney biopsies were examined for the presence of iNOS and endothelial nitric oxide synthase (eNOS) and citrulline by using immunohistochemical methods. Additionally, iNOS and eNOS mRNAs were examined by reverse transcription -PCR amplification of total renal RNA.
Results: All biopsies expressed constitutive eNOS, but in contrast to normal kidney biopsies, 70 % of the lupus biopsies also expressed iNOS mRNA and the cognate protein. Eight positive biopsies corresponded to class IV lupus nephritis, which also had a high degree of citrullination.
Conclusions: The data indicate that functional iNOS activity is present in glomeruli as part of the inflammatory process in the kidney; therefore the products of iNOS could play a role in the pathogenesis of lupus nephritis.

Key words: iNOs – Lupus nephritis – Free radicals and tissue damage – Inflammatory mediators – Citrulline

Introduction

Nitric Oxide (NO) is a free radical that behaves as a diffusible gas, and it has known effects on blood flow and renal function. Among its multiple effects, NO induces vasodilatation in response to acetylcholine and bradykinin. Moreover, NO agonists augment renal blood flow, whereas antagonists decrease it, therefore modulating renal arterial pressure and natriuresis [1].

NO is the product of nitric oxide synthase (NOS), which has three isoforms: NOS I or neuronal (nNOS), originally discovered in neurons, with splice variants found in other tissues. NOS II (iNOS) is the inducible form linked to inflammation and it is expressed by macrophages and other cells in several tissues. Finally, endothelial NOS III or endothelial (eNOS) is constitutively expressed by endothelial cells [2]. The kidney express the three isoforms of the enzyme, with NOS I present in macula densa cells whereas iNOS is expressed by smooth muscle cells of pre-glomerular afferent arterioles, and eNOS is present in arterioles [1].

The pathway of NO production is marked by the conversion of arginine to citrulline, and one of the three isoforms is converted by the enzyme nitric oxide synthases. The eNOS dependent NO production is a physiologic process whereas iNOS produces NO-related inflammation. Citrulline is an amino acid that possesses an asymmetric carbon with two enantiomers, the natural form being the L form. Citrulline has reactivity like other aminoacids; it may form peptide bonds and is present in certain proteins, however as this amino acid lacks a genetic codon, its presence in a given protein is necessarily the result of post-translational modification [1, 2].

During inflammatory processes, iNOS replaces the constitutive eNOS expression, which is of particular importance because as in the case of inflammatory glomerulonephritis, such a replacement is linked to tissue damage [3]. The consequences of NO generation in systemic lupus erythematosus (SLE) has been studied, and the levels of serum nitrate/nitrite

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and nitrotyrosine has been correlated with lupus disease activity, and renal disease activity scores such as glomerular proliferation, leukocyte exudation, karyorrhexis/fibrinoid necrosis, cellular crescents, hyaline deposits, and interstitial inflammation [3–5]. In the murine New Zealand Black x New Zealand White F1 (NZB/NZW F1) model of SLE, the kidney damage is blocked by aminoguanidine, a potent inhibitor of NO [6].

Since iNOS expression may be an indicator of inflammation with particular clinical importance in lupus nephritis, NO may be a potential therapeutic target. The current studies were designed to determine whether the iNOS/eNOS expression ratio is altered in lupus nephritis. Moreover, we examined the presence of citrulline in kidney tissues as an indirect indicator of iNOS function. These studies are important because the activity of iNOS has not been previously reported.

Methods

Kidney biopsies

Biopsies from seventeen patients with systemic lupus erythematosus (SLE) (14 females and 3 males) were studied. The patients had a mean of age of 25.9 years (range 14–40) with a mean disease duration of 4.8 years (range 1–10). All patients met the revised American College of Rheumatology (ACR) classification criteria for SLE [7], and they had one or more serologic criteria, such as positive antinuclear antibody test, low serum complement, or anti-dsDNA antibodies (*Crithidia. luciliae* test). Some patients had a positive lupus skin band test. In all patients kidney biopsies were performed percutaneously, and a segment of each biopsy was stained for hematoxilin and eosin (H&E), and evaluated under light microscopy. Biopsies were classified according to the ISN/RPS (The 2003 International Society of Nephrology (ISN)/Renal Pathology Society (RPS) 2003 classification of lupus nephritis) [8] as follows: Class I minimal mesangial lupus nephritis (LN). Class II mesangial proliferative. Class III Focal (50 % of glomeruli), active lesions (III A), active and chronic lesions (III A/C), Class IV diffuse (50 % of glomeruli), diffuse segmental (IV-S) or global (IV-G), IV A (active lesions) or IV A/C (active and chronic lesions), IV C (chronic lesions). Class V or membranous. Class VI (advanced sclerosing (90 % globally sclerosed glomeruli without residual activity).

Activity and chronicity were determined by the indicators proposed by Austin et al [9]. The Activity Index (AI) was based on the following histology features: glomerular proliferation, leukocyte exudation, karyorrhexis/fibrinoid necrosis (×2), cellular crescents (×2), hyaline deposits, and interstitial inflammation. A scale of 0 (absent), 1 (mild), 2 (moderate), and 3 (severe) was applied to each histological feature for biopsy specimens, in which six or more glomeruli were examined. Accordingly, the maximum AI score was 24. The Chronicity Index (Cl) was the sum of individual scores of the following features: glomerular sclerosis, fibrous crescents, tubular atrophy, and interstitial fibrosis, the scale of 0 (absent), 1 (mild), 2 (moderate), and 3 (severe) was applied. The maximum CI score was 12.

Seventeen control normal kidney biopsies were obtained during necropsy from subjects without renal pathology, who died in accidents as a result of craneo-encephalic trauma. The Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) was measured when the kidney biopsy was obtained; SLEDAI is an instrument that measures disease activity by weighting the importance of each organ system involved [10].

This investigation was conducted in accordance with the Declaration of Helsinki; biopsies were performed with the consent of the human subjects and or relatives. In the case of control biopsies, and the protocol was approved by the Ethics Committee of each institution participating in this study.

Immunohistochemistry

eNOS, iNOS and citrulline were detected in tissues by immunohistochemistry of 4 μm thick sections of renal tissues mounted on microscope slides. The specimens were dewaxed, permeabilized with 0.01 % Triton X-100/Phosphate buffer saline (PBS) and then washed three times with PBS. Endogenous peroxidase was blocked with horse serum heat inactivated at 56°C. After a further wash, tissues were incubated for 12 hour with rabbit anti-iNOS (sc-651 Santa Cruz, Biotechnology, Inc. Santa Cruz.CA), anti-eNOS (sc-654), or anti-citrulline antibody (cat 231246, Calbiochem, Darmstadt, Germany) diluted 1:1000 in 10 % BFS-PBS. The tissues were then washed in several changes of PBS and bound antibodies were identified with peroxidase-coupled goat anti-rabbit IgG. Color reaction was induced by 2, 2 diaminobenzydine-0.06 % H_2O_2 (Sigma, San Louis, MO) and the reaction stopped with 2N sulphuric acid. The slides were then examined under light microscope. The assays were performed in triplicate and evaluated by two pathologists in a blinded fashion. The intensity of the color reaction obtained by immunohistochemistry was analyzed by the KDS1 program (Kodak) that measures the sum of color intensity in selected pixels.

NO production and citrulline presence

With the rationale that both NO and citrulline are the products in the reaction catalyzed by NOS, the presence of free citrulline was tested by immunohistochemistry. We used an anti-citrulline antibody from Calbiochem (Cat. 231246) that was originally developed for ELISA. Therefore, it was necessary to demonstrate that the cellular production of NO was *bona fide* and was correlated to the intensity of anti-citrulline antibody staining in a well-defined *in vitro* system as follows: Kidneys were excised from adult Balb/c mice and cultured for 12 h in DMEM media in presence of 20 μl of TNF or IL-1 (Sigma, St Louis MO). The nitrite production was measured by Griess method [11]. In addition, tissues were fixed, embedded in paraffin and processed by immunohistochemistry to detect citrulline. Control kidneys cultured without cytokine exposure were included as controls. A mean and standard deviation (SD) was done in ten replicas of this experiment, differences between control and problems was calculated by Student t test.

RNA extraction

Total RNA was extracted from several 4 μm kidney sections (containing a mean of 10 glomeruli per section) by acid guanidium thiocyanate-phenol-chloroform method (TRIzol, GIBCO-BRL Gaithersburg MD). RNA was quantitated by OD at 260 nm.

Oligonucleotides

The primers used for polymerase chain reaction (PCR) were: eNOS forward 5'-CAGTGTGTCCAACATGCTGCTGGAAATTG-3', and backward 5'-TAAAGGTCTTCTTCCTGGTGATGCC-3' of the coding sequence that amplify a 485-bp product; iNOS forward 5'-GGCCTCGCTCTGGAAAGA-3', backward 5'-TCCATGCAGACAACTT-3' of the sequence coding that amplify a 485-bp product [12]. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) forward 5'-TGA AGG TCG GTGTGA ACG GAT TTG GC-3' and backward 5'-CATGTA GGC CAT GAG GTC CAC CAC-3' (Clontech, Palo Alto, CA)

Reverse-transcription/polymerase chain reaction (RT-PCR)

cDNA synthesis was achieved from 250 ng total RNA incubated with 200 mM dNTP, and 0.7 μM of the forward and backward primers were mixed with 5 μl of rTth/DNA polymerase (SuperScript One-Step, Invitrogen LifeTechnology, Carlsbad, CA). Reverse transcription was per-

Table 1. Clinical and histological data of lupus nephritis patients.

Gender	age	Disease duration	SLEDAI	Nephritis Class	Nephritis Activity	Nephritis Chronicity
1-Female	33	5	8	IV-A/C	4	4
2-Male	19	8	6	IV-A/C	4	5
3-Male	40	8	5	III-A/C	4	1
4-Female	26	7	4	IV-A/C	8	4
5-Female	15	7	6	III-A/C	6	3
6-Female	35	5	6	III-A/C	6	2
7-Female	24	10	4	III-A	8	0
8-Female	34	9	5	IV-A/C	14	12
9-Female	32	4	7	IV-A/C	4	3
10-Female	14	1	7	III-A/C	16	11
11-Female	16	4	8	IV-A	6	0
12-Female	30	4	6	IV-A/C	4	5
13-Female	36	1	4	IV-A/C	5	2
14-Female	26	2	8	III-A/C	8	1
15-Male	16	2	8	IV-A/C	10	1
16-Female	26	2	10	III-A/C	8	5
17-Female	19	4	8	IV-A/C	7	5
Mean ± sd	25.9 ± 8.3	4.8 ± 2.8	6.4 ± 1.7	10 = IV, 7 = III	7.2 ± 3.4	3.7 ± 3.4

A = active C = chronic Sd = Standard deviation

Table 2. Immunohistochemistry in kidneys of eNOS and iNOS protein, the intensity of the color reaction was expressed in pixels.

Group	Positive biopsies	Mean + sd	t Test
1- eNOS Lupus Nephritis	17/17	639.8 ± 181.2	1 vs 2 = P < 0.0001; 1 vs 3 = P < 0.0001
2- eNOS Control	17/17	12170 ± 4016	2 vs 3 = P < 0.0001
3- iNOS Lupus Nephritis	12/17*	26210 ± 7146	3 vs 4 = P < 0.0001
4- iNOS Control	0/17	235.4 ± 61.68	4 vs 2 = P < 0.0001
5- Citrulline Lupus Nephritis	12/17*	38170 ± 9115	5 vs 6 = P < 0.0001
6- Citrulline Control	0/17	188.0 ± 58.85	6 vs 4 = 0.0001

*p < 0.001 by Fisher's exact test

formed at 55°C for 30 min. Reaction tubes containing 25 µl of sample mixture were amplified in a thermal cycler (Perkin Elmer, GeneAmp PCR system 2400, Foster City, CA) with 30 cycles under the following conditions: 94°C for 2 min, 55°C for 2 min and 72°C for 1.4 min. At the end of the PCR reaction, the samples were electrophoresed in 2 % agarose containing 0.5 µl ethidium bromide. PCR products were observed under UV light. Band migration and density were documented using an image analysis system by BioRad. Pro-apoptotic transcript levels were determined by comparing with the G3PDH densitometry.

Statistical analysis

Data were processed by Student t test using the Prisma program, and Fisher exact test, p < 0.05 was considered statistically significant (http://www.physics.csbsju.edu/stats/exact2.html).

Results

Histology

The lupus nephritis biopsies included ten that had Class IV and seven with Class III. Patients with Class IV nephritis displayed the highest activity and chronicity scores while control kidneys had no histological evidence of renal disease. The background data of individual patients is shown in Table 1.

eNOS is expressed in all glomeruli

Control kidney tissues broadly expressed the physiologic eNOS, which was mainly detected along the tubules, in

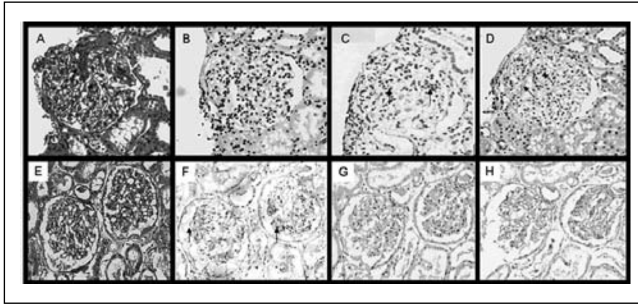

Fig. 1. Immunohistology of lupus nephritis (A, B, C and D) and control (E, F, G and H) kidney biopsies processed by hematoxilin & eosin staining (A and E), and by immunohistochemistry to detect eNOS (B and F), iNOS (C and G) and citrulline (D and H). The expression of eNOS is demonstrated mainly in control biopsies (F), shown as brown color scattered in glomerular loops) and iNOS and citrulline is exclusively present in lupus nephritis (C, arrows pointing out the cell infiltration and proliferation surrounded by iNOS) and citrulline (D) is present in glomerular loops and mesangium.

glomerular endothelial cells and in the mesangium. Although lupus nephritis biopsies showed similar distribution of eNOS, their staining intensities were lower (Table 2). Because the staining color exhibited slight differences from one experiment to another, each experiment was performed with positive and negative controls, and color intensity was measured by an image analyzer program, and values were expressed as sum of intensities in pixels in one hundred fields. Using this approach, significant differences of eNOS between controls and nephritis biopsies were observed (Table 2).

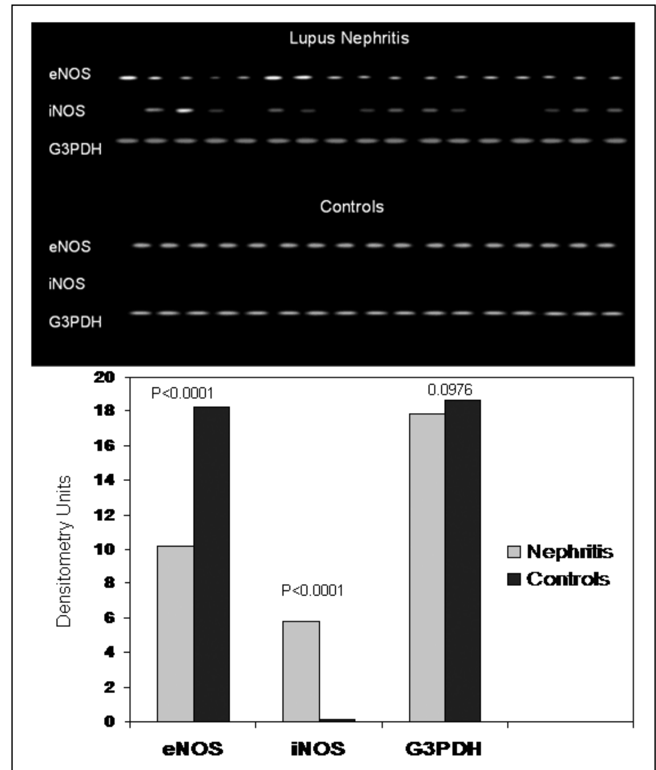

Fig. 2. Agarose gel electrophoresis showing the bands correspondent to reverse transcription–PCR amplification of eNOS, iNOS and G3PDH mRNA from total RNA isolated from lupus nephritis and control kidney biopsies. The presence of iNOS amplificates was observed exclusively in the twelve lupus nephritis specimens.

iNOS expression in lupus nephritis

Since none of the control biopsies displayed a defined iNOS signal, it was assumed that iNOS activity was absent. In sharp contrast, lupus nephritis kidneys broadly expressed iNOS along the glomeruli, tubular epithelia, as well as around cellular infiltrates (Table 2).

iNOS expression in the kidney correlates with the presence of citrulline

NO production is the result of the conversion of L-arginine to L-citrulline, and one indirect way to demonstrate NO production in tissues is to examine the presence of citrulline with anti-citrulline antibodies [13]. Thus, positive staining for citrulline probably reflects iNOS activity. Figure 1 shows that citrulline was not detected in normal kidney tissues, despite the possibility that endothelial NO synthase (eNOS) is available to generate citrulline. This unexpected negative result could be due to NO activity that was lower than eNOS activity. As a consequence, it was almost not detectable by anti-citrulline antibodies used in the present assay. In sharp contrast, citrulline was abundantly detected in glomeruli and tubules of lupus nephritis tissues, and, particularly around the inflammatory infiltrates. Seventy percent of all lupus nephritis biopsies showed the presence of both messenger (m) iNOS and iNOS protein and the presence of citrulline; the remaining 30 % biopsies was negative. (Table 2)

Messengers of iNOS are increased in lupus nephritis

By RT-PCR amplification, iNOS mRNA was detected in 70 % of lupus nephritis biopsies, all class IV and two class III exhibited RT-PCR products, which were associated with the presence of iNOS protein (Table 2 and Figure 2). As expected, all biopsies had detectable physiologic eNOS. G3PDH was used as a housekeeper gene because it is constitutive in eukaryotic cells and is relatively unaffected by external factors. As expected G3PDH expression was not different between lupus nephritis and controls.

Citrulline in tissues means NO activity

Since the present investigation utilized tissue biopsies included in paraffin, it was technically difficult to demonstrate the *in vitro* NO activity as detected by nitrite production. As a consequence, an experiment using normal murine kidneys was performed. When NO production was triggered by TNF and IL-1, an increase of NO concentration as measured by Griess reagent was observed in kidney tissues accompanied by widespread citrulline production and deposition in tubular epithelia and glomeruli. Control kidney tissues were negative in both assays (Figure 3). This *in vitro* finding suggested that the presence of citrulline in kidneys of lupus nephritis is linked to NO production.

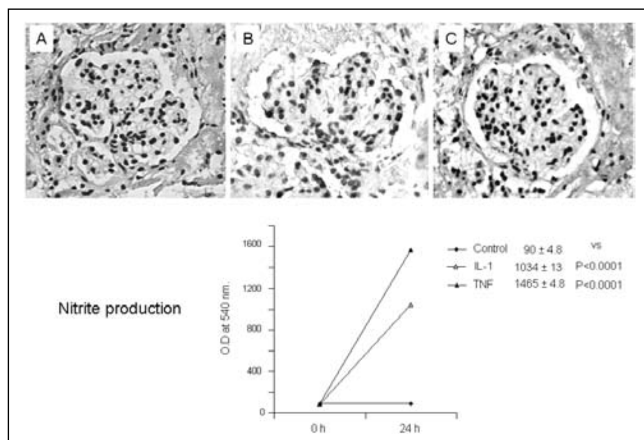

Fig. 3. Citrulline detection by immunohistochemistry in kidneys of Balb/c mice, stimulated with IL–1 and TNF. A. Control at 0 h is negative. B. Kidneys stimulated with IL–1 and C. Kidneys stimulated with TNF are positive for citrulline presence, which is evident in glomerulus. On the inferior panel a graphic showing the nitrite production triggered by these cytokines after 24 hours, and measured by spectrophotometry at 560 nm, by the Griess method. Mean and standard deviation was obtained in ten experiments, and the P value was calculated by Student t test.

Discussion

The aim of the present studies was to determine whether iNOS is present during inflammatory processes of lupus nephritis. The major findings of our studies are:

1) iNOS was detected in 70 % of lupus nephritis biopsies;
2) All class IV lupus nephritis classified according to the ISN/RPS displayed iNOS;
3) the presence of citrulline was correlated with NO production and iNOS expression. These findings suggest that the NO pathway could play a role in the pathogenesis of lupus nephritis.

The participation of the NO pathway in autoimmune glomerulonephritis has been addressed by other investigators who found that normal kidneys constitutively express eNOS, whereas the iNOS species is found exclusively in inflammatory glomerunonephritis [14, 15]. Based on these observations, therapies based on deviation of inflammatory to physiologic NO have been experimentally used. Among these is hemin, a molecule that triggers the inducible form of haem oxygenase (HO-1), which protects tissues by switching inflammatory NO to the physiologic form and is expressed in response to various stimuli such as hydrogen peroxide, heat, heavy metal ions, hyperoxia, hypoxia, endotoxin and inflammatory cytokines [16]. HO-1 prevents the development of inflammatory kidney disease by suppressing the synthesis of IL-1, IL-6 and TNF [17], all of which are also involved in the pathogenesis of lupus nephritis [17]. Moreover, all-trans-retinoic acid (ATRA), a drug that blocks iNOS, has beneficial effects on nephritis and improves renal function of NZB/NZW mice [18].

Citrulline is an amino acid which is a key piece of the NO pathway. Citrulline metabolism in mammals can be found in two forms: free citrulline that is the product of one of the three NOS isoenzymes. The second species of citrulline, is the result of post-translational modification of certain proteins at arginine residues by peptidylarginine deiminases (PADs), which are a group of enzymes that citrullinate (deiminate) protein arginine residues, yielding citrulline residues. Protein citrullination is NOS independent and is important in rheumatoid arthritis [19].

Citrulline can be detected by two different methods. The first one uses diacetylmonoxim and requires the removal of urea prior to the assay [19]. The second, which we used, employs anti-citrulline antibodies, an excellent method to detect citrulline in tissues, which can be used to detect NOS activity [20]. The only weakness of this technique is that it also detects citrullyl residues in tissue proteins [21]. The high association observed in our studies between the presence of citrulline in areas of iNOS expression suggests that iNOS is functionally active and hence the likely result that NO is produced *in situ* in lupus nephritis.

In conclusion, the present studies suggest that NO-related responses may be results of lupus nephritis, and could play a pathogenic role by increasing inflammation and kidney damage. Although the presence of iNOS was previously reported in lupus nephritis [14, 15], the main contribution of the present investigation is the demonstration of significant iNOS activity in lupus nephritis that is accompanied by the presence of citrulline, a finding which was not previously reported. The importance of this work implicated NO as a potential therapeutic target to improve renal function in lupus nephritis.

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