



## Chromatin modified with Nitric Oxide and its Role in Systemic Lupus Erythematosus

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### ABSTRACT

Freshly isolated chromatin from goat liver, exposed to nitric oxide (generated by the reduction of sodium nitrite with sodium dithionite) caused structural alterations and loss of tertiary structure of chromatin along with strand breaks and base modifications in the DNA of chromatin. The NO-chromatin was highly immunogenic in experimental animals as compared to its native form. Induced antibodies were highly specific for their respective immunogen. However, delineating the antigenic specificity of anti-NO-chromatin antibodies by competition ELISA, multiple cross-reactivity was observed. Anti-NO-chromatin antibodies recognized human blood proteins, modified tyrosine polymers and histones. Moreover, NO modified conformers were better recognized than their native forms. The visual detection of immune complex formation with native and NO-chromatin reiterated preferential binding with modified chromatin. Chromatin modified by nitric oxide presents unique epitopes which may be one of the factors in antigen driven autoimmune response in SLE.

**Keywords :** autoantibodies; chromatin; NO-chromatin; nitric oxide; SLE.

### Introduction

Nitric oxide (NO), a multifaceted bioregulatory agent (vasodilator, neurotransmitter) produced by many cell types is known to play a critical role in both regulatory processes and cell defense. It also participates in collateral reactions, leading to DNA damage and cell death in both NO-generating and neighboring cells [1]. It can accelerate DNA base deamination, strand breaks and cause deamination-induced genetic changes in lung cells [2]. Besides these effects, NO also causes tissue injury by attacking the Fe-S centers in various key proteins [3], inhibiting ribonucleotide reductase [4] and several other DNA repair/replication enzymes [5, 6].

SLE is an autoimmune disorder of unknown etiology. Antibodies to dsDNA is a classic antibody that characterizes SLE[7]. However, the dominant presence of antibodies against the exposed conformational epitopes on chromatin strongly suggests that the pathogenic immune response in lupus is driven by chromatin [8,9,10,11,12,13]. The nucleosomes(or chromatin)is emerging as the most reactive substrate among nuclear antigens, 70%-80% of SLE patients being positive. Also, anti-chromatin (or nucleosomes) antibodies might be a good marker for anti-DNA antibodies negative SLE cases [14].

Nitric oxide is generated in vivo by many types of cells through the nitric oxide synthase(NOS) pathway[15,16]. It is an important mediator of the inflammatory response. Activated macrophages produce nitric oxide which damages DNA resulting in mutations and strand breaks[1]. Retrospective studies have suggested a role of nitric oxide in the pathogenesis of SLE by demonstrating elevated levels of nitric oxide in these patients[17,18].In the present study, we have investigated immunogenicity of native chromatin and nitric oxide modified chromatin(NO-chromatin). The notable feature of this investigation is the high degree of recognition of nitric oxide modified conformers of histones, nucleosomes and human blood protein by anti-NO-chromatin antibodies.

### Materials and methods

#### Materials

Sodium nitrite and sodium dithionite were purchased from Bio-Rad laboratories (California, USA). Calf thymus DNA, nuclease S1,methylated bovine serum albumin(BSA), anti-

rabbit IgG alkaline phosphatase conjugate,Freund's complete and incomplete adjuvants, ethidium bromide, Ficoll-400,bromophenol blue p-nitrophenyl phosphate, Tween-20, Triton X-100 were procured from Sigma-Aldrich (St.Louis, MO, USA). Protein A-Sepharose CL-4b was purchased from Genei (India). Polystyrene microtitre flat bottom ELISA plates were procured from NUNC (Denmark).Agarose was purchased from Pharmacia (Sweden). All other chemicals were of analytical grade.

### Methods

#### Isolation of chromatin

Chromatin was isolated from fresh goat liver by the method of Bonner et al [19] with slight modifications. Briefly, 10 g of goat liver was homogenized with 200ml of saline-EDTA (0.075M NaCl, and 0.024M EDTA, pH 8.0). The homogenate was strained through 6-8 layer of cheese cloth. The filtrate was centrifuged at 1500g for 15 min. the pellet was homogenized in 40 ml of Tris buffer (0.05M, pH8.0) and sedimented at 10,000g for 15 min. This step was repeated once. The final pellet was suspended in 30 ml of Tris buffer (0.05 M, pH 8.0). Five milliliter aliquots of the above suspension were layered on 25 ml portions of 1.7 M sucrose (in 0.01 M Tris buffer, pH 8.0) contained in centrifuge tubes. The upper two thirds of each tube were gently mixed and the tubes were then centrifuged at 21,000 rpm for 3h in F0650 rotor (Beckman) at 4°C. The pellets were suspended in 0.01 M Tris buffer, pH8.0, and dialyzed overnight against the same buffer. The dialyzed suspension was sheared in Viritis homogenizer for 90 s, stirred for 30 min and then centrifuged at 10,000g for 30 min. The supernatant of the above is referred to as sheared liver chromatin.

#### Modification of chromatin by nitric oxide

Isolated chromatin was modified with nitric oxide generated by the reduction of sodium nitrite with sodium dithionite [20, 21]. Aqueous solution of chromatin in PBS (A260=2), 20 mM Tris-HCl buffer, pH 7.5, sodium nitrite (100 mM) and sodium dithionite (10 mM) was kept at 37°C for 3 h. Extensive dialysis of the sample was done immediately at the end of incubation time to remove the salts. Native chromatin incubated with salts alone served as control. The UV absorption characteristics of native and NO-chromatin were recorded on a Shi-

madzu UV-240 spectrophotometer. All the other samples like nucleosomes, histones, tyrosine polymers and human serum albumin were similarly modified.

#### Agarose gel electrophoresis

Native and NO-chromatin were mixed with a one-tenth volume of sample buffer (0.125% bromophenol blue, 30% Ficoll-400, 500 mM EDTA in 10x electrophoresis buffer). The samples were loaded in the wells and electrophoresed for 2 h at 30 mA. The gels were stained with ethidium bromide (0.5 µg/ml), viewed by illumination under UV light and photographed.

#### Nuclease S1 treatment

Nitric oxide induced modification was characterized by nuclease S1 treatment [22] followed by agarose gel electrophoresis [23]. One microgram each of native and modified chromatin in acetate buffer (30 mM each of sodium acetate and zinc chloride, pH 5.0) were treated with nuclease S1 (20 units/ µg DNA) for 30 min at 37°C. The reaction was stopped by adding one tenth volume of 200 mM EDTA, pH 8.0. The digested and control samples were subjected to agarose gel electrophoresis.

#### Fluorescence emission spectroscopy

Fluorescence emission spectroscopy of native and NO-chromatin samples was done using Fluorimeter (Hitachi, Japan). Two different excitation wavelengths were chosen a) 280 nm and b) 260 nm. At excitation wavelength, 260 nm, fluorescence emission spectroscopy of native and modified samples were recorded using ethidium bromide. While ethidium bromide alone in sample buffer served as control and was used to set the basal line.

#### Immunization schedule

The immunization of random bred, female, New Zealand white rabbits was performed as described previously [24]. Briefly, rabbits (n= 4; two each of native and NO-chromatin antigens) were immunized subcutaneously at multiple sites with 50 µg of antigen emulsified with an equal volume of complete Freund's adjuvant. The animals were boosted intramuscularly in Freund's incomplete adjuvant at weekly intervals for 5 weeks with the same amount of antigen. Test bleeds were performed 7 days post boost which gave appropriate titre of the antibody. The animals were bled and the serum separated from the blood (preimmune and immune) was decomplexed by heating at 56°C for 30 min.

#### Purification of antibodies

Immunoglobulin G was isolated from immune sera on a Protein A-Sepharose 4B affinity column [25]. The homogeneity of isolated IgG was checked by performing 7.5% SDS-polyacrylamide gel electrophoresis.

#### ELISA

An enzyme linked immunosorbent assay (ELISA) was performed on flat bottom 96 well polystyrene immunoplates [26]. Briefly, the plates were coated with 100 µl of respective antigen (2.5 µg/ml) for 2h at room temperature and overnight at 4°C. After washing three times with TBS-T (20mM Tris, 2.68 mM KCl, 150 mM NaCl, pH 7.4 containing 0.05 % Tween 20), unoccupied sites were blocked with 1.5% bovine serum albumin in TBS (10 mM Tris, 150 mM NaCl, pH 7.4) for 4-6 h at room temperature. The test serum serially diluted in TBS-T (100 µl/well) was adsorbed for 2 h at room temperature and overnight at 4°C. Bound antibodies were assayed with anti-human IgG alkaline phosphatase conjugate using p-nitrophenyl phosphate as substrate. The absorbance of each well was monitored at 410 nm on an automatic microplate reader. Equal number of wells devoid of antigen coating served as controls and the plate was thereafter processed similarly.

#### Competition ELISA

Antibody specificity was ascertained by competition binding assay [27]. Varying concentrations of inhibitors (0-20 µg/ml) were allowed to interact with a constant amount of antibody (1:100 diluted serum) for 2 h at room temperature and over-

night at 4°C. The mixture was added to antigen coated plates and the bound antibody was detected as in the direct binding ELISA.

#### Band shift assay

Band shift assay was performed for the visual detection of antigen antibody binding and immune complex formation [28]. Electrophoresis was performed on 1 % Agarose gel in 40 mM Tris-acetate buffer (TAE), pH 8.0. A constant amount of antigen (2.0 µg) was incubated with increasing concentration (0-80 µg) of affinity purified IgG for 2h at room temperature and overnight at 4°C before loading onto the gel. The gel was run at 30 mA for 2h, stained with ethidium bromide and visualized under UV light.

#### Results

##### Characterization of NO-chromatin

Freshly isolated chromatin was modified by nitric oxide exposure and the induced changes in NO-chromatin were analyzed by UV absorption spectroscopy. The absorption spectra of NO-chromatin showed loss of peak sharpness and hyperchromicity (51%) at 260 nm relative to native chromatin. The modifications incurred on chromatin were also analyzed by UV-difference spectroscopy. The spectral curve exhibited increased absorbance at 240 nm (data not given).

Agarose gel electrophoresis of native and NO-chromatin was performed on 1% gel. Single homogenous bands were observed, though there was more stretching seen in case of modified chromatin as compared to its native form (Fig. 1). The generation of single strand breaks in NO-chromatin was demonstrated by nuclease S1 digestibility followed by agarose gel electrophoresis. On digestion, the data (Fig. 2) showed substantial decrease in intensity in case of NO modified sample. However, the intensity loss in case of unmodified chromatin was substantially less as compared to its modified sample.

Fluorescence emission spectra of native and NO-chromatin was taken at two different excitation wavelength, 260 nm and 280 nm. At 260 nm, using ethidium bromide in the samples, there was marked decrease in the fluorescence intensity of NO-chromatin as compared to native chromatin (Fig. 3a), indicating generation of DNA strand breaks and alterations in the structure of chromatin due to the modification by nitric oxide. However, at wavelength 280 nm, there was marked increase in fluorescence intensity of NO-chromatin as compared to native chromatin (Fig. 3b). This may be due to the exposure of chromophoric groups and/or unfolding of proteins in chromatin leading to overall structural alterations occurring due to radical modification.

##### Antigenicity of NO-chromatin

Native chromatin was found to be immunogenic in rabbits with a titre of at least 1:1600. While NO-chromatin induced a high titre antibodies of >1:12800. Protein A-Sepharose isolated (purified) IgGs exhibited strong binding to the respective immunogen (Fig. 4). Pre-immune IgGs as negative control showed negligible binding.

Competition ELISA was performed using immunogen: DNA, histones, blood proteins, tyrosine polymer and modified form of nucleosomes as inhibitors to ascertain the antigenic binding specificity of induced antibodies.

##### Immuno-cross reactivity of anti-native chromatin antibodies

A maximum of 73% inhibition of anti-native chromatin antibodies with immunogen as inhibitor was observed. 50% inhibition was achieved with only 13.7 µg/ml of native chromatin. While 33% inhibition with NO-chromatin as inhibitor for induced antibodies was seen. Native calf thymus DNA (nCT DNA) and reactive oxygen species (hydroxyl) ROS-400bp DNA showed only 20% and 11% inhibition respectively. On the other hand, native nucleosomes as inhibitor showed maximum inhibition of 57% with 17.5 µg/ml for 50% inhibition. While ROS-

nucleosome and NO-nucleosome showed 22% and 24% inhibition respectively. Also 18% and 12% maximum inhibition with histone(H2A) and its nitric oxide modified form(NO-HSA) respectively was achieved. Poly-L-tyrosine, nitric oxide modified poly-L-tyrosine (NO-poly-L-tyrosine) and peroxyxynitrite modified poly-L-tyrosine (ONOO<sup>-</sup> -poly-L-tyrosine) gave only 14%, 11% and 12% inhibition at 20 µg/ml inhibitor concentration respectively. Human serum albumin (HSA) and ROS-HSA showed 54% and 37% inhibition respectively with 18.5 µg/ml HSA required for 50% inhibition (Table 1).

**Table 1**  
**Antigen binding specificity of anti-native chromatin and NO-chromatin antibodies**

Inhibitor	Native chromatin Maximum % inhibition at 20 µg/ml	Con. For 50% inhibition (µg/ml)	NO-chromatin Maximum % inhibition at 20 µg/ml	Con. For 50% inhibition (µg/ml)
Native chromatin	73	13.7	58.5	17.0
NO-chromatin	33	-	99.4	10.16
Native calf thymus DNA	20	-	15	-
ROS-400bp DNA	11	-	19	-
Native nucleosome	87	17.5	52	19.2
ROS-nucleosome	22	-	59	16.9
NO-nucleosome	24	-	68	14.7
Histone (H2A)	18	-	29	-
NO-Histone(H2A)	12	-	48	-
Poly-L-tyrosine	14	-	20	-
NO-poly-L-tyrosine	11	-	38	-
ONOO <sup>-</sup> -poly-L-tyrosine	12	-	45	-
Human serum Albumin (HSA)	54	18.5	62	16.1
ROS-HSA	37	-	65	15.3

#### Immuno- crossreactivity of anti-NO- chromatin antibodies

The anti-NO-chromatin antibodies exhibited wide range of heterogeneity as demonstrated by inhibition assay using various inhibitors. A maximum of 98.4% inhibition with immunogen as inhibitor was observed with 50% inhibition achieved at 10.2 µg/ml of NO-chromatin. The induced antibodies recognized native chromatin and showed 58.5% maximum inhibition with 17 µg/ml required for 50%inhibition of antibody binding. Native calf thymus DNA and ROS-400bpDNA showed only 15% and 19% inhibition respectively. On the other hand, moderate to high inhibition was seen with native nucleosome, ROS-nucleosome and NO-nucleosome which was 52%, 59% and 68% respectively, while 50% inhibition was achieved at 19.2 µg/ml, 16.9 µg/ml and 14.7 µg/ml respectively. Also, 29% and 48% inhibition was achieved with histone (H2A) and NO-H2A as inhibitors respectively. While poly-L-tyrosine, NO-poly-L-tyrosine and ONOO<sup>-</sup> -poly-L-tyrosine as inhibitors showed 20%, 38% and 46% inhibition respectively. Blood proteins like HSA and ROS-HSA showed maximum inhibition of 62% and 65% with anti-NO-chromatin antibodies respectively. 50% inhibition was achieved at 16.1 µg/ml and 15.3 µg/ml concentration respectively (Table 1).

The formation of immune complex of NO-chromatin and anti-NO-chromatin antibodies was visualized by band shift assay. As clearly evident with increasing amount of IgG the formation of high molecular weight immune complex increased, as judged by their fluorescence intensity in the wells, whereas retardation of chromatin bands with decreased intensity of unbound antigen was seen( Fig. 5a). The recognition of native chromatin by anti-NO-chromatin IgG was similarly demonstrated by shift in electrophoretic mobility with proportional decrease in intensity of unbound chromatin upon immune complex formation which is consistent with the data in table 1 (Fig. 5b).

#### Discussion

Nitric oxide (NO), a multifaceted bioregulatory agent, shows an unusual divergence of action. It is known to play a major role as neurotransmitter and a regulator of blood pressure. Also, nitric oxide is an important mediator of the inflammatory response. Over expression of NOS2 and nitric oxide are parallel with the development of an autoimmune syndrome with variety of inflammatory manifestations like SLE, rheumatoid arthritis and multiple sclerosis [29,30,31]. Nitric oxide is known to cause cell injury via variety of mechanisms including disruption of mitochondrial respiration, enzyme inhibition, lipid peroxidation and genetic mutation. Toxicity is largely mediated through intermediates such as N<sup>o</sup>O<sup>o</sup> and peroxyxynitrite, involving nitric oxide with either molecular oxygen or

ROS. Though, a high concentration of nitric oxide generated by the induction of NOS is required [32,33,34]. Macrophages use nitric oxide as a cytotoxic agent which may lead to DNA damage. The effects of nitric oxide at the DNA level are complex and involve formation of N-nitrosamines, deamination of purines and pyrimidines or damage induced by peroxyxynitrite [5,6,35]. There is also possibility of intrastrand cross-linking via attack of any diazonium ion of one purine on the free amino group of a second purine in the matching strand [36].

Nitric oxide radical caused substantial damage to chromatin, as evident from the loss of peak and hyperchromicity observed at 260 nm as compared to its native form. This could be attributed to strand breaks and base modifications in DNA of chromatin. No change was observed in chromatin incubated with either sodium nitrite or sodium dithionite alone.

Formation of single stranded regions in modified chromatin was confirmed by nuclease S1 digestibility of native and modified chromatin. The intensity loss in case of native chromatin can be attributed to some single stranded regions already present in unmodified sample (sheared liver chromatin). However; the intensity loss in modified sample was more, showing the formation of single stranded regions on exposure to nitric oxide. The data is compatible with earlier reports indicating nitric oxide radical result in strand breaks [22, 37]. Also, fluorescence studies of NO-chromatin compared with native chromatin showed prominent changes in spectral studies. It confirmed the alterations in structural organization of chromatin incurred by nitric oxide exposure.

Chromatin itself is immunogenic but in diseases like SLE, it becomes more immunogenic[38,39,40]. This might be due to the exposure of chromatin to various free radicals including nitric oxide radicals altering its structure and rendering it more immunogenic. Both native and modified chromatin was found to be immunogenic in rabbits. But NO-chromatin was found to be highly immunogenic as compared to its unmodified form. Antibodies raised against native and NO-chromatin were further determined for their antigenic binding characteristics with various inhibitors. Besides showing strong recognition for their respective immunogen, anti-NO-chromatin antibodies showed cross-reactivity with native chromatin as well. This may be due to the common antigenic determinant to both NO-chromatin and native chromatin. Gel retardation data reiterated the preferential recognition of NO modified chromatin over native chromatin by the anti-NO-chromatin antibodies. However, pure ds DNA or ROS modified DNA fragment (ROS-400 bp) did not appreciably recognized the induced antibodies. The data suggests that DNA alone may not be responsible for antibody generation rather quaternary DNA-protein complex is the neoepitope recognized by the antibodies against native and modified chromatin. Also, native nucleosomes, ROS-nucleosome and NO-nucleosomes showed moderate to high percent cross-reactivity for antibodies generated against native and NO-chromatin.

On the other hand, histone(H2A), a core binding protein in nucleosomes, showed negligible inhibition with anti-native chromatin IgG as compared with anti NO- chromatin IgG(moderate inhibition). It is reported that antigenicity of histones bound to chromatin is markedly diminished as compared to histones free in solution[41]. However, histones on modification with nitric oxide generate similar epitopes as recognized by anti-NO-chromatin antibodies. 3-Nitrotyrosine is a biological marker of oxidative damage by nitric oxide[42,43]. Therefore, in case of anti-NO-chromatin IgG, native tyrosine polymer (poly-L-tyrosine) used as inhibitor, showed negligible inhibition compared to tyrosine polymer modified with nitric oxide and one of its radical, peroxyxynitrite. Thus, tyrosine on initiation generates epitopes for antibody recognition similar to epitopes generated by NO-chromatin. Similarly human blood protein like human serum albumin (HSA) and its ROS modified forms show moderate to high recognition for antibodies indicating similar epitope sharing or cross-reactivity. Analysis of the data indicates that anti-NO-chromatin IgG is immunogenic specific

and the varying degree of cross-reactivity is due to the sharing of common antigenic determinants.

Several studies have reinforced the concept that SLE is an anti-chromatin disease i.e. the auto immune response is against the chromatin with development of antibodies directed to various epitopes on chromatin including but not limited to DNA alone[40,41,44]. It has been proposed that a defect in the catabolism of chromatin may render it immunogenic. The change in chromatin's antigenicity by environmental factors and genetic background may be the common pathway to SLE pathogenesis [13]. Our preliminary studies using protein A-Sepharose 4B purified SLE autoantibodies from SLE sera showed higher inhibition by NO-chromatin in comparison to native chromatin and native calf thymus DNA, when the immobilized antigen was native calf thymus DNA. The present study clearly shows the perturbations in chromatin by nitric oxide radical rendering it highly immunogenic and the neo-epitopes might play a role in the induction of circulating autoantibodies in SLE.

**Legend**



Figure 1. Agarose gel electrophoresis of native chromatin (lane 1) and NO-chromatin (lane2). Electrophoresis was carried out on 1% agarose gel for 1 hr at 30 mA.

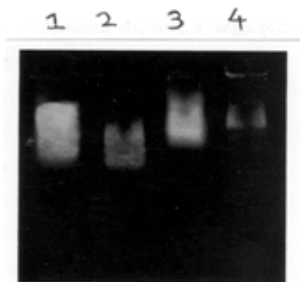


Figure 2. Nuclease S1 digestibility of native and NO-chromatin. Lane1 contained native chromatin, while lane 2 contained native chromatin treated with nuclease S1. Lane3 contained NO-chromatin; while lane4 contained NO-chromatin treated with nucleaseS1 for 30min. Electrophoresis was carried out on 1% agarose gel for 2hr at 30mA.

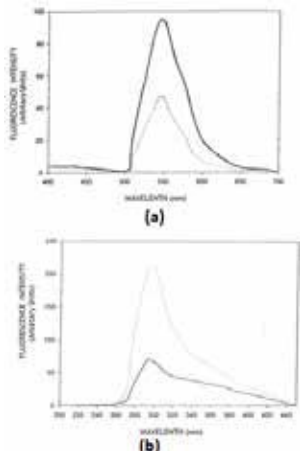


Figure 3. Fluorescence emission spectrum of native chromatin (—) and NO-chromatin (...). Excitation wavelength was (a) 260nm and (b) 280nm.

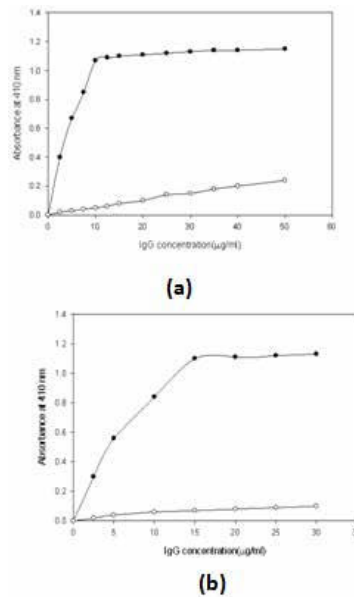


Figure 4. Direct binding ELISA of affinity purified (a) anti-native chromatin immune IgG (•) and preimmune IgG (.) to native chromatin. (b) anti-NO-chromatin immune IgG (•) and preimmune IgG (.) to NO-chromatin. Microtitre plates were coated with respective immunogen (2.5 µg/ml).



Figure 5. Band shift assay of anti-NO-chromatin IgG binding to NO-chromatin and native chromatin. (a) NO-chromatin (2 ug) was incubated with buffer (lane1) and 20, 30, 50 and 60ug IgG through lanes 2 to 5 respectively. (b) Native chromatin (2 ug) was incubated with buffer (lane1) and 20, 30, 50 and 60ug IgG through lanes 2 to 5 respectively for 2 hr at 37°C and overnight at 4°C. Electrophoresis was carried out on 1% agarose gel for 2 hr at 30 mA.

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