Immunological studies of oxidized superoxide dismutase in patients with systemic lupus erythematosus

Correlation with disease induction and progression

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ABSTRACT

Objectives: To investigate the status and contribution of oxidized superoxide dismutase (SOD) in systemic lupus erythematosus (SLE), and to explore whether oxidized-SOD has a role in disease induction and progression.

Methods: This study was performed in the College of Medicine, Qassim University, Buraidah, Kingdom of Saudi Arabia between October 2011 and May 2012. The study was designed to explore the role of oxidized-SOD in SLE autoimmunity. The SOD was modified by reactive oxygen species (ROS) and characterized. Binding characteristics of autoantibodies in SLE patients (n=50) with varying levels of disease activity according to the SLE Disease Activity Index (SLEDAI) against ROS-modified-SOD (ROS-SOD) were screened by immunoassays and the results were compared with healthy age-matched controls (n=34).

Results: The ROS caused extensive damage of SOD. Serum analysis showed significantly higher levels of anti-ROS-SOD antibodies in SLE patients compared with controls. Interestingly, not only was there an increased number of subjects positive for anti-ROS-SOD antibodies, but also the levels of these antibodies were significantly higher among SLE patients, whose SLEDAI scores were ≥20. In addition, a significant correlation was observed between the levels of anti-ROS-SOD antibodies and the SLEDAI score (r=0.796).

Conclusion: Our findings show an association between oxidized-SOD and SLE. The stronger response observed in patients with higher SLEDAI scores suggests that oxidized-SOD may be a useful biomarker in evaluating the progression of SLE and in elucidating the mechanisms of disease pathogenesis.


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Systemic lupus erythematosus (SLE; also referred to as “lupus”) is a chronic autoimmune, multisystem, and multifactorial disease characterized by the presence of autoantibodies to nucleic acid, proteins, and nucleoprotein complexes. The initial immunizing immunogen(s) that drive the development of SLE are unknown, but characteristics of the immune response in SLE suggest that it is an antigen-driven condition. The current therapies for SLE are only based on the concept of nonspecific immunosuppression, and consist of nonsteroidal anti-inflammatory drugs (NSAIDS), corticosteroids, anti-malarials, and cytotoxic drugs, despite given relief, all of which have serious adverse side effects including organ damage. In recent years, free radical-mediated biomolecular damage has drawn considerable attention as the potential mechanism of SLE pathogenesis. Findings from studies using an autoimmune-prone MRL+/+ mouse model have also suggested an association between oxidative stress and autoimmunity. Now, it is well documented that the number of proteins were found to be oxidatively modified leading to the formation of neoantigens, which could in turn initiate SLE autoimmunity. However, the potential role of oxidized proteins in the pathogenesis and progression of SLE remains unresolved.

Superoxide dismutase (SOD) is an important antioxidant enzyme that detoxifies the effects of superoxide, thus limiting the deleterious effects of reactive oxygen species (ROS). Hence, SOD has been considered an important regulator of oxidative stress. It is documented that sera from patients with SLE contain high levels of antibodies to native SOD, but it is not clear why this prevalent extracellular protein becomes antigenic in this chronic condition. Therefore, we thought that SOD may be continuously exposed to oxidative stress, so that alterations in conformation and function of SOD may occur, which may result in modification of its biological properties. In view of these, we hypothesized that oxidative by-products, like hydroxyl radical damage SOD, help to initiate autoimmunity in SLE. To test this hypothesis, we studied the presence of circulating autoantibodies in SLE patients sera directed against ROS-modified SOD (ROS-SOD), and analyzed their relationship to the extent of disease activity according to the SLE Disease Activity Index (SLEDAI). Our results not only support an association between ROS-SOD and SLE, but also suggest that ROS damaged to SOD may be an important biomarker for the evaluation of SLE progression and in the elucidation of the mechanisms of disease pathogenesis.

Methods. Study design and human subjects. The study was performed in the College of Medicine, Qassim University, Buraidah, Kingdom of Saudi Arabia between October 2011 and May 2012. The present study was designed to investigate the role of oxidized SOD in SLE autoimmunity, and to explore whether oxidized SOD has a role in disease induction and progression. The study group included 49 Saudi female patients and one Saudi male with SLE (age 29.5±6.5 years). Inclusion criteria of the patients were based on the clinical diagnosis of SLE as defined by the American College of Rheumatology 1997 criteria, and patients must have SLEDAI greater or equal to 4, must not be pregnant, 18 years of age or older, and updated vaccinations prior to the blood collection. Exclusion criteria of the patients were based on the following points: patients have active peripheral neurologic disease, have evidence, or test positive for hepatitis B, hepatitis C, or human immunodeficiency virus (HIV) positive, and have evidence of active or latent tuberculosis. The SLEDAI score was determined using the Systemic Lupus Activity Measure, and the SLEDAI scores among SLE patients ranged from 9-43 (20.8±8.01). The SLE patients were divided into 2 groups based on lower versus higher SLEDAI scores, in which the group of patients with low SLEDAI scores (SLEDAI score <20) comprised 21 SLE patients, and those with high SLEDAI scores (SLEDAI score≥20) comprised 29 SLE patients. The control group comprised 34 healthy female subjects (age 24.1±24.5 years). The racial or ethnic compositions of the SLE groups were comparable with those of the control group. The study was carried out in accordance with the code of ethics of the World of Medical Association (Declaration of Helsinki) for humans and was approved by the ethical review board committee, College of Medicine, Qassim University.

Modification of superoxide dismutase. Superoxide dismutase (catalog # S7571, Sigma-Aldrich, St Louis, MO, USA) was modified in phosphate buffer solution (PBS) (10 mM sodium phosphate buffer containing 150 mM NaCl, pH 7.4) by our published procedure. Briefly, an aqueous solution of SOD (1 mg/ml) was modified by hydroxyl radicals, generated by the irradiation (30 minutes) of hydrogen peroxide (30 μM) at 254 nm. Excess hydrogen peroxide (Sigma, St Louis, MO, USA) was removed from the samples by extensive dialysis (dialysis tubing was from Sigma) against PBS, pH 7.4.

Assay of carbonyl formation. Carbonyls contents of native and ROS-modified SOD were analyzed as described previously. Briefly, the reaction mixture containing 15 μM of protein samples, 0.5 ml of 10
mM 2, 4-dinitrophenylhydrazine (DNPH)/2.5 M HCl was added and thoroughly mixed. Oxidized proteins in the reaction mixture were precipitated by the addition of 20% (w/v) trichloroacetic acid (TCA, Sigma, St Louis, MO, USA) and the pellet was collected by centrifugation (Eppendorf Centrifuge, Hamburg, Germany). Ethanol and ethyl acetate mixture (1:1 of 1ml) were used to wash the pellet (3 times). The pellet was then dissolved in 1 ml of 6 M guanidine solution and was incubated for 15 minutes at 30°C. After incubation, the reaction mixture was centrifuged and the supernatant was collected for carbonyl contents estimation. Carbonyl contents were calculated by the absorbance difference between test and control using the molar absorption coefficient of 22,000 M⁻¹cm⁻¹ at 370 nm. Protein concentration was determined in the samples and carbonyl contents were expressed as nmol/mg protein.

Fluorescence measurements. Fluorescence measurements were performed on Anthos Zenyth 3100 Multimode Detectors (Salzburg, Austria). Different excitation and emission slits were set, and intrinsic fluorescence was recorded by using native and ROS-modified SOD (1 mg/ml). Decrease of fluorescence intensity (FI) was calculated using the following equation:

\[
\% \text{ Decrease of FI} = \left[ \frac{(\text{FI}_{\text{native SOD}} - \text{FI}_{\text{ROS-SOD}})}{\text{FI}_{\text{native SOD}}} \right] \times 100
\]

Enzyme-linked immunosorbent assays (ELISA). Direct binding ELISA was performed on flat bottom 96-well, polystyrene maxiSorp immunoplates (Catalog # P8616; Nunc-ImmunoTM MicroWell, Sigma-Aldrich, St Louis, MO, USA) as described previously with some modifications. Briefly, polystyrene polysorp immunoplates were coated with 100 µl of native or modified SOD (10 µg/ml) in carbonate buffer (0.05 M, pH 9.6). The plates were coated for 2 hours at room temperature (RT) and overnight at 4°C. Each sample was coated in duplicate and half of the plates served as control devoid of only antigen coating. Unbound antigen was washed with PBS-T (10 mM, 150 mM NaCl, pH 7.4 containing 0.05% Tween-20; Sigma, St. Louis, MO, USA) and unoccupied sites were blocked with block buffer (PBS containing 1% BSA) for 1-2 hours at RT. After incubation, the plates were washed with PBS-T. The test serum (1:100 in PBS-T (100 µl/well)) was adsorbed for 2 hour at RT and overnight at 4°C. Bound antibodies were analyzed with anti-human HRP linked conjugate (catalog # sc2769, Santa Cruz Biotechnology, CA, USA) using 3,3′,5,5′-Tetramethylbenzidine substrate (TMB, catalog #206697A, Santa Cruz Biotechnology, USA). Reaction was stopped by stop solution (2M H2SO4) and the absorbance of each well was recorded at 405 nm on an automatic microplate reader (Anthos Zenyth 3100 Multimode Detectors, Salzburg, Austria).

Competitive binding assays. Antibody specificity was determined by competitive inhibition ELISA as previously described. Inhibitors (20 µg) were allowed to interact with a constant amount of serum samples for 2 hours at 37°C and overnight at 4°C. Formed immune complexes were coated in the wells instead of serum. The remaining steps were the same as in direct binding ELISA. Percent inhibition was calculated using the formula:

\[
\% \text{inhibition} = \left[ 1 - \left( \frac{A_{\text{inhibited}}}{A_{\text{uninhibited}}} \right) \right] \times 100
\]

Statistical analysis. Results are expressed as the mean±SD unless stated otherwise. One-way ANOVA of variance followed by Tukey-Kramer multiple comparisons test, and two-way ANOVA of variance followed by Bonferroni comparisons test. P<0.05 was considered significant. Spearman’s rank correlation was used to calculate correlation coefficients for associations between serum levels of anti-ROS-SOD antibodies and SLEDAI scores. All statistical analysis was carried out using Graph Pad Prism version 5.0 (Graph Pad Software Inc., San Diego, CA, USA) or Origin software package version 5.0 (Origin Lab Corporation, Northampton, MA, USA).

Results. Characterization of reactive oxygen species modified SOD. Native SOD was modified by hydroxyl radicals, generated by ultraviolet (UV) irradiation of hydrogen peroxide and the induced changes in SOD were analyzed by UV absorption spectroscopy. The UV absorption spectra of ROS-SOD revealed a marked hypochromicity (42.9%) at 280 nm (Figure 1A). The generation of hydroxyl radicals in the reaction mixture was confirmed by using dimethyl sulfoxide (DMSO; 20 mM), specific quenchers of hydroxyl radicals. The data showed decreased absorbance at the 240-320 nm range in the presence of DMSO, whereas high absorbance in this range occurred in the absence of this quencher (Figure 1). Oxidation of SOD was further evident by the increase of 64.2% carbonyls contents (P<0.0001). The generation of hydroxyl radicals was further confirmed by using DMSO (20 mM). Our novel results showed that carbonyl formation was significantly reduced in the presence of DMSO (P<0.05; Figure 1B). The ROS-induced oxidative modification of SOD was also studied by tryptophan intrinsic fluorescence...
using an excitation wavelength 295 nm. Our results showed that FI at 340 nm of emission wavelength was 47.35% decreased, when the protein was excited at 295 nm ($p<0.05$; Figure 2A). Oxidative modification in tryptophan residues of SOD was further confirmed by the loss of 61.2% FI at the 320 nm of emission wavelength using an excitation wavelength 280 nm ($p<0.05$; Figure 2B). Complete characterization of native and ROS-modified SOD has been summarized in Table 1.

Detection of anti-ROS-SOD antibodies in the sera of SLE patients. In an attempt to understand the role of oxidative damage of SOD in the pathogenesis of SLE, we determined the serum levels of ROS-SOD specific antibodies in patients with SLE (Figure 3). Sera from 50 SLE patients and 34 normal human (NH) subjects were tested for binding to SOD and ROS-SOD by specific ELISAs. Our data shows that the majority of SLE sera showed strong binding to ROS-SOD over native SOD at 1:100 serum dilution ($p<0.0001$) (Figure 3A). The average absorbance at 405 nm (±SD) of 50 SLE sera binding to ROS-SOD and SOD was 0.61±0.38 and 0.21±0.19. Single-stranded DNA (ssDNA) was used as immunochemical markers for SLE. Almost all tested SLE sera showed strong binding with ssDNA (Figure 3A). Whereas, NH sera showed negligible binding with native and ROS-modified SOD or with nucleic acid antigen. The absorbance at 405 nm (±SD) of 34 NH sera binding with ROS-SOD was 0.17±0.17, SOD was 0.16±0.15, and ssDNA was 0.10±0.09 (Figure 3B). The SLE autoantibodies directed to ROS-SOD were further evaluated by competitive binding assays. The specificity of SLE serum autoantibodies from SLE patients was screened towards ROS-SOD and SOD (used as inhibitors), whereas microtiter plates were coated with ssDNA. The average percent inhibition (±SD) of SLE autoantibodies towards ssDNA by ROS-SOD was 30.35±18.5, and SOD was 17.9±10.3 (Figure 4A). The data reveal striking differences in the recognition of ROS-SOD and SOD by SLE autoantibodies ($p<0.0001$). Similar experiments with normal human (NH) sera were performed, NH sera showed negligible binding with either of the antigen ($p>0.05$; Figure 4B).

The SLEDAI-related increase in serum levels of anti-ROS-SOD antibodies in SLE patients. To validate our hypothesis, we assessed the increases in serum levels of anti-ROS-SOD antibodies as a function of the SLE disease activity index (SLEDAI) score (Figure 5). As evident in Figure 5A, levels of anti-ROS-SOD antibodies in all of the SLE patients (both those with SLEDAI scores≥20 and those with SLEDAI scores<20) were significantly higher in comparison with healthy controls ($p<0.0001$). Interestingly, levels of anti-ROS-SOD antibodies in SLE patients sera (both in those with SLEDAI scores≥20 and in those with SLEDAI scores<20) were significantly higher than those with the levels of anti-nSOD antibodies ($p<0.0001$) (Figure 5A). Remarkably, an increase in levels of anti-ROS-SOD antibodies in SLE patients sera (both in those with SLEDAI scores≥20 and in those with SLEDAI scores<20) was significantly higher than those with the levels of anti-nSOD antibodies ($p<0.0001$) (Figure 5A).

**Figure 1** - Characterization of reactive oxygen species modification of superoxide dismutase. A) Ultraviolet (UV) absorption spectra of native superoxide dismutase (nSOD = black circle), reactive oxygen species (ROS)-modified SOD (ROS-SOD; black rectangle) and ROS-SOD in the presence of dimethyl sulfoxide (ROS-SOD+DMSO; black triangle). The SOD (1 mg/ml) was modified by ROS, generated by the UV irradiation (30 min) of hydrogen peroxide (10 µM) at 254 nm. B) Carbonyl contents in nSOD, ROS-SOD, and ROS-SOD in the presence of DMSO. Protein concentration in all samples was 1 mg/ml. Each histogram represents the mean±SEM of 5 independent assays. *$p<0.0001$ versus carbonyl contents present in nSOD; †$p<0.023$ versus carbonyl contents present in ROS-SOD.
antibodies in patients with SLEDAI scores≥20 was greater in comparison with those in patients with SLEDAI scores<20, suggesting a positive association between the increase in anti-ROS-SOD antibodies, and SLE disease activity.

Correlation of serum anti-ROS-SOD antibodies with SLEDAI. To further evaluate the significance of ROS-modified SOD in SLE, the relationship between the increases in the serum levels of anti-ROS-SOD antibodies and the SLEDAI scores was analyzed (Figure 5B). A significant correlation was observed between the serum levels of anti-ROS-SOD antibodies and the SLEDAI scores (r=0.642, p<0.0001) (Figure 5B).

To further strengthen our findings, we also investigated the relationship between the serum levels of anti-native (n) SOD antibodies and the SLEDAI scores. Our results showed that no correlation was observed between the serum levels of anti-nSOD antibodies and the SLEDAI scores (r=0.161, p>0.05) (Figure 5C). These results not only further support the potential role of oxidized SOD in SLE, but also suggest that the serum levels of antibodies against oxidized SOD may be useful in predicting the progression of SLE.

Discussion. Oxidative modification of proteins, nucleic acid, and lipids has been implicated in the etiology of numerous disorders including SLE. In fact, proteins are better candidates than plasma lipids, and nucleic acids for use in detecting specific pathways of oxidative stress, due to the accessibility of plasma proteins for sampling, their relatively long half-lives, and their well-defined biochemical pathways. Therefore, oxidized plasma proteins can serve as important in-vivo biomarkers of oxidative stress.

In the present study, commercial SOD was modified by hydroxyl radical, generated by the UV irradiation of hydrogen peroxide, resulting in extensive damage of SOD protein as evident by 42.9% UV hypochromicity at 280 nm. The observed hypochromicity could be due to the modification of chromophoric groups, modification of aromatic amino acid residues of SOD, or due to structural alterations. The ROS-induced alterations in tryptophan residues of SOD were studied by tryptophan intrinsic fluorescence using excitation wavelengths of 295 nm and 280 nm. Oxidation of tryptophan residues of SOD upon ROS modification was evident by the loss of intrinsic fluorescence intensity, when the protein was excited at 295 nm, these oxidative modifications in tryptophan residues were further confirmed by exciting the protein at 280 nm. The SOD upon ROS modification was confirmed by the changes in the fluorescence intensity after exciting the protein at different wavelengths (Table 1). Protein carbonyl contents are the most commonly used biomarker

Table 1 - Characterization of native and reactive oxygen species (ROS)-modified superoxide dismutase (SOD) under identical experimental conditions.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Native SOD (mean±SEM)</th>
<th>ROS-SOD (mean±SEM)</th>
<th>Modifications %</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV absorbance at 280 nm</td>
<td>0.6880±0.12</td>
<td>0.3931±0.04</td>
<td>42.9 hypochromicity</td>
</tr>
<tr>
<td>Fluorescence intensity, AU (Em. 295 nm; Ext. 340 nm)</td>
<td>330±24.6</td>
<td>179±34.1</td>
<td>47.35 decreased</td>
</tr>
<tr>
<td>Fluorescence intensity, AU (Em. 280 nm; Ext. 320 nm)</td>
<td>411±21.7</td>
<td>159±17.7</td>
<td>61.2 decreased</td>
</tr>
<tr>
<td>Carbonyl contents (nmol/mg protein)</td>
<td>5.4±0.9</td>
<td>15.1±1.9</td>
<td>64.2 increased</td>
</tr>
</tbody>
</table>

UV and fluorescence studies were performed in duplicate independent experiments, whereas, carbonyl contents were estimated in 5 independent experiments. SEM - standard error of the mean, UV - ultraviolet, AU - arbitrary unit, Ext - excitation wavelength, Em - emission wavelength.
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Figure 3 - Binding of systemic lupus erythematosus (SLE) autoantibodies to reactive oxygen species (ROS)-modified superoxide dismutase (SOD). A) Levels of SLE patients (SLE, n=50) binding to ROS-modified SOD (ROS-SOD), native SOD (nSOD) and single-stranded DNA (ssDNA). #p<0.0001 versus anti-ROS-SOD antibodies in SLE patients. B) Levels of circulating antibodies of normal human subjects (NH, n=34) binding to ROS-SOD, nSOD and ssDNA. ##p=0.1422 versus anti-ROS-SOD antibodies in healthy control. The SLE or NH sera (1:100 diluted) were analyzed by direct binding ELISA. The ELISA plates were individually coated with ROS-SOD (10µg/ml), nSOD (10µg/ml) and ssDNA (2.5 µg/ml).

Figure 4 - Competitive binding assays of systemic lupus erythematosus (SLE) autoantibodies. Inhibition of serum SLE antibodies (A) and serum normal human (NH) antibodies B) binding to single stranded DNA (ssDNA) by reactive oxygen species (ROS)-modified superoxide dismutase (SOD) and native SOD. Inhibitors used in the assays were ROS-SOD and unmodified SOD (nSOD). Inhibitors concentration used in the competitive binding assays was 20 µg/ml. Microtitre plates were coated with ssDNA (2.5 µg/ml). #p<0.0001 versus SLE autoantibodies. Inhibited by ROS-SOD; ##p<0.05 versus for protein oxidation.17,21,23 The oxidation of ROS-modified SOD was further evident by the significant increase (p<0.0001) in carbonyl contents. Superoxide dismutase is a major enzyme, and the first line of defense against ROS in various pathological conditions.12,24 In SLE patients, SOD dysfunction has been reported; it behaves not only as an anti-oxidant enzyme, but also as a putative antigen for SLE autoantibodies.13,25 However, it is still unclear how antibodies are generated against SOD in SLE; therefore, we hypothesized that if SOD is continuously exposed to oxidative stress, this may cause alterations in its conformation and function and may help to generate autoimmunity against oxidized SOD in SLE.

To validate our central hypothesis that the initiation of autoimmunity may also be mediated by increased formation of ROS-SOD following excessive ROS generation and oxidative stress, anti-ROS-SOD antibodies were quantitated in the sera of SLE patients in comparison with the sera from age- and gender-matched healthy control subjects. Our results showed a significantly increased prevalence of anti-ROS-SOD antibodies in patients with SLE as compared with anti-SOD antibodies. An increased prevalence of these antibodies in SLE patients suggests that ROS-SOD...
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is increased in SLE. Increased oxidatively modified proteins have previously been detected in SLE, but the significance of oxidized proteins in the initiation and development of SLE remains largely unexplored.

In this study, when the SLE patients were divided into 2 groups based on their SLEDAI scores (≥20 versus <20), both groups showed higher serum levels of anti-ROS-SOD antibodies than were observed in healthy controls, but the levels were much greater in the group of SLE patients with higher SLEDAI scores (SLEDAI score ≥20), suggesting an ongoing involvement of oxidation of SOD in SLE. The highly positive correlation between serum levels of anti-ROS-SOD antibodies and the SLEDAI score observed in the current study further suggests a strong association of oxidative stress and formation of anti-ROS-SOD antibodies, and SLE disease activity, namely, the greater the oxidative stress, the higher the SLEDAI. The enhanced anti-ROS-SOD antibodies observed in SLE patients in this study drew our attention to investigate the status of anti-oxidant function of SOD in SLE. Our literature survey revealed that SOD activity was significantly lower in the SLE patients as compared with the healthy controls. Additionally, it was also well reported that SLE patients with higher SLEDAI scores showed greater reductions in the SOD activity in comparison with SLE patients with lower SLEDAI scores. In the present study, we also found lower levels of SOD activity in SLE patients compared with the controls, and the group of patients with SLEDAI scores ≥20 showed greater reductions in comparison with SLE patients with SLEDAI scores <20 (data not shown). In contrast to the present or the previously published studies, few studies also showed the increase of SOD activity in SLE patients. We may suggest that this increase of SOD activity may only be in the initial phase of the disease, and it may decrease with the increase of disease activity. Despite an increase or decrease in the levels of SOD activity in SLE, all of these studies showed dysfunctioning of SOD. This dysfunctioning of SOD strongly supports our hypothesis. However, this study has a few limitations; the most obvious limitation of the study is the sample size and region of sample collection. It would have been better to include 75-100 patients, and not to confine the sample collection to only one region. In addition, diverse antigen characteristics of in-vivo generated anti-ROS-SOD antibodies with SLE associated antigens would further strengthen our findings.

In conclusion, this study demonstrates ROS-induced SOD damage in SLE, which may play an active role in the progression and/or progress of the disease. The present study proposed that in addition to SOD in serum concentration, the quality of SOD molecules may be not only a crucial factor affecting its protective effects, but also a risk factor as a pro-oxidant in lupus patients. We conclude that SOD after modification...
with ROS presents unique epitopes, which may play a role in the induction of circulating autoantibodies in SLE.

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