Expression of neutrophil SOD2 is reduced after lipopolysaccharide stimulation: a potential cause of neutrophil dysfunction in chronic kidney disease

Jenny Olsson1,5, Therese A.S. Jacobson2, Josefín M. Paulsson3, Elham Dadfar2, Ali Moshfegh2, Stefan H. Jacobson4,5 and Joachim Lundahl3

1Department of Nephrology, Skåne University Hospital, Malmö Sweden, 2Department of Oncology and Pathology, Karolinska Biomic Center, Karolinska University Hospital, Stockholm, Sweden, 3Department of Clinical Immunology, Karolinska University Hospital, Stockholm, Sweden, 4Department of Nephrology, Danderyd University Hospital, Stockholm, Sweden and 5Department of Clinical Sciences, Danderyd University Hospital, Karolinska Institutet, Stockholm, Sweden

Correspondence and offprint requests to: Jenny Olsson; E-mail: jenny.k.olsson@skane.se

Abstract

**Background.** Neutrophils from patients with chronic kidney disease (CKD) are dysfunctional and thus a contributing factor to the risk of infections. The mechanisms for leucocyte dysfunction in CKD are not fully understood. It is known that lipopolysaccharide (LPS) activates transcription of several genes encoding proinflammatory cytokines. We therefore aimed to study the effect of LPS on neutrophil expression of genes related to the inflammatory response to address the hypothesis that LPS-induced gene transcriptions are altered in CKD patients.

**Methods.** We analysed gene expression of LPS-stimulated neutrophils from 30 patients with CKD and 15 healthy controls. Superoxide dismutase-2 (SOD2), IL1A, IL-1R1, IL-1R2 and IL8RA gene expression from both neutrophils and differentiated HL60 cells were measured by quantitative polymerase chain reaction. Differentiated HL60 cells were stimulated with phorbol-12-myristate-7-acetate (PMA) after inhibition of SOD2 by small interfering RNA followed by respiratory burst assessment using flow cytometry.

**Results.** LPS stimulation induced a significant mobilization of CD11b on neutrophils from CKD and healthy controls. Upregulation of SOD2, IL1A, IL-1R1 and IL-1R2 gene expression in neutrophils from healthy controls after LPS stimulation was contrasted by no change in gene transcription (IL-1R1 and IL-1R2) or even a downregulation in patients with CKD (SOD2 and IL1A). Inhibition of SOD2 reduced the PMA-induced respiratory burst and IL1A, IL-1R1, IL-1R2 and IL8RA gene expression in neutrophil-differentiated HL60 cells.

**Conclusions.** Because of the critical role of SOD2 in the generation of hydrogen peroxide during phagocytosis, downregulation of SOD2 gene expression after LPS stimulation in neutrophils from patients with CKD indicates a potential mechanism for neutrophil dysfunction and cytokine dysregulation in these patients.

**Keywords:** chemokines; chronic kidney disease; leucocyte dysfunction; respiratory burst; SOD2

Introduction

Chronic kidney disease (CKD) is associated with a high prevalence of cardiovascular and infectious diseases with high morbidity and mortality rates [1,2]. The enhanced susceptibility to infections in patients with CKD is multifactorial.

Much research has focused on the function of circulating neutrophils in patients with advanced CKD and the potential causes of neutrophil dysfunction. Some metabolic and functional abnormalities of neutrophils have been suggested to be caused by the accumulation of toxins that inhibit leucocyte function [3–6]. Neutrophils from CKD patients have a reduced capacity to generate hydrogen peroxide that impairs the neutrophil intracellular killing capacity [7–10]. There is also evidence of neutrophil priming in CKD [11,12].

Neutrophils have generally been regarded as fully differentiated cells that exert their function based on preformed receptors and inflammatory modulators, acting primarily by phagocytosis of invading microorganisms. This view has recently been challenged in publications that suggest a second activation of biosynthesis after extravasation [13]. The transcriptional activation occurs at the inflammatory site and engages genes involved in a variety of neutrophil functions, such as production of oxygen radicals, cytokines and chemokines [14,15]. Hence, neutrophils gain competence to orchestrate the inflammatory response...
through communication with immune-modulating cells [15–17].

Lipopolysaccharide (LPS) activates neutrophils by interacting with LPS-binding molecules, e.g. CD14 and Toll-like receptor 4 (TLR4) [18,19], and promotes respiratory burst [20]. LPS-primed neutrophils release a number of proinflammatory mediators, including tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-8, superoxide dismutase 2 (SOD2), IL-12, macrophage inflammatory protein-1α (MIP-1α), MIP-1β and other inflammatory mediators [16,19,21–24]. Moreover, it has been shown that LPS can activate gene transcription in neutrophils in a similar manner as in the transmigration process, which would prime the cells to a hyper-responsiveness to a second stimulus [25,26].

SOD2 (MnSOD) is present in mitochondria of several cell types, including neutrophils, and promotes neutrophil respiratory burst by converting superoxide ions to hydrogen peroxide, the latter of which contributes to the intracellular killing of microorganisms [27,28].

Giving neutrophils a potential immunoregulatory role and LPS a relevant physiological trigger in CKD patients, we hypothesized that the gene expression after LPS stimulation may be substantially altered in neutrophils from CKD patients, which might be a direct cause of a dysfunctional state of neutrophils at the actual inflammatory site.

Materials and methods

Study population

Thirty patients with CKD stages 3–5 (glomerular filtration rate, GFR <60 mL/min/1.73 m²) were recruited from the outpatient clinic of the Department of Nephrology at Karolinska University Hospital between May and August 2006. Fifteen age-matched healthy subjects served as controls.

Patients with chronic kidney disease

The mean age of the 30 CKD patients was 52 ± 11 years (18 were men and 12 were women). The mean GFR was 34 ± 20 mL/min/1.73 m² measured with isohexol clearance. The main causes of renal failure were glomerulonephritis in 18 patients, polycystic kidney disease in 8 patients and nephrosclerosis in 4 patients. One patient had diabetes mellitus. Two of the patients were smokers: six had stopped smoking >6 months earlier. Two of the patients had signs of ischaemic cardiac disease detected by exercise test, and one had impaired systolic left ventricular function measured with echocardiography. Twenty-one patients had hypertension requiring pharmacological treatment. All but two of the patients were treated with ACE inhibitor and/or angiotensin blockers. Eight patients were on treatment with echocardiography. Twenty-one patients with CKD had hypertension.

Healthy controls

The mean age of the 15 healthy controls was 50 ± 12 years (6 were men and 9 were women). All had a normal plasma creatinine and other laboratory parameters, and according to the Modification of Diet in Renal Disease (MDRD) equation, they had a mean GFR of 91 ± 17 mL/min/1.73 m², and according to the Cockcroft–Gault equation 97 ± 19 mL/min/1.73 m² [29]. Two were smokers, and one had stopped smoking >6 months earlier. None of the healthy controls had any clinical signs of infections and was not on any medication at the time of the study. The mean BMI of the healthy subjects was 22.5 ± 2.0 kg/m², and there was no significant difference in BMI between CKD patients and healthy subjects.

Informed consent was obtained from all participants, and the study was approved by the Regional Ethics Committee, Stockholm, Sweden.

Isolation of neutrophils from peripheral blood

Twenty millilitre of peripheral blood from the patients with CKD and healthy controls was collected (Vacutainer, 5-mL tubes, with 0.129 M Na citrate, Becton Dickinson AB, Stockholm, Sweden), diluted 1:1 in phosphate-buffered saline (PBS) (pH 7.4) and layered onto Percoll solution (GE Healthcare Biosciences AB, Uppsala, Sweden) diluted in physiological NaCl and H₂O (Milli-Q). After centrifugation (1000 g, 30 min, 20–22°C), the mononuclear cells at the interface were removed, and the neutrophils and erythrocytes in the pellets were collected. The erythrocytes were haemolysed by the addition of 45 mL of isotonic buffer [154 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM ethylene diaminetetraacetic acid (EDTA), pH 7.2] and then incubated for 10 min at 4°C before centrifugation at 300 g for 12 min at 4°C. A second haemolysis was performed in 15 mL of NH₄Cl–EDTA for 3 min at 4°C, and the tubes were then centrifuged at 300 g for 8 min at 4°C. The neutrophil preparations were thereafter washed in PBS (at 4°C), centrifuged and finally resuspended in 2 mL of RPMI 1640 supplemented with HEPES (HEPES-RPMI 1640, Gibco Ltd, Paisley, Renfrewshire, UK/Invitrogen AB) and 5% heat-inactivated fetal bovine serum (FBS). The sample was separated into two tubes after measuring the cell count by flow cytometry, one tube for LPS stimulation and the other for unstimulated cells.

LPS stimulation

This method has previously been used by Malcolm et al. to stimulate gene expression in neutrophils [24]. Stimulation with LPS at a final concentration of 100 ng/mL was performed in six-well cell culture plates in HEPES-RPMI 1640 supplemented with 5% FBS at a cellular concentration of 4 × 10⁶ cells/mL for 4 h at 37°C. The concentration of LPS used is higher than the normal serum levels during sepsis, but previous experience has demonstrated that this is necessary to activate the cells in a culture in absence of LPS-binding protein and other activating substances [19,25].

After incubation, neutrophils were collected by washing the wells with 5 mL cold PBS, after which the cell suspension was transferred to a 15-mL collecting tub. PBS was added up to 15 mL, and 100 μL was removed for CD11b immunostaining. The cell suspension was then centrifuged at 300 g for 8 min at 4°C. After additional washing with PBS, the number of neutrophils was counted by flow cytometry. The sample was centrifuged at 300 g for 8 min at 4°C, and the pellet was immediately stored at –80°C for further analysis.

CD11b immunostaining

Expression of CD11b was measured in 100 μL of cell suspensions with unstimulated or LPS-stimulated neutrophils by the addition of 5 μL of phycocerythrin-conjugated mouse monoclonal anti-human CD11b (Clone 2L2PM19c, Dako AS, Glostrup, Denmark). The cells were incubated for 30 min on ice, then washed with 2 mL of cold PBS and centrifuged at 300 g for 5 min at 4°C. The pellet was resuspended in 300 μL of cold PBS before analysis by flow cytometry. The expression of CD11b was measured to verify that the cells were adequately activated by the LPS stimulation.

HL60 cell differentiation

We used neutrophil-differentiated HL60 cells in order to study the impact of SOD2 inhibition on gene transcription and neutrophil function in an experimental model. This approach was chosen to minimize the impact of potential confounders such as other transcriptional regulatory factors in neutrophils from CKD patients. HL60 is a leukaemic promyelocytic cell line that can be differentiated into different inflammatory cells by incubation with certain substances. The differentiation of HL60 cells into neutrophils by incubation with DMSO is an established method for studying neutrophil functions in vitro [30–32].
HL60 cells were cultured in RPMI 1640 with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine at 37°C in humidified air containing 5% CO2 and were induced to differentiate towards the neutrophil lineage by incubation with dimethyl sulphoxide (DMSO) (1.176 g/mL) for 7 days [30,31] before they were collected for transfection.

Transfection with small interfering RNA

Differentiated HL60 cells were washed with PBS (Mg^{2+}/Ca^{2+} free) at room temperature and resuspended in RPMI 1640 before 250 nM small interfering RNA (siRNA) (SOD2, s13269, Applied Biosystems, Foster City, CA, USA) was added. The cells were electroporated using 0.4-cm cuvettes (Bio-Rad Laboratories, CA, USA) by the Gene Pulser MXcell electroporation system (Bio-Rad Laboratories, Hercules, CA, USA). RPMI 1640 with 20% FCS was directly added to the electroporated cells after 10-min incubation at room temperature before further incubation in six-well plates for 72 h at 37°C in a humidified air containing 5% CO2. Before RNA extraction, the differentiated HL60 cells were divided and either exposed to phorbol-12-myristate 7-acetate (PMA) for 15 min at 37°C or remained unstimulated.

RNA extraction

RNA was extracted from neutrophils and differentiated HL60 cells using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). The assay was used according to the manufacturer's instructions. RNA quality and quantity were measured using the Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE, USA). RNA samples with an absorbance (OD) ratio between 1.8 and 2.0 were considered of high purity and taken into account for further analysis. Samples were stored at −80°C for quantitative polymerase chain reaction (PCR).

Expression analysis using quantitative PCR

We analysed the amount of mRNA of SOD2, IL1A (IL-1α), IL-1R1, IL-1R2 (IL-1 receptors 1 and 2) and IL8RA (IL-8 receptor α) present in neutrophils from patients with CKD and healthy controls, and in differentiated HL60 cells by using quantitative PCR (qPCR).

First-strand cDNA was synthesized by Superscript II reverse transcriptase (Invitrogen Corporation, Carlsbad, CA, USA) using 100 ng extracted RNA. ABI TaqMan Gene Expression assays for SOD2 (Hs00167309_m1), IL-1R2 (Hs01030384_m1), IL-1R1 (Hs00168392_m1), IL1A (Hs99999082_m1) and IL8RA (Hs00174146_m1) were used in a 20-μL reaction mix with ABI FAST MasterMix (Applied Biosystems Foster City, CA, USA) and synthesized cDNA. The qPCR reaction was performed using the ABI 7900 HT FAST Real-Time PCR system (Applied Biosystems Foster City, CA, USA). Analysis of the data was performed using Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Inc, Redwood City, CA, USA). As an internal control, expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used.

The changes in mRNA following stimulation of the cells with LPS (neutrophils from CKD patients and healthy subjects) were expressed as fold change of gene transcription. In HL60 cells, changes in gene transcription were made between cells pre-treated or not pre-treated with siRNA against SOD2 before and after PMA stimulation. Data are expressed as fold change in Figure 3 and Table 1. We set a cut-off of fold change ±2.0, and the results within this level (≥ 2.0 and ≤ 2.0) were considered as no change (NC) signifying no transcriptional up- or downregulation.

Measurement of respiratory burst

The respiratory burst was assessed by the dichlorofluorescein diacetate (DCFH-DA) method. DCFH-DA (Eastman Kodak Company, Rochester, NY, USA) permeates the neutrophil membrane to become hydrolysed intracellularly, and if hydrogen peroxide is available, the hydrolysed DCFH-DA is oxidized to highly fluorescent 2′, 7′-dichlorofluorescein. The amount of hydrogen peroxide produced can be quantified by measuring the fluorescence intensity by flow cytometry. Differentiated HL60 cells were incubated with PBS-glucose supplemented with 5 μM (5 μmol/L) DCFH-DA for 15 min at 37°C. During this time, the tubes were stirred several times to achieve a proper permeabilization of DCFH-DA. The H2O2 production during cellular activation was determined by adding a receptor-independent stimulus, 2.5 × 10^{-7} M PMA (Sigma) for 15 min at 37°C. Cells incubated in PBS-glucose for 15 min at 4°C served as controls. The activation was stopped by addition of 1 mL PBS–acetic acid supplemented with 0.1 mM EDTA. The cells were immediately subjected to flow cytometry to measure their H2O2 production.

Statistical analysis

The Mann–Whitney U-test was used to determine differences between groups regarding neutrophil CD11b expression, count and gene transcription fold change after LPS stimulation. The Student’s t-test was used for statistical analysis between populations of differentiated HL60 cells (SOD2 inhibited/uninhibited) in the analysis of gene transcription fold change and respiratory burst after PMA stimulation. Statistical significance was determined at P < 0.05.

Results

Neutrophil activation with LPS and CD11b mobilization

Results for neutrophil count and CD11b expression are shown in Figure 1 and 2. The CD11b expression on unstimulated neutrophils from the peripheral circulation of patients with CKD was higher when compared with cells from healthy controls (P < 0.001). After in vitro stimulation with LPS, there was a significant mobilization of CD11b on neutrophils from both patients with CKD and healthy controls (P < 0.001 for both comparisons). The expression of CD11b increased ~5–6 times after stimulation with LPS in both patients with CKD and healthy controls (Figure 2). The mean recovery percentage of neutrophils after LPS stimulation was 75 ± 11% for cells from healthy controls and 79 ± 12% for cells from CKD patients. No significant difference was observed between the recovery percentages between the study groups.

LPS stimulation and changes in gene expression

We observed a significant difference between healthy controls and patients with CKD as to the change in proinflammatory gene expression in response to LPS stimulation (Figure 3). Neutrophils from patients with CKD did not display any change in IL-1R2 or IL-1R1, and SOD2 and IL1A expression were downregulated. This contrasted neutrophils from healthy subjects in which SOD2 and IL1A were upregulated (P < 0.01 for both comparisons, Figure 3). There was a similar upregulation of IL8RA transcription in neutrophils from CKD patients and healthy subjects after LPS stimulation.

Gene transcription in differentiated HL60 cells

To explore the consequence of downregulated SOD2 in neutrophils from CKD patients, we inhibited SOD2 with siRNA in neutrophil-differentiated HL60 cells and compared gene transcription in these cells with differentiated HL60 cells without prior inhibition of SOD2, before and after stimulation with PMA. Before PMA stimulation, there was no transcriptional fold change of IL1A, IL-1R1, IL-1R2 or IL8RA in these cells. After PMA stimulation, we noted a significant downregulation of IL1A, IL-1R1, IL-1R2 and IL8RA (P < 0.001 for all comparisons, Table 1) in HL60 cells with inhibited SOD2 transcription.
compared with HL60 cells with maintained SOD2 transcription. At baseline (before PMA stimulation), there was no difference in SOD2 transcription between siRNA-treated and non-siRNA-treated HL60 cells. After PMA stimulation, SOD2 was significantly downregulated (P < 0.001) in HL60 cells with inhibited SOD2 transcription.

Fig. 1. Number of neutrophils in samples from patients with chronic kidney disease and healthy controls before and after stimulation with LPS. Results are expressed as median and percentiles.

Fig. 2. CD11b expression on neutrophils from patients with chronic kidney disease and healthy controls before and after stimulation with LPS. Results are expressed as median mean fluorescence intensity (MFI) and percentiles.
tion compared with HL60 cells without prior inhibition of SOD2. These data indicate a central role of SOD2 for the upregulation of important proinflammatory markers in neutrophils.

Respiratory burst in differentiated HL60 cells stimulated with PMA before and after inhibition of SOD2

Given that hydrogen peroxide production is an essential function in neutrophils, we examined the impact of SOD2 inhibition in differentiated HL60 cells. We found a significant increase in neutrophil hydrogen peroxide production after stimulation with PMA both in cells with maintained SOD2 production and in SOD2-inhibited cells (Figure 4, \( P < 0.01 \)). There was no significant difference in the hydrogen peroxide production of unstimulated differentiated HL60 cells with or without SOD2 inhibition. After stimulation with PMA, however, there was a significantly higher respiratory burst response in neutrophils with normal SOD2 \textit{de novo} production than in SOD2-inhibited differentiated HL60 cells (Figure 4, \( P < 0.01 \)).

Discussion

We demonstrate, for the first time, that neutrophils from CKD patients downregulate SOD2 gene expression after LPS stimulation. Given that SOD2 plays a central role in respiratory burst and the interleukin-1 axis, this finding points to a potential underlying mechanism for neutrophil dysfunction and the increased susceptibility to infections in CKD patients.

We chose to study the impact of SOD2 inhibition because of its proposed central role in neutrophil respiratory burst. The consequence of a reduced SOD2 gene transcription was further analysed in our study of differentiated HL60 cells, a model used for studies of neutrophil functions [33,34]. Using siRNA, which blocks \textasciitilde90\% of the newly transcribed SOD2 mRNA in the cells, according to our previous experience, we demonstrate in the present study that \textit{de novo} synthesis of SOD2 is necessary for neutrophils to generate optimal amounts of hydrogen peroxide. A reduced capacity in this respect might impair the intracellular killing of invading microbes.
In parallel to reduced SOD2 gene expression, we also noted a reduced fold change in gene transcription of IL1A, both in LPS-stimulated neutrophils from patients with CKD and in PMA-stimulated differentiated HL60 cells with inhibited SOD2 production. IL-1α is a proinflammatory cytokine produced by activated macrophages and neutrophils, which regulates both the innate and adaptive immune response through binding to the IL-1 receptor 1 and 2 on several inflammatory cells [35]. Binding to IL-1 receptor 1 creates a physiological response as opposed to binding to IL-1 receptor 2, which blocks the IL-1α response [36,37]. It is well established that differences in IL-1 function play an important role in several diseases, including CKD [38].

Our study indicates a central role of SOD2 in the upregulation of IL1A, IL-1R1, IL-1R2 and IL8RA in neutrophils. The explanation for the more pronounced downregulation of IL-1R1 after SOD2 inhibition in differentiated HL60 cells following PMA stimulation than in LPS-stimulated neutrophils from CKD patients could be that SOD2 is almost completely inhibited by the siRNA treatment, while in neutrophils from CKD patients, traces of SOD2 can be active.

IL-8 (CXCL8) is the principal chemokine that modulates neutrophil function [39]. It binds to the IL-8 receptor α and β (CXCR1 and CXCR2) that cause a different conformation of integrins. This conformational change allows neutrophils to bind to the endothelial cells, which is the first step in the transmigration process [40]. Neutrophils from CKD patients and healthy controls upregulated IL8RA upon LPS stimulation to the same degree. However, SOD2 inhibition by siRNA resulted in a reduced IL8RA transcription in differentiated HL60 cells as a response to PMA stimulation. The interpretation is that SOD2 might be involved in the regulation of this principal chemokine receptor for neutrophil extravasation and transmigration into an inflammatory site.

In the present study, we used LPS as a trigger for neutrophil activation. The rationale for this approach is that LPS constitutes an important physiological stimulus, especially in view of the increased susceptibility against Gram-negative bacteria in this patient group.

The setbacks of the present study include the potential confounding factors related to transcriptional regulation in neutrophils from CKD patients, a wide distribution of some mRNA values and the difficulties to use a proper experimental model for studying neutrophil functions. The glomerular filtration rate was broad, including CKD stages 3–5, but due to the small sample size, it was not possible to analyse the results according to different stages of CKD. However, we feel that these potential drawbacks do not substantially impact the general interpretations made in the present study.

To summarize, we report a significant reduced LPS-induced neutrophil gene expression of SOD2 and IL1A in CKD patients. Because SOD2 plays a key role in the immune response, both for neutrophil respiratory burst and for the upregulation of other proinflammatory markers, we propose that this finding may have implications for the high susceptibility to infectious disease reported in patients with CKD.
Definitions of neutrophil SOD2

Expression of neutrophil SOD2

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