Extracellular superoxide dismutase and glomerular mesangial cells: its production and regulation

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Abstract Extracellular superoxide dismutase (EC-SOD) is synthesized in mesenchymally derived cells and prevents the oxygen radical-induced injury. We studied whether kidney mesangial cells (MCs) produce EC-SOD and how its production is associated with chemokine secretion. Under unstimulated condition, MCs produced EC-SOD, and its production was correlated positively with cyclic adenosine monophosphate (cAMP), but negatively with interleukin (IL)-6 or IL-8 production. By prednisolone or phorbol myristate acetate treatment, EC-SOD levels were correlated negatively with levels of IL-6 and IL-8. The presence of adenylate cyclase inhibitor 2′,3′-dideoxyadenosine lost the prednisolone effect. The stimulation of EC-SOD production might be one of the important effects of prednisolone via cAMP pathway in MCs. **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Superoxide dismutase; Glucocorticoid; Interleukin 8; Interleukin 6; Mesangial cell; Reactive oxygen species

1. Introduction

The oxygen radicals generated by activated neutrophils, macrophages and glomerular cells have been implicated in the progression of glomerulo-nephritis, and they are removed by superoxide dismutase (SOD) [1,2]. Extracellular SOD (EC-SOD) is the major SOD isozyme acting in the extracellular space localized on the surface of the endothelium and vascular smooth muscle cells [3,4]. Intracellularly it exists in much smaller amounts than copper, zinc-SOD or manganese-SOD [3]. EC-SOD, which is an anti-inflammatory enzyme against oxygen radical-related inflammation in extracellular space, is produced from mesenchymally derived cells such as fibroblasts, smooth muscle cells and glioma cells [3,5,6]. For the regulation of EC-SOD production, the cyclic adenosine monophosphate (cAMP) pathway is conceived to be important in vivo and vitro [7].

EC-SOD is a secretory tetrameric copper- and zinc-containing glycoprotein with a subunit molecular weight of about 30 kDa and is mainly localized on the surface of endothelial cells to express its activity effectively in the vascular system [8]. It was also confirmed by in vivo and in vitro studies that binding of EC-SOD to heparan sulfate residues on the cell membrane or matrix is an important feature in expressing the enzyme activity and protecting from oxygen radicals generated by activated leucocytes [4,9]. Moreover, EC-SOD plays a role in prolonging the lifespan of nitric oxide [10] and decreases the net production of powerfully oxidizing peroxynitrite [11]. In renal tissue, heparan sulfate proteoglycans are essential components of the glomerular basement membrane and mesangial matrix as well as cell membrane carrying a strong anionic charge, which is partly produced by the glomerular mesangial cells (MCs) [12]. It is still unclear from in vivo or vitro studies whether MCs, which are derived from mesenchyme cells, are able to produce EC-SOD.

MCs are in a pivotal position to organize the glomerular function in the process of immune injury, because MCs have been shown to produce oxygen radicals, cytokines and extracellular matrix by immunological and pharmacological stimulation [13–15]. MCs have a direct or indirect role in the initiation and propagation of inflammatory events, partly by producing chemotactic cytokine interleukin (IL)-6- and IL-8-recruiting neutrophils and macrophages. In inflammation, the activated neutrophils and macrophages are the major source of oxygen radicals [16–18].

In vitro, MCs in culture show several interesting actions under glucocorticoid or phorbol myristate acetate (PMA) stimulation [19]. Glucocorticoid constitutes the first-line of therapy in human nephrotic syndrome [20] and puromycin aminonucleoside nephrosis [21] in rats in which oxygen radicals are important factors in the pathogenesis [22]. In contrast, PMA stimulation causes the hyperproduction of extracellular matrix component through the modulation of MCs due to protein kinase C (PKC) activation [19]. PKC activation is associated with the hyperproduction of chemokines and oxygen radical production under high glucose [23] or angiotensin II stimulation [24] in vitro, thus mimicking the pathological condition.

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Abbreviations: EC-SOD, extracellular superoxide dismutase; cAMP, cyclic adenosine monophosphate; IL-6, interleukin-6; MC, mesangial cells; PMA, phorbol myristate acetate; DDA, 2′,3′-dideoxyadenosine

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This study was designed to examine first, whether MCs produce EC-SOD and second, how the production is influenced by corticosteroid or PMA. We also examined the relationship between the production of EC-SOD and that of IL-6, IL-8 or cAMP by MCs.

2. Materials and methods

2.1. MC culture

MCs were immediately isolated by a differential sieving method from the normal part of the human kidney cortex after surgical nephrectomy of 15 renal carcinoma cases as previously described [25]. The MCs were grown in tissue culture flasks or 6-well tissue culture plates in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal calf serum and antibiotics. The MC identity was confirmed by the appearance of characteristic hillock structures under the microscope. By using a series of cell markers, intermediate filament desmin, myosin and vimentin, the purity of MCs was over the 98%. The MCs used in this study were between passages 3 and 5.

2.2. MC stimulation with prednisolone and PMA

To clarify the change in EC-SOD production from stimulated MCs, the EC-SOD content in culture media was analyzed for 3 days after the addition of prednisolone or PMA. The added contents in the culture media of MCs were 0.1, 0.5, 1 and 10 nM of PMA and 0.1, 0.5, 1, 5 and 10 μM of prednisolone.

2.3. Inhibition of adenylate cyclase activity by 3,5-deoxyadenosine

To study the participation of cAMP system in EC-SOD production, MCs was analyzed using 2′,3′-dideoxyadenosine (DDA) as the adenylate cyclase inhibitor [26]. The DDA contents in culture media were 1, 5, 10, 50 and 100 μM.

2.4. Enzyme-linked immunosorbent assay (ELISA) for cAMP

A competitive ELISA was used for the measurement of cAMP. The cAMP ELISA system was the Titer Zyme Dual Range cAMP EIA, purchased from PerSeptive Biosystem (Framingham, MA, USA). The working range for measurement of cAMP was from 0.25 pmol/ml to 100 pmol/ml.

2.5. Measurement of EC-SOD, IL-6 and IL-8 levels

A two-step ELISA method was used for the measurement of EC-SOD and IL-8. The EC-SOD ELISA system was our original system as described previously [27]. IL-6 and IL-8 were measured using an ELISA system purchased from Fuji-Rebio, Tokyo, Japan. The working ranges for the measurement of EC-SOD, IL-6 and IL-8 were from 50 pg/ml to 50 ng/ml, 15 pg/ml to 40 ng/ml and 20 pg/ml to30 ng/ml, respectively. We confirmed that this ELISA system for EC-SOD showed no cross-reactivity with other SOD isozymes.

3. Results and discussion

3.1. EC-SOD production from kidney glomerular cells

The kidney glomerulus is composed of three kinds of embryologically different cells: endothelial cells, epithelial cells and MCs. MCs are mesenchymally derived cells like smooth muscle. MCs are involved in a number of important functions including glomerular filtration, antigen presentation, cytokine production, phagocytosis, matrix synthesis and superoxide production, as well as anatomical support for the glomerular capillary loops [14].

In this study, the amount of EC-SOD production by MCs was studied in the culture supernatant, which was changed every 2 days. MC growth reached an optically confluent state in 10 days. The EC-SOD production was recognized in the early stage, reached a plateau of 3.42 ± 0.59 ng/ml/mg of protein in average about 20 days from the start of culture and showed stable production thereafter. To confirm the EC-SOD contents in the culture media reflects the majority of EC-SOD production by MCs, we compared the EC-SOD content in the culture media with that in MCs cell fraction. Over 95% of EC-SOD exists in the culture media, not stuck in the MCs surface. In vivo, most part of EC-SOD is localized in the surface of cells binding to heparan sulfate residues, however, in vitro, the major part of EC-SOD is secreted in the medium, presumably because cultured cells possess limited amount of heparan sulfate residues on the surface.

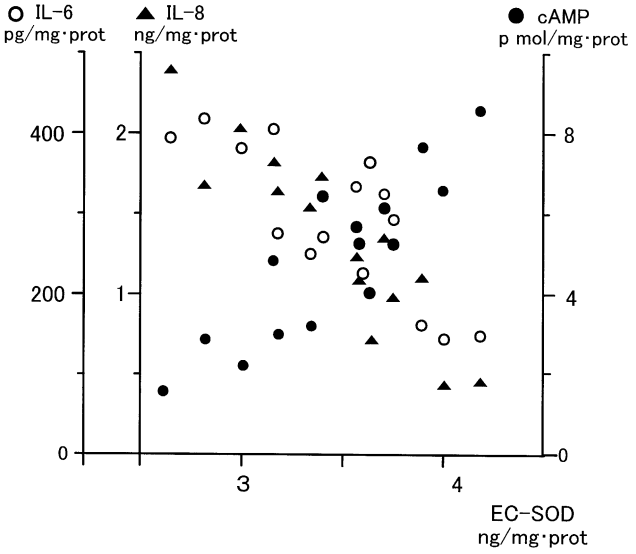

Fig. 1. Production of EC-SOD, IL-6, IL-8 and cAMP of MCs in individual cases. MCs production of cAMP, IL-6 and IL-8 for EC-SOD production in individual cases under the unstimulated conditions. Data were determined in triplicate. The dose value shows IL-8 (▲), IL-6 (●) and cAMP (○).

We also studied the EC-SOD production by kidney interstitial cells: fibroblasts and renal tubular epithelial cells. Primary cultured human kidney fibroblasts and Green Monkey kidney fibroblasts: COS7 cells expressed EC-SOD, while human cultured renal tubular epithelial cells did not (data not shown). Our results confirmed that MC in culture continuously produce EC-SOD. It has been reported that smooth muscle cells, glioma cells, skin fibroblasts, osteoblastic cells and lipid-laden macrophages are capable of producing EC-SOD [3,28].

3.2. The relation of EC-SOD production with cAMP and chemokines in MCs

To study the relation between the EC-SOD and chemokines production, the culture media of MCs were analyzed under the conventional unstimulated conditions in 20 days. As Fig. 1 shows, the EC-SOD levels showed a positive correlation with cAMP and a negative correlation with IL-6 and IL-8 production, indicating that EC-SOD production is closely related to the inflammation regulatory system through the cAMP system.

3.3. Suppression of EC-SOD production due to PMA stimulation

In vitro, PMA induces the stimulation of PKC that promotes the production of matrix components [19,29], oxygen radical generation [1,30] and chemokine production [31,32], thus mimicking the state of glomerular injury. As Fig. 2A shows, PMA stimulation to MCs results in a decrease in

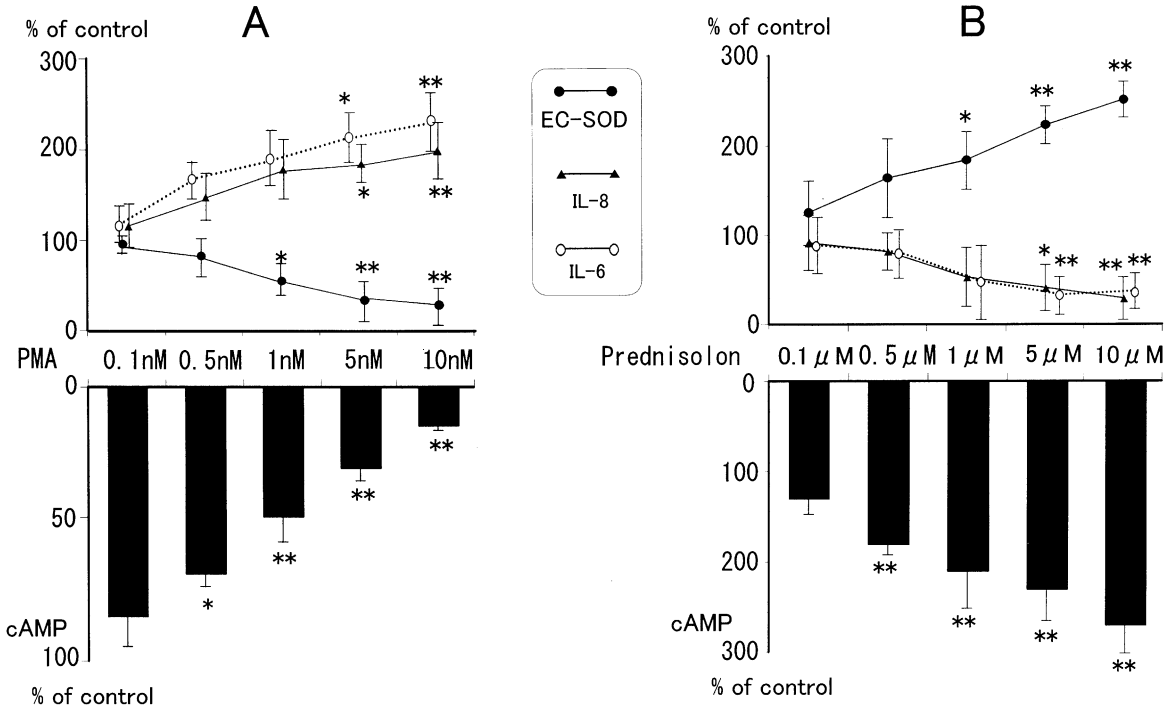

Fig. 2. Production of EC-SOD, IL-6, IL-8 and cAMP of MCs by stimulated condition. A: Dose-dependent suppression of EC-SOD and cAMP, induction of IL-6 and IL-8 by PMA stimulation of MCs ($n=15$). B: Dose-dependent induction of EC-SOD and cAMP, suppression of IL-6 and IL-8 by prednisolone of MCs ($n=15$). Values are shown compared to the control, and were determined in triplicate. Statistical significance was calculated by ANOVA with the Bonferroni post test relative to the control value with $*P<0.05$ and $**P<0.01$.

EC-SOD with cAMP production and an increase in IL-6 and IL-8 production in a dose-dependent manner.

In the process of glomerular injury, where local production of inflammatory cytokines such as IL-6 and IL-8 by MCs and recruitment of inflammatory cells are involved [16,17], oxygen radicals are over-produced. Against these reactive oxygen radicals, SOD acts as a protective protein. Our data suggest that MCs suppress the production of EC-SOD when they are activated by PMA, resembling the state of inflammation. The decrease of EC-SOD production by the PKC activation due to the inflammatory stimulation may play a pathophysiological role in the progression of glomerular injury. In the long term this situation may lead to the glomerular and vascular malfunction. We reported in a clinical study in chronic hemodialysis patients with diabetes, that those patients with genetic substitution in the EC-SOD molecule are prone to vascular injury and have a poor prognosis, suggesting that the prolonged defect of EC-SOD action affects the prognosis [33].

3.4. *Effect of prednisolone on EC-SOD, IL-6, IL-8 and cAMP generation by MC*

The regulatory mechanisms of EC-SOD production in MCs on glucocorticoid stimulation were studied together with the production of IL-6 and IL-8. As Fig. 2B shows, glucocorticoid treatment increased the EC-SOD and cAMP production in a dose-dependent manner, and decreased the IL-6 and IL-8 production. These results indicate the suppression of chemotactic cytokines by prednisolone occurred concomitantly with the enhanced EC-SOD production through the cAMP regulation.

The activation of gene transcription by glucocorticoids is widely known to involve the binding of the hormone–receptor complex to a consensus sequence within the cis transcriptional control element of the gene (glucocorticoid responsive element; GRE) [34]. A half-site for the GRE is located at −189 bp (TGTCCT) of the transcription initiation site in the human EC-SOD genome sequence [35]. Steroid-induced gene regulation in the various tissues and vascular wall is achieved through the interaction of specific receptor proteins

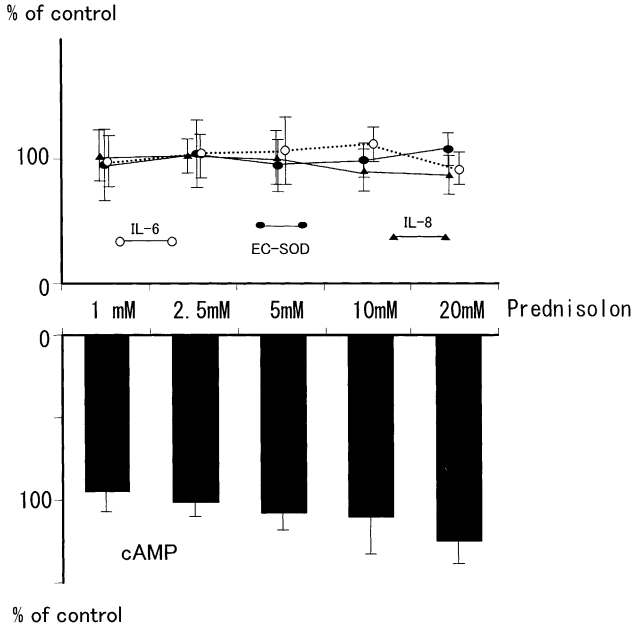

Fig. 3. Under the presence of adenylate cyclase inhibitor (DDA), EC-SOD, IL-6, IL-8 and cAMP production of MCs stimulating by prednisolone. Data were determined in triplicate. The dose value shows IL-8 (▲), IL-6 (●) and cAMP (○).

and promoters of the target genes. Prednisolone enhances the production of cAMP in renal tubular cells under the stimulated conditions and reduces the production of inflammatory cytokines [36]. cAMP also regulates IL-6 and IL-8 production in IL-1-stimulated human MCs [37]. Our data demonstrated that the promotion of EC-SOD production by prednisolone treatment was associated with the elevation of cAMP content and the decrease of IL-6 and IL-8. This study cannot clarify whether the enhancement of EC-SOD production was a direct or indirect role of cAMP and/or glucocorticoid.

Our results suggest that the anti-inflammatory effect of prednisolone treatment is not only due to the suppression of cytokines but also to the enhancement of EC-SOD production. A novel hypothesis has recently been postulated based on the results of a series of animal studies indicating that the therapeutic effect of steroids is modulated through their capacity to up-regulate renal antioxidant enzyme. The glomeruli isolated from rats given a daily injection of methylprednisolone were found to have significantly higher activities of antioxidant enzymes than those from untreated rats [38]. This result is consistent with our data that prednisolone promotes the EC-SOD production in MCs. Yoshioka et al. reported that manganese-SOD, which is one of the intracellularly located isozymes of SOD, was induced by glucocorticoid in glomerular cells [39]. Mn-SOD and catalase activities, but not Cu, Zn-SOD or glutathione peroxidase activities, were found to increase significantly in response to methylprednisolone. We assume that the enhancement of EC-SOD production is an important effect of prednisolone as well as the regulation of cytokines in MCs in glomerular disease.

3.5. The effect of prednisolone on EC-SOD production under the presence of adenylate cyclase inhibitor

Under the stimulated conditions by prednisolone, the EC-SOD, IL-6 and IL-8 production were associated with cAMP levels. These results suggest that the production of inflammatory cytokines and EC-SOD production were regulated in MCs. To clarify the participation of cAMP in production of EC-SOD, IL-6 and IL-8 production, we studied the effect of the adenylate cyclase inhibitor in MCs. Fig. 3 shows that in the presence of adenylate cyclase inhibitor, DDA, the effect of prednisolone was studied in EC-SOD, IL-6 and IL-8 production. In the presence of adenylate cyclase inhibitor, prednisolone stimulation showed no effects on EC-SOD, IL-6 or IL-8 production. These results indicate that EC-SOD production is closely related to the inflammation regulatory system through the cAMP system.

Little is known about the regulatory factors of EC-SOD production in vivo or in vitro. Cyclic nucleotides as the second messengers are one of the regulatory factors of cytokine production in glomerular injury [36], which is also the major EC-SOD regulatory factor [7]. EC-SOD production in fibroblasts is induced by treatment of interferon-gamma, cAMP and ATP [7], but is not induced by oxygen radicals, tumor necrosis factor, IL-1, IL-6 or IL-8 [40]. The nucleotide sequence of the human EC-SOD gene has many transcription and regulation elements including a cAMP response element and a glucocorticoid response element, as reported by Foltz et al. [35]. In renal tissue, cAMP is involved in the various regulatory systems and its action is regulated by the balance between adenylate cyclases and cyclic 3′,5′ nucleotide phosphodiesterases. Thus, it is difficult to clarify the precise action of cAMP in the regulation of EC-SOD production with or without the presence of prednisolone. It is conceivable that the effect of prednisolone on EC-SOD production is via a cAMP-dependent pathway. We assume that the enhancement of EC-SOD production is an important effect of prednisolone as well as the regulation of cytokines in MCs in glomerular diseases.

4. Conclusion

It was concluded that kidney glomerular MCs produce EC-SOD like other mesenchymally derived cells. The production of EC-SOD could be via a cAMP-dependent system and was regulated in negative correlation with IL-6 and IL-8 when stimulated by prednisolone or suppressed by PMA. We concluded that the enhancement of EC-SOD production is one of the important functions of prednisolone.

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