FEBS Letters 458 (1999) 370–374 FEBS 22627

Increase of urinary extracellular-superoxide dismutase level correlated with cyclic adenosine monophosphate

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Received 23 August 1999

Abstract Extracellular superoxide dismutase (EC-SOD) is a secretory protein that is the major SOD isozyme in extracellular fluids. Plasma EC-SOD levels are distributed in two discrete groups with the rare group having an enzyme with glycine instead of arginine-213, which causes a 10-fold higher serum level. Within the common phenotype group, the urinary EC-SOD level was significantly correlated with the urinary excretion of *N*-acetyl-β-D-glucosaminidase (NAG), but not with serum EC-SOD. EC-SOD appears not to be leaked from the plasma by glomerular filtration, but rather to be secreted from the renal tubule or its surrounding tissues. The urinary EC-SOD level was also significantly correlated with the urinary cyclic adenosine monophosphate (cAMP) level. cAMP analogues and adenylate cyclase modulators significantly stimulated the expression of EC-SOD but not other SOD isozymes in cultured fibroblast cell lines. Moreover, injection of parathyroid hormone, in Ellsworth-Howard tests, increased urinary EC-SOD accompanied with the elevations of urinary cAMP and NAG. Together these observations suggest that factor(s) that stimulate the adenylate cyclase-cAMP system regulate the urinary EC-SOD level.

Key words: Extracellular superoxide dismutase; cAMP; *N*-acetyl-β-D-glucosaminidase; Parathyroid hormone; Renal tubule

1. Introduction

Superoxide and other secondarily generated active oxygen metabolites are continuously generated as normal by-products of cellular metabolism. These oxygen metabolites are highly reactive and can attack many biochemical components in the cells. Normal cells have a number of enzymatic endogenous antioxidant mechanisms, such as superoxide dismutase (SOD, EC. 1.15.1.1), catalase and glutathione peroxidase. These enzymes quickly eliminate toxic oxygen metabolites under physiological conditions. When the flux of active oxygen metabolites exceeds the capability of endogenous antioxidant mechanisms, tissue injury ensues, because the generation of highly toxic metabolites of oxygen is greatly increased under pathological conditions [1].

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Abbreviations: EC-SOD, extracellular superoxide dismutase; Cu,Zn-SOD, copper- and zinc-containing SOD; Mn-SOD, manganese-containing SOD; cAMP, cyclic adenosine monophosphate; dbcAMP, N^6-$O^{2'}$-dibutyryl cAMP; 8BrcAMP, 8-bromo cAMP; IBMX, 3-isobutyl-1-methylxanthine; CRE, creatinine; NAG, *N*-acetyl-β-D-glucosaminidase; PTH, parathyroid hormone

There are three SOD isoforms, copper- and zinc-containing SOD (Cu,Zn-SOD), manganese-containing SOD (Mn-SOD) and extracellular-SOD (EC-SOD). Cu,Zn-SOD and Mn-SOD are intracellular SOD isozymes with molecular weights of 32 kDa and 85 kDa and are found predominantly in the cytoplasm and mitochondria, respectively. EC-SOD, the most recently discovered SOD isozyme, is a secretory, copper- and zinc-containing glycoprotein with an apparent molecular weight of 135 kDa [2,3]. Blood vessel walls contain large amounts of EC-SOD and this enzyme is the principal enzymatic scavenger of superoxide in the vascular system, whereas EC-SOD is the least abundant enzyme in other tissues [4,5]. A prominent feature of EC-SOD is its affinity to heparin-like substances [2,6] and more than 99% of this enzyme is anchored to heparan sulfate proteoglycans in the interstitium of tissues, especially blood vessel walls [4]. The less than 1% of EC-SOD is present in the circulation in equilibrium between the plasma phase and the glycocalyx of the endothelium [4,7]. Molecular genetic studies of EC-SOD have shown that a single base substitution causing exchange of glycine for arginine-213 (R213G) in the heparin-binding domain of this enzyme causes an extremely high plasma level of EC-SOD [8–10]. The high plasma EC-SOD phenotype is found in about 2 to 6% of healthy subjects in Japanese, Swedish and Australian populations [8,10–14]. We have reported that the plasma levels of the common phenotype EC-SOD and the frequency of the R213G mutation were higher in hemodialysis patients than in healthy persons [10,15]. On the other hand, little is known about urinary EC-SOD. We reported that urinary EC-SOD levels in patients with renal diseases were, in contrast to the serum levels, lower than those in healthy subjects [15]. However, it is still unclear how urinary EC-SOD changes. In the present study, our findings suggest that the urinary EC-SOD level is regulated by cyclic adenosine monophosphate (cAMP)-mediated renal tubule function.

2. Materials and methods

2.1. Subjects

The subjects studied were healthy individuals who had participated in an annual health checkup. They were free of any abnormality on physical examination, blood chemical screening, electrocardiogram, chest X-ray and urinalysis. Blood samples were taken without anticoagulant. The blood samples were centrifuged within 2 h and sera were stored at −70°C in aliquots until analysis. The urinary samples were first morning urine.

2.2. Assay

The EC-SOD concentration was determined by an enzyme-linked immunosorbent assay (ELISA) as described previously [11]. Cu,Zn-SOD and Mn-SOD were determined using ELISA kits purchased from NOF (Tokyo, Japan). The other assays were performed by the standard clinical examination methods. Data obtained by urinalysis were normalized by the urinary creatinine (CRE) concentration.

PII: S0014-5793(99)01185-0

2.3. Fibroblast cell culture

Human skin fibroblast cell lines were initiated from skin punch biopsy specimens obtained from four volunteers, using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 4 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin as medium. They were kept in 5% CO_2/95% air at 37°C. Cells (2×10^5 cells/well) were plated in six-well plates and cultured for 3 days; then the medium was changed to FCS-free culture medium and the cells were cultured for another 24 h. The cells were then cultured for 4 days in FCS-free culture medium with or without reagents such as adenosine triphosphate (ATP, Oriental Yeast, Tokyo, Japan), N^6-2′-*O*-dibutyryl cAMP (dbcAMP, Nacalai Tesque, Kyoto, Japan), 8-bromo cAMP (8BrcAMP, Sigma St. Louis, MO), forskolin (Wako Pure Chemical Industries, Osaka, Japan) and 3-isobutyl-1-methylxanthine (IBMX, Wako Pure Chemical Industries). The medium was then removed for the assay of EC-SOD, and the cells were washed twice with 2 ml of cold phosphate buffered saline (PBS). The cells were then detached by sonication and collected into 0.5 ml of PBS. Cell protein was assayed by the Bradford method [16].

2.4. Ellsworth-Howard test

The Ellsworth-Howard test was done in four healthy laboratory personnel members according to the standard protocol of the Ministry of Health and Welfare investigation research group in Japan. Before and after the intravenous injection of 100 U of teriparatide acetate, Human parathyroid hormone (PTH) (Asahi Kasei, Tokyo, Japan), urine was collected every hour.

2.5. Statistical analyses

Data are presented as means ± S.D., and changes within groups were analyzed by paired two-tailed Student's *t*-test.

3. Results and discussion

3.1. Urinary EC-SOD level

Normal individuals are divided into two groups; one with common phenotype EC-SOD and one with the rare phenotype EC-SOD with R213G exchanged enzyme. We measured serum and urinary EC-SOD levels of 10 subjects (male $n=3$, female $n=7$, age range 20 to 59) with the rare phenotype of EC-SOD and 77 subjects (male $n=20$, female $n=57$, age range 21 to 71) with the common EC-SOD phenotype in the current study. Similar to our previous findings [11], the rare phenotype group showed significantly (10-fold) higher serum EC-SOD levels than the common phenotype group (741 ± 145 μg/l vs 74.6 ± 22.1 μg/l, $P<0.0001$). The EC-SOD levels of these two groups were distributed in completely discrete ranges of 580 to 1023 μg/l and 37.7 to 145 μg/l, respectively. We found that the urinary level of the EC-SOD of the rare phenotype group (24.8 ± 10.9 μg/g CRE) was also significantly higher than that of the common phenotype group (14.5 ± 5.6 μg/g CRE, $P=0.015$); however, their distribution ranges overlapped. It is known that the R213G variation in EC-SOD decreases the affinity to heparin-like glycosaminoglycans on the endothelial cell surface, and this causes the high plasma level of this enzyme [8,17]. It is possible that such EC-SOD with low heparin-affinity is highly distributed into the urine due to inefficient trapping within the interstitium between the expressing cells and the urinary space.

3.2. Correlations between urinary EC-SOD and other chemical values

The aim of the current study was to identify the factor(s) regulating the urinary EC-SOD level in common phenotype subjects. Within the common phenotype group, there was no significant correlation between urinary EC-SOD and serum EC-SOD levels ($r=0.124$, $P=0.281$). It is known that Cu,Zn-SOD in plasma is excreted rapidly by glomerular filtration [18,19] Compared to Cu,Zn-SOD, with molecular weight of 32 kDa, EC-SOD is larger (135 kDa) and would not pass through the capillary basement of the glomerular filtration apparatus of the kidney. Moreover, the high affinity to the endothelial cell surface decreases the rate of loss of EC-SOD from the circulation [20]. Karlsson and Marklund reported that human EC-SOD intravenously injected into rabbits was distributed mainly to the liver and slightly to the kidney and spleen and other organs [21]. Although the major site of EC-SOD degradation was not deduced, the liver is likely candidate for this [21]. Under physiological conditions, proteins found in the urine are classified into two groups: plasma proteins leaked from the glomerulus by filtration, such as albumin, and proteins derived from the renal tubule, such as *N*-acetyl-β-D-glucosaminidase (NAG). In healthy individuals studied here, the urinary total protein concentration was significantly correlated with urinary NAG ($r=0.342$, $P=0.0023$). It has been reported that some exogenous factors transiently increased the urinary excretion of NAG without any renal tubular damage [22], however an increase of urinary NAG is generally inferred to represent a leakage of the enzyme from the damaged renal tubular cells. Urinary EC-SOD was significantly correlated with NAG ($r=0.289$, $P=0.0107$), but not with urinary total protein ($r=0.101$, $P=0.381$) (Fig. 1). We found that Green Monkey kidney fibroblasts expressed EC-SOD, while human cultured renal tubule epidermal cells did not (data not shown). Together, these findings suggest that EC-SOD in urine might be not leaked from the plasma, but may rather be secreted from fibroblasts in the renal tubule or its surrounding tissues.

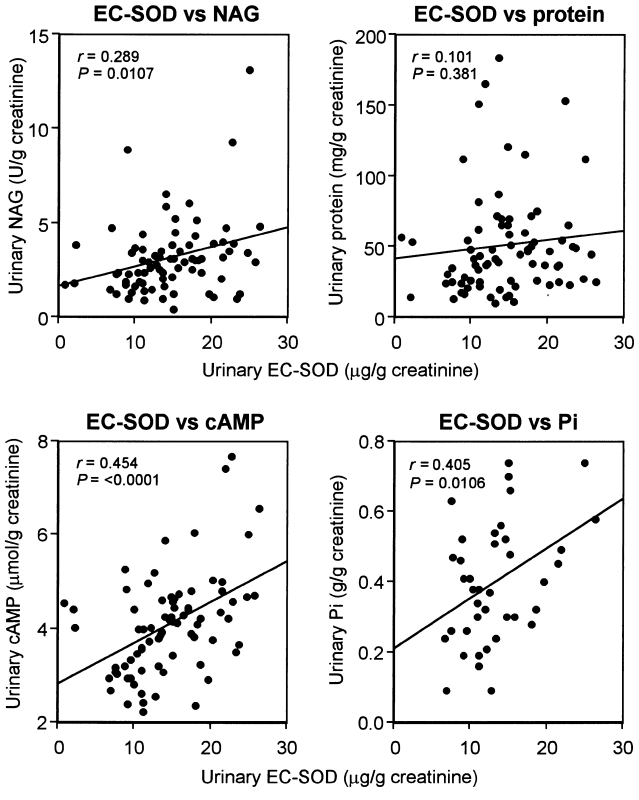

Fig. 1. Correlation between EC-SOD and other substances in urine.

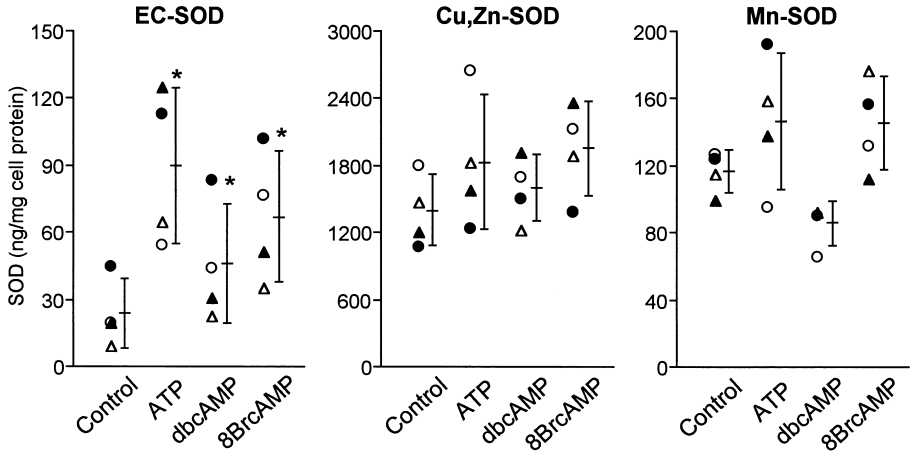

Fig. 2. Effect of ATP, dbcAMP and 8BrcAMP on the expression of SODs by fibroblast cells. Fibroblast cells (●,○,▲,△) obtained from four volunteers were cultured for 4 days in the absence or presence of ATP (500 μM), dbcAMP (500 μM) and 8BrcAMP (500 μM) by the methods described in Section 2. SODs were assayed by the methods described in Section 2. Each cross is the mean ± S.D. of the four fibroblast cell lines. *: $P < 0.05$ compared to control.

In humans, EC-SOD is reported to be expressed by fibroblasts, glial cells, smooth muscle cells and lipid-laden macrophages [23–26]. The synthesis of EC-SOD by fibroblasts is regulated by some inflammatory cytokines [27] and not by oxidative stress [28]. Nicolaï et al. reported that EC-SOD transcription and secretion were enhanced by cAMP in a rat glioma cell line [29]. cAMP is known to be an intracellular second messenger that regulates the expression of proteins including nitric oxide synthase [30] and many other proteins. A cAMP-producing enzyme, adenylate cyclase, is a component of the cAMP-dependent signaling pathway in renal tubules and is activated by many substances, such as PTH, glucagon, catecholamine and prostaglandins. The renal excretion of phosphate (Pi) is also stimulated by PTH through this pathway. Since part of the cAMP synthesized in tubules leaks into the urine and can be detected there, the assay of urinary cAMP concentration is used as a diagnostic test of the hormonal function of PTH. In samples tested in the current study, urinary Pi was significantly correlated with urinary cAMP ($r = 0.526$, $P = 0.0006$). In agreement with a previous report [22], urinary NAG was also correlated with urinary cAMP ($r = 0.507$, $P < 0.0001$), while urinary protein was not ($r = 0.150$, $P = 0.1925$). We found that urinary EC-SOD was significantly correlated with urinary cAMP ($r = 0.454$, $P < 0.0001$) and urinary Pi ($r = 0.405$, $P = 0.0106$) (Fig. 1). These results suggest that the factor(s) that elevate the cellular cAMP level enhance the expression of EC-SOD in the renal tubule tissue and enhance the urinary excretion of this enzyme. To extend the above observations, we carried out in vitro experiments on the effect of cAMP on the expression of EC-SOD in cultured cell lines, and performed an in vivo experiment with volunteers to test the effect of PTH injection on the urinary excretion of EC-SOD.

3.3. Effect of cAMP on EC-SOD expression by fibroblasts

We investigated the effect of elevation of the cellular cAMP level on the expression of EC-SOD in fibroblasts. Extracellular ATP and cAMP analogues such as dbcAMP and 8BrcAMP have been reported to elevate the intracellular cAMP level in several different cell types [29–31]. We confirmed the elevation of cAMP by the addition of above reagents. (data not shown). As shown in Fig. 2, the level of EC-SOD expression varied a lot among the cell lines. However, the responses for reagents were in a similar manner. The addition of ATP, dbcAMP and 8BrcAMP to the medium significantly increased the expression of EC-SOD, 4.63-fold, 2.03-fold and 3.13-fold, respectively. The stimulation of EC-SOD synthesis is not due to stimulation of cell growth, since the protein content in the cell layer did not increase. On the other hand, these reagents did not induce the significant changes in the levels of Cu,Zn-SOD and Mn-SOD. The nucleotide sequence of the human EC-SOD gene has been reported, and a cAMP-responsive element was found at position −438 bp [32]. In fact, it was reported that EC-SOD mRNA transcription was enhanced dose-dependently by cAMP analogues [29]. We next tested the effect of modulators of adenylate cyclase-cAMP system on the EC-SOD expression. The adenylate cyclase activator forskolin and the phosphodiester-

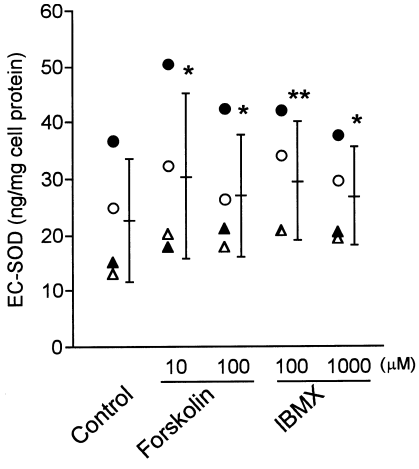

Fig. 3. Effect of forskolin and IBMX on the expression of SODs by fibroblast cells. Fibroblast cells (●,○,▲,△) obtained from four volunteers were cultured for 4 days in the absence or presence forskolin (10 μM and 100 μM) and IBMX (100 μM and 1 mM) by the methods described in Section 2. Each cross is the mean ± S.D. of the four fibroblast cell lines. *: $P < 0.05$; **: $P < 0.01$ compared to control.

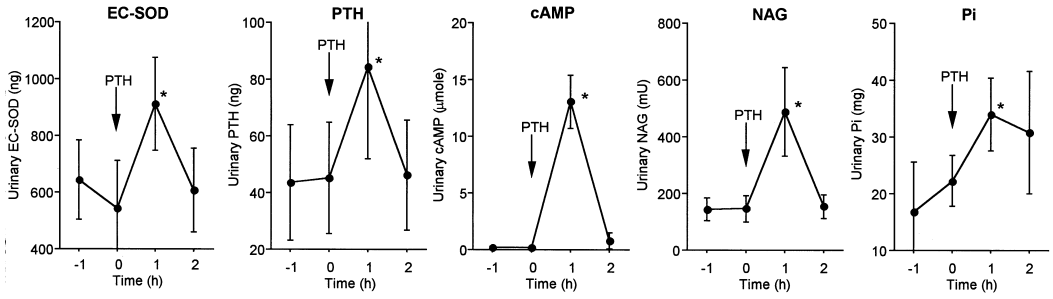

Fig. 4. Changes in urinary excretion of EC-SOD, PTH, cAMP, NAG and Pi after the injection of 100 U of human PTH in four healthy personnel members. All data are shown as mean ± S.D. *: $P < 0.05$ compared to before PTH injection.

ase inhibitor IBMX significantly induced the expression of EC-SOD by fibroblasts, as shown in Fig. 3. These results imply that EC-SOD expression would be constantly regulated by several factors through the cAMP-dependent signaling system.

3.4. Effect of cAMP elevation on urinary EC-SOD level in vivo

The Ellsworth-Howard test is a PTH load test which monitors the PTH hormonal function mediated through the PTH-receptor on renal tubules. After the injection of PTH according to the protocol of the Ellsworth-Howard test, PTH, cAMP, NAG and Pi in the urine were significantly elevated (Fig. 4), whereas urinary protein and CRE were only slightly changed (data not shown). A positive correlation between the increase of urinary NAG and the increase of cAMP excretion after PTH injection has been reported [22]. Moreover, a cAMP-dependent mechanism has been suggested to contribute to the urinary NAG excretion [22]. The EC-SOD in urine was also significantly increased after the injection of PTH (Fig. 4). The results of the Ellsworth-Howard test thus suggest that the urinary excretion of EC-SOD might be due to the stimulation of the adenylate cyclase-cAMP system and were in agreement with the population study described above.

We have reported that the plasma level of common phenotype EC-SOD was higher in patients with renal diseases, while urinary EC-SOD levels in patients were lower than those in healthy subjects [15]. In this study, we found that urinary common phenotype EC-SOD correlated with urinary cAMP and Pi, but not with serum EC-SOD and urinary protein. We therefore tested whether cAMP contributes to the regulation of EC-SOD expression by in vitro experiments with fibroblast cell lines and in vivo experiment to test the effect of PTH injection to healthy laboratory personnel members. The elevation of cAMP in fibroblast cell lines by the addition of the substrate of adenylate cyclase, cAMP analogues, adenylate cyclase activator, and phosphodiesterase inhibitor increased the expression of EC-SOD especially but not other SODs. In Ellsworth-Howard test, injection of PTH induced the elevation of urinary EC-SOD accompanied with the elevation of cAMP, NAG and Pi in the urine. Taken together, our data suggest that urinary EC-SOD does not come from the plasma EC-SOD. Whilst the mechanism remains speculative, it is possible that expression of EC-SOD by the fibroblasts in or surrounding the renal tubule is enhanced by the elevation of cellular cAMP and this leads to the increase of the urinary EC-SOD level.

Acknowledgements: This project was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and by a grant from the Gifu Life Science Research Promotion Council (TA).

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