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The uremic solute indoxyl sulfate acts as an antioxidant against superoxide anion radicals under normal-physiological conditions

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ABSTRACT

The effect of the uremic solute indoxyl sulfate (IS) on scavenging superoxide anion radicals ($O_2^{\cdot-}$) generated from both the xanthine/xanthine oxidase (X/XO) system and activated neutrophils was investigated by electron paramagnetic resonance spectroscopy, combined with 2-ethoxycarbonyl-2-methyl-3,4-dihydro-2H-pyrrole-1-oxide (EMPO). The findings show that the presence of normal-physiological serum concentrations of IS (0.1–10 μM) resulted in decreased formation of EMPO-superoxide adduct without affecting XO activity. Furthermore, IS showed scavenging activity against cell-derived $O_2^{\cdot-}$ generated from activated neutrophils. In addition, IS also eliminated hydroxyl radicals. These findings suggest that IS acts as a novel endogenous antioxidant under normal-physiological conditions.

1. Introduction

When kidney function is impaired, a variety of pathological changes occur that are collectively referred to as uremic syndrome. A part of this syndrome involves elevated serum levels of a number of substances [1]. In patients with chronic kidney diseases (CKD), uremic toxins accumulate in the serum by a combination of the following four mechanisms: (1) a decrease in renal clearance (indoxyl sulfate (IS)); (2) the accumulation of abnormal metabolites (methylguanidine); (3) an increase in hormone production (parathyroid hormone); and (4) a decreased rate of catabolism by the kidney (β2-microgloblin) [2].

IS, an extensively investigated uremic toxin, is derived from dietary protein including tryptophan and contains an indole ring. It is present at high levels in the serum of patients with CKD (the serum concentration of CKD patients is 100 μM–1 mM versus 0.1–10 μM in healthy subjects). Recent findings indicate that IS is likely involved in both the progression of CKD and the development of complications. In fact, we and other researchers have recently reported that IS causes tubulointerstitial injuries in rodent models [3,4] and produces oxidative stress in various cell systems, such as renal proximal tubular cells, mesangial cells and endothelial cells [5–8]. Moreover, excessive dietary supplementation of tryptophan, a precursor of IS, enhanced plasma lipid peroxidation in rats [9]. These observations suggest that IS has an adverse effect via the enhancement of oxidative stress in vivo.

On the other hand, from a structural perspective, it is possible that endogenous indole derivatives have the potential to function as an antioxidant against various reactive oxygen species (ROS), attributed to the electron-attracting nitrogen atom (–NH) in the pyrrole ring [10]. For example, melatonin (MLT) precursor

Abbreviations: IS, indoxyl sulfate; X, xanthine; XO, xanthine oxidase; $O_2^{\cdot-}$, superoxide anion radicals; ·OH, hydroxyl radicals; CKD, chronic kidney diseases; EPR, electron paramagnetic resonance; EMPO, 2-ethoxycarbonyl-2-methyl-3,4-dihydro-2H-pyrrole-1-oxide; EMPO-OOH, EMPO-superoxide adduct; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; DMPO-OH, DMPO-hydroxyl radical adduct; DTPA, diethylene-triamine-pentaacetic acid; ROS, reactive oxygen species; Cu, Zn-SOD, Cu, Zn-superoxide dismutase; ALP, allopurinol; pterin, 2-amino-4-hydroxypteridine; isoxanthopterin, 2-amino-4,7-dihydroxypteridine; IA, indolacetate; HA, hippurate; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropionate

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tryptophan (Trp) was reported to scavenge both hydroxyl radicals (·OH) and superoxide anion radicals ($O_2^{\cdot-}$) [11,12]. In addition, MLT, serotonin and the MLT metabolite 6-OH-MLT also showed antioxidant activity against various free radicals [13,14]. Interestingly, Chyan et al. reported that indole-3-propionic acid scavenges ·OH and also showed potent neuroprotective properties against the Alzheimer β-amyloid [15]. Based on these reports, we hypothesize that, although under high serum levels, IS functions as a prooxidant due to an enhancement in oxidative stress, it may also function as an antioxidant under normal-physiological conditions. However, the detailed information on the role of IS under normal-physiological conditions does not appear to be available.

In the current study, using a luminol-dependent chemiluminescence assay and electron paramagnetic resonance (EPR) spectroscopy, combined with a 2-ethoxycarbonyl-2-methyl-3,4-dihydro-2H-pyrrole-1-oxide (EMPO)-spin trapping technique, we examined the issue of whether normal-physiological concentrations of IS (0.1–10 μM) show scavenging activity for $O_2^{\cdot-}$, generated by both the xanthine/xanthine oxidase (X/XO) system and neutrophils treated with LPS. The findings were compared with the data for other antioxidant compounds, such as Cu, Zn-superoxide dismutase (Cu, Zn-SOD, an $O_2^{\cdot-}$ scavenger) and allopurinol (ALP) (a XO inhibitor), and were also compared with that for other uremic toxins under normal non-uremic concentrations [16]. In addition, the effect of IS on ·OH generated by a H_2O_2/UV system was also investigated.

2. Materials and methods

2.1. Chemicals and materials

X, XO, IS, uric acid sodium salt, Cu, Zn-SOD and ALP were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Diethylene-triamine-pentaacetic acid (DTPA) was purchased from Dojindo Laboratories (Kumamoto, Japan). EMPO and 5,5-dimethyl-1-pyrroline N-oxide (DMPO) were purchased from Alexis Biochemicals (Lausen, Switzerland). Luminol, indolacetate (IA), hippurate (HA) and *p*-cresol were purchased from Nakalai Tesque Inc. (Kyoto, Japan). 3-Carboxy-4-methyl-5-propyl-2-furanpropionate (CMPF) was synthesized as described previously (Tsutsumi et al., 1999). All other reagents used were of the highest grade available from commercial sources.

2.2. EPR spectroscopy in combination with EMPO and DMPO spins trapping technique

$O_2^{\cdot-}$ was assayed by EPR spin trapping with EMPO and the scavenging activity was calculated from the relative intensity of the peak corresponding to the EMPO-superoxide adduct (EMPO-OOH) EPR signal. The reaction mixtures contained 100 μg/ml X, 0.02 U/ml XO, 100 μM DTPA and 20 mM EMPO in the absence or presence of varying concentrations of IS and were immediately transferred to quartz EPR flat cells. EPR spectra were recorded at room temperature on a JES-TE 200 EPR spectrometer (JEOL, Tokyo, Japan) under the following conditions: modulation frequency, 100 kHz; modulation amplitude, 50 Gauss; scanning field, 335.2 ± 5 mT; receiver gain, 300–500; response time, 0.03 s; sweep time, 2 min; microwave power, 40 mW; and microwave frequency, 9.43 GHz. In order to confirm the specificity of the reaction, 100 μg/ml ALP and 5 U/ml Cu, Zn-SOD were added to the above mixture.

·OH was assayed by EPR spin trapping with DMPO and the scavenging activity was calculated from the relative intensity of the peak for the DMPO-hydroxyl radical adduct (DMPO-OH) EPR signal. The reaction mixtures contained 100 μM DTPA, 9 mM DMPO and 500 μM H_2O_2 in the absence or presence of varying concentrations of IS and were immediately transferred to the EPR flat cell and irradiated with UV (254 nm) for 1 min. EPR spectra were obtained immediately after the UV-irradiation. This analysis was done under the same conditions as were used for the previous EPR experiments. After recording the EPR spectra, the signal intensities of the DMPO-OH or the EMPO-OOH adducts were normalized against that of a manganese oxide (Mn^{2+}) signal, in which Mn^{2+} served as an internal control.

2.3. Chemiluminescence assay using activated neutrophils

The scavenging activity of IS against $O_2^{\cdot-}$ from the neutrophils was determined using a MiniLumat LB 9506 luminometer (Berthold Technologies, Bad Wildbad, Germany). The neutrophils (1.0×10^7 cells/ml) were pre-treated with LPS (1 μg/ml) for 30 min at 37 °C to activate the cells and generate $O_2^{\cdot-}$. Aliquots of this cell suspension were combined with 100 μM DTPA and 500 μM luminol in HBSS in the absence or presence of varying concentrations of IS and 1 U/ml Cu, Zn-SOD. Chemiluminescence was obtained from a graph of the slope of the counts versus time. Isolation of polymorphonuclear neutrophils are given in Supplementary data.

2.4. Chemiluminescence assay using X/XO system

The chemiluminescence response, induced by $O_2^{\cdot-}$ generated by an X/XO system, was measured by using a MiniLumat LB 9506 luminometer. The reaction was initiated by adding 40 μg/ml X to a sodium phosphate buffer (pH 7.4) containing 0.004 U/ml XO, 250 μM DTPA and 500 μM luminol in the absence or presence of each physiological concentration of IA, HA, CMPF, uric acids, and *p*-cresol (Fig. 3). In order to confirm the specificity of the reaction, a solution containing 40 μg/ml ALP and 5 U/ml Cu, Zn-SOD were added to the above mixture. The Chemiluminescence response was continuously recorded for 10 min at room temperature.

2.5. Statistics analyses

Statistical analyses were performed using the Student's *t*-test. A probability value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. IS scavenges $O_2^{\cdot-}$ generated by X/XO system

Fig. 1A shows data for the radical scavenging activity of IS examined by EPR using an EMPO spin trapping technique. EMPO was generally considered to be a good candidate for quantifying and trapping $O_2^{\cdot-}$ and has been extensively used as an efficient spin trapping agent for applications in vitro and in vivo [17,18]. In these experiments, Cu, Zn-SOD ($O_2^{\cdot-}$ scavenger) and ALP (XO inhibitor) were used as positive controls. These EPR signals generated by the X/XO system were completely inhibited by the action of both Cu, Zn-SOD and ALP. In the presence of 10 μM IS, the EPR signals were also significantly decreased. When the production of $O_2^{\cdot-}$ was calculated using the EPR spectra (Fig. 1B), the concentration of $O_2^{\cdot-}$ was found to be about 2.1 μM in the control and IS markedly inhibited $O_2^{\cdot-}$ production. These results demonstrate that normal-physiological concentration of IS, exhibited radical scavenging activity against $O_2^{\cdot-}$.

3.2. IS does not affect XO activity

Generally, XO uses X/hypoxanthine and oxygen as substrates to generate uric acid and $O_2^{\cdot-}$. Although decreases in the EPR signal

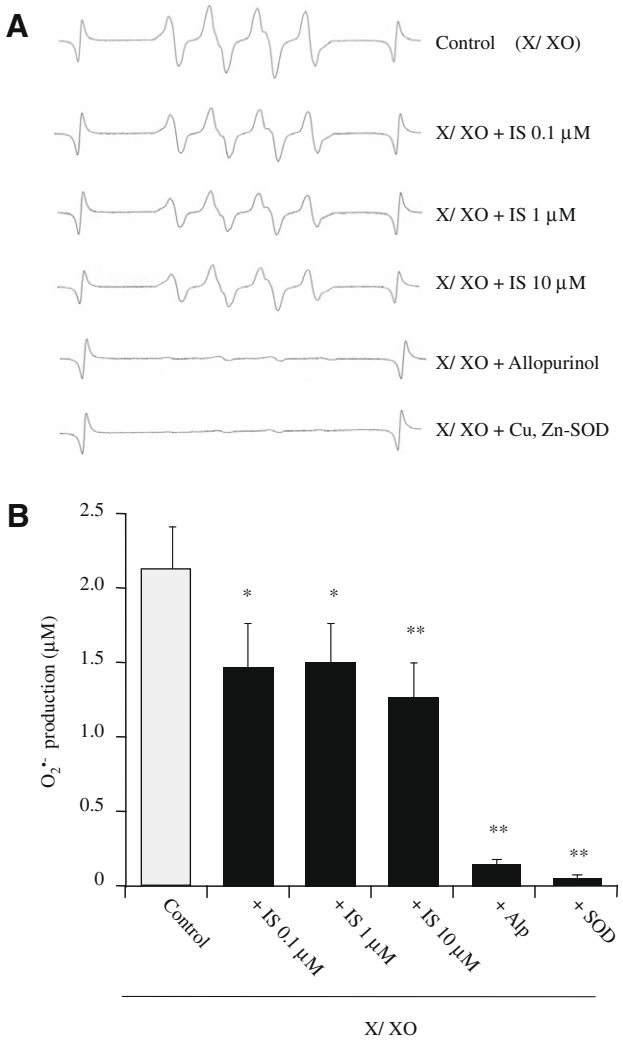

Fig. 1. Indoxyl sulfate scavenges superoxide anion radicals generated by a xanthine/xanthine oxidase system. (A) The EPR spectrum of EMPO spin adducts of $O_2^{\bullet-}$ generated in the xanthine/xanthine oxidase system; (B) the quantitation of the $O_2^{\bullet-}$ concentration. Values are expressed as the mean ± S.D. (n = 3–4). $^{*}P < 0.05$, $^{**}P < 0.01$ in comparison to the control.

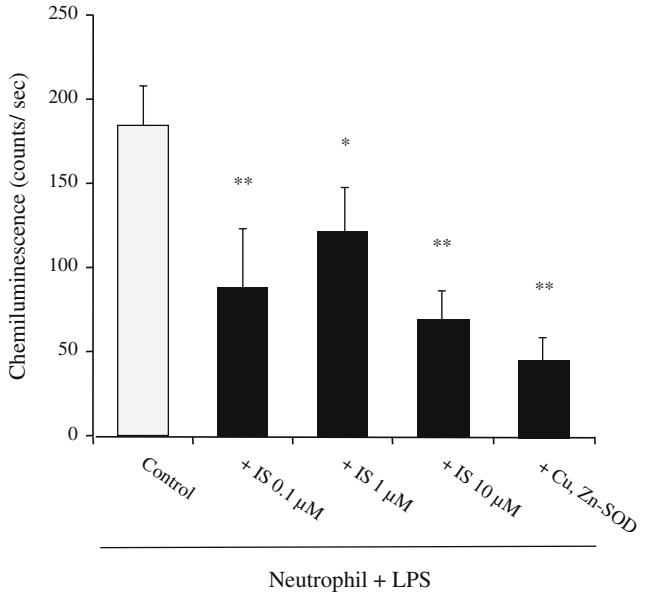

Fig. 2. The antioxidant properties of IS to LPS-stimulated neutrophils, as assessed by luminol chemiluminescence. Values are expressed as the mean ± S.D. (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$ in comparison to the control.

intensities was observed in the IS treatment (Fig. 1), it was necessary to examine whether this phenomenon was due to the inhibitory action of IS on XO or to direct $O_2^{\bullet-}$ scavenging activity. The formation of uric acid and 2-amino-4,7-dihydroxypteridine (isoxanthopterin), adducts catalyzed by XO from X and 2-amino-4-hydroxypteridine (pterin), and oxygen consumption were not affected by the IS treatment. Further details are given in Supplementary data. Based on these results, since the normal-physiological serum levels of IS had no effect on XO activity, it can be concluded that IS directly scavenges $O_2^{\bullet-}$ generated by the X/XO system.

3.3. IS scavenges $O_2^{\bullet-}$ generated by activated neutrophils

We also examine whether IS shows scavenging activity against $O_2^{\bullet-}$ in a cell system. $O_2^{\bullet-}$ was also generated from LPS-stimulated neutrophils by means of luminol-dependent chemiluminescence, because white corpuscle cells such as monocytes and neutrophils are major sources of various free radicals within blood vessels. As shown in Fig. 2, IS at concentrations of 0.1–10 μM also significantly decreased the chemiluminescence. It is especially noteworthy that the quenching effect of IS at 10 μM was comparable to that of Cu, Zn-SOD. These data suggest that IS also functions as a potential antioxidative compound against cell-derived $O_2^{\bullet-}$.

3.4. IS has strong $O_2^{\bullet-}$ - scavenging activity compared with other uremic toxins

As shown in above, within the concentrations observed under normal-physiological conditions, IS would be a potent endogenous scavenger of $O_2^{\bullet-}$. Thus, we investigate whether other uremic toxins also exhibit antioxidative activity within the concentration range for non-CKD conditions. IA, HA, CMPF, uric acids, and *p*-cresol, were used, as representative toxins (Fig. 3A) and their scavenging activities against $O_2^{\bullet-}$ under normal-physiological concentrations, respectively were evaluated using the luminol chemiluminescence system. As shown in Fig. 3B, significant decreases in chemiluminescence were observed in the cases of IS, uric acids, and *p*-cresol, whereas IA and HA showed no effects. Recent evidences demonstrated that in human, *p*-cresol was mainly circulated in the form of its sulfated conjugate, *p*-cresyl sulfate, and it increased leukocyte oxidative burst activity [19,20]. Thus, it is interesting to know whether *p*-cresyl sulfate also possesses radical scavenging activity as well as *p*-cresol. However, from a structural perspective, it is possible to deduce that the antioxidative potential of *p*-cresyl sulfate may be weaker than that of *p*-cresol because conjugate looses the phenolic OH which is generally corresponded to the radical scavenging property of phenolic compounds [21]. If this is correct, the radical scavenging activity of *p*-cresyl sulfate against $O_2^{\bullet-}$ may be weaker than that of IS since this activity was comparable to *p*-cresol (Fig. 3B). Of course, further studies using *p*-cresyl sulfate would be required to provide precise information regarding the roles of *p*-cresol as a radical scavenger. Collectively, these results and the data shown in Fig. 1, indicate that IS is an effective scavenger of $O_2^{\bullet-}$, among the endogenous uremic toxins.

3.5. IS scavenges $^{\bullet}OH$ generated by H_2O_2/UV system

As mentioned in Section 1, MT, another endogenous indole derivative, was an effective scavenger of, not only $O_2^{\bullet-}$, but also

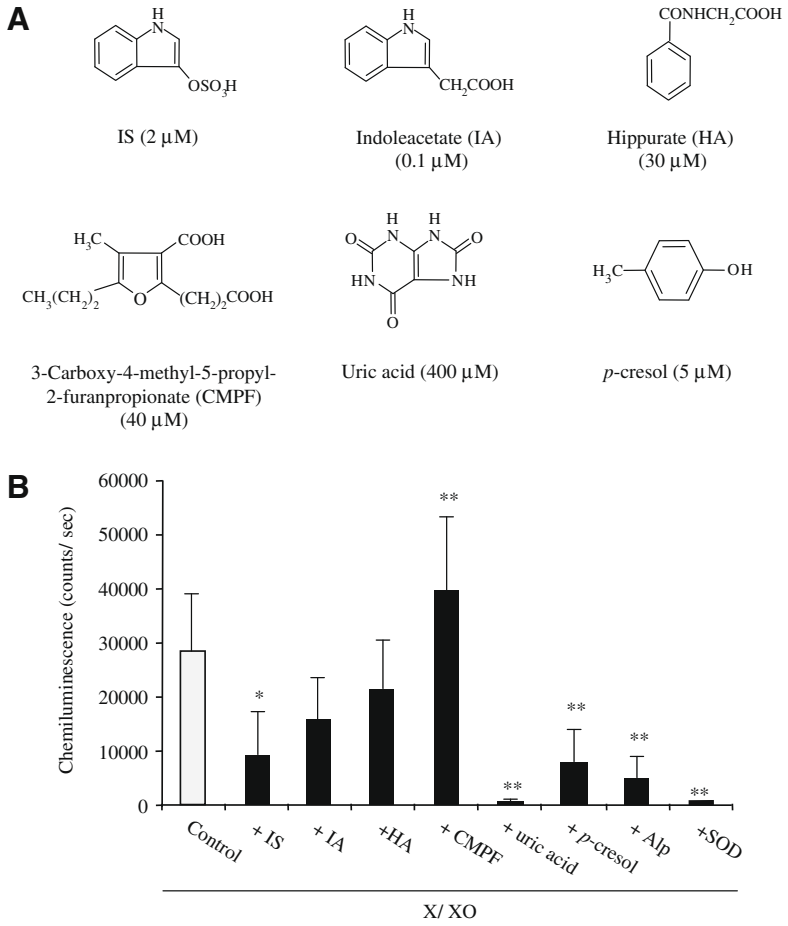

Fig. 3. Indoxyl sulfate has a strong scavenging activity compared to other uremic toxins. (A) Chemical structures of the uremic toxins used in this study; (B) the antioxidant properties of uremic toxins assessed by luminol chemiluminescence. Values are expressed as the mean ± S.D. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ in comparison to the control.

·OH. Therefore, we also investigated whether IS showed scavenging properties ·OH. In this study, ·OH was generated by means of an H_2O_2/UV system. As shown in Fig. 4, IS at 1–10 μM concentrations also eliminated ·OH significantly, suggesting that, IS also acts as a scavenger of ·OH at normal-physiological concentrations.

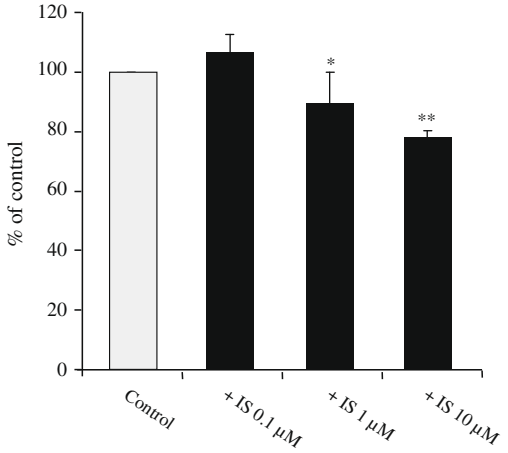

Fig. 4. Scavenging activity of IS against ·OH generated by a H_2O_2/UV system. Relative antioxidant activity of DMPO spin adducts of ·OH generated by H_2O_2/UV system. Values are expressed as the mean ± S.D. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ in comparison to the control.

4. Discussion

The present study demonstrated, for the first time, that IS, at normal serum concentrations, exhibited radical scavenging activity, especially against $O_2^{\cdot-}$. It is well known that $O_2^{\cdot-}$ species is an important initiator, in that it is capable of generating various reactive oxygen species, such as ·OH and peroxynitrite, at physiological conditions. In particular, $O_2^{\cdot-}$ produced by the X/XO system was known to be a significant activator of endothelial NADPH oxidase, one of other major sources of $O_2^{\cdot-}$. Such $O_2^{\cdot-}$ mediate pro-oxidative, proinflammatory, and procoagulatory changes in primary endothelial cell lines. Its damage induced by $O_2^{\cdot-}$ can disrupt the balance between vasodilation and vasoconstriction, which affects arterial remodeling, inflammatory mediators, and hemostasis and thrombosis. Thus, oxidative stress induced by $O_2^{\cdot-}$ produced in vascular cells will cause accumulative injuries to the endothelium and consequently are likely involved in the pathogenesis of cardiovascular and metabolic diseases, including atherosclerosis, ischemia–reperfusion injury, diabetes, and hypertension [22–24], if they are not effectively controlled. In other words, an adequate scavenger for $O_2^{\cdot-}$ is necessary to prevent the damage to the endothelium induced by $O_2^{\cdot-}$.

Our study clearly showed that, within the concentrations observed under normal-physiological conditions, IS is a potent endogenous scavenger of $O_2^{\cdot-}$ generated not only from X/XO system, but also from activated neutrophils. In addition, when the normal-physiological concentration of IS at 1 μM was added to human umbilical vein endothelial cells (HUVECs), a significant de-

crease in intracellular ROS was found, even though IS at levels in CKD conditions such as 100 or 500 μM significantly enhanced the intercellular ROS in HUVECs as previously reported [8]. Further details are given in Supplementary data. These observations predict that, in addition to extracellular SOD, IS may be one of the important endogenous compounds acting as an O_2^- scavenger in the blood circulation and also serves to protect endothelial functions. Furthermore, IS also exhibited radical elimination properties against ·OH. These findings suggest that IS plays a role as a novel endogenous antioxidant under normal-physiological conditions. Although the differences of antioxidant activity between IS and other indole compounds remained unclear, the sulfate might be favorable to enhance the reactivity of the electron-attracting nitrogen atom (–NH) in the pyrrole ring rather than other substituents because this nitrogen atom was likely to be responsible for the antioxidative potential of indoles.

As mentioned in Section 1 and Supplementary data, IS at levels in CKD conditions, significantly produced oxidative stress in various cell systems. At a glance, this may seem to be in conflict with the present results in which IS was found to act as an antioxidant. If this is correct, IS has both pro- and antioxidant properties, depending on its concentration. These interesting phenomena may imply that under non-CKD condition, antioxidative activity of IS greatly exceeded the intracellular ROS produced by IS, while under CKD condition, it was well over the radical scavenging activity of IS. In fact, such complex redox alternation is not an exception and has been also observed for other endogenous compounds. For example, uric acid functions as an antioxidant at low concentrations, while it causes ROS production at high concentrations [25]. Although further in vitro and in vivo studies will be needed to clarify the dual roles of IS with respect to its pro- or antioxidant properties, our results also explain, at least in part, the protective role of IS against oxidative stress under normal-physiological conditions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.04.046.

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