Review

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Daily hydroxyl radical scavenging capacity of mammals

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Summary **Both the formation and reactions of hydroxyl radical (•OH) are quantitative chemical reactions even in mammalians, and so we can reproduce such *in vivo* reactions in test tubes. Daily urinary excretions of some reaction products have been used to estimate the amount of •OH produced daily. Although urinary 8-hydroxydeoxyguanosine (8-OHdG) is a well-known marker of •OH, we have shown that creatol (CTL: 5-hydroxycreatinine), an •OH adduct of creatinine (Crn), and its metabolite, methylguanidine (MG), are better markers, because the amount of •OH scavenged by deoxyguanosine (dG) in the body is negligible. We measured CTL and MG together with Crn in 24-h urine, and calculated their molar sum, CTL + MG, providing a daily estimate of moles of •OH scavenged with Crn, and, from the molar ratio (CTL + MG)/Crn, we can calculate the percentage of Crn that was used to scavenge •OH. Healthy subjects and normal rats were indicated to use *circa* (ca.) 0.2 and 0.3% of Crn in order to scavenge •OH, respectively, because the corresponding ratios, scavenged •OH/Crn, were 2.2 and 3.0 mmole/mole (24-h urine) (Crn scavenged ca. 20-25 μmole and ca. 200 pmole of •OH in healthy subjects and normal rats, respectively). Since 8-OHdG/Crn has been reported to be 1.9 μmole/mole (24-h urine), the daily scavenging capacity with Crn is 10^3-fold more than dG. In patients with chronic renal failure (CRF) or chronic kidney disease (CKD) at stages 3-5: glomerular filtration rate (GFR) < 60 mL/min/1.73 m^2, •OH levels increased in proportion to the severity of CKD: up to ca. 3% of Crn was used daily in order to scavenge •OH. Although the accumulation of MG in organs has not been reported except for the brain and skin tissues in normal animals, •OH increases markedly and MG becomes detectable in all organs such as the kidney, liver, and heart in CRF rats.**

Keywords: Hydroxyl radical, creatinine, creatol, methylguanidine, 8-hydroxydeoxyguanosine

1. Introduction

Since creatinine (Crn) is one of main intrinsic hydroxyl radical (•OH) scavengers (*1*), we aimed to quantitatively show how much Crn scavenges •OH daily. Crn reacts with •OH to scavenge •OH and produce creatol (CTL: 5-hydroxycreatinine; •OH adduct to Crn, which partially decomposes to methylguanidine (MG) or demethylcreatinine (DMC)) (Figure 1A), and then they are excreted together with Crn into urine (*1-10*). Although we can detect *in vitro* creatones A and B as intermediates from CTL to MG (*11*), we do not introduce them herein, because they are not detectable *in vivo*. CTL and MG in serum and urine have been recognized as *in vivo* markers of •OH (*1-3,5-9*), although they were initially known as markers for chronic renal failure (CRF) (*12-14*). We can estimate their daily scavenging capacity for •OH using the urinary Crn-related metabolites of CTL, MG, and DMC. Since we know that urinary (CTL + MG) and DMC are roughly in a one to one ratio (*1,5*), we estimated the amount of DMC from the corresponding measured amount of CTL plus MG: the total sum might be nearly equal to 2 × (CTL + MG). Furthermore, we wanted to show in this mini-review that the well-known urinary level of 8-hydroxydeoxyguanosine (8-OHdG) (Figure 1B) (*15,16*) is not suitable as an *in*

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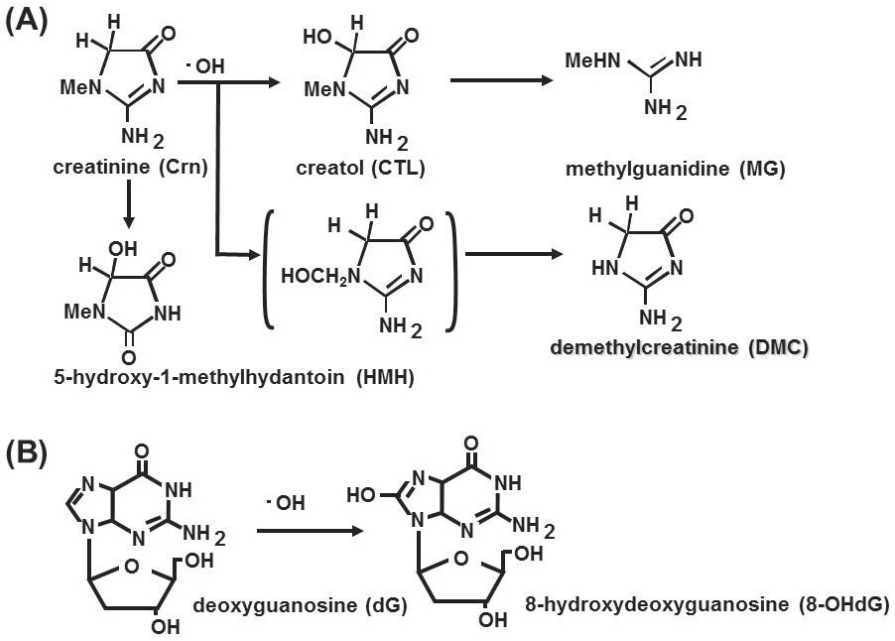

Figure 1. Metabolic pathways of Crn (A) and dG (B).

vivo marker of •OH in comparison with those of Crn-related markers, because their level is negligible (*17,18*). However, we wanted to cite papers reporting that 8-OHdG could be a useful marker of •OH inside the nucleus and mitochondria (*19*).

In order to show sites where •OH is detectable, we referred to reports on the distribution of MG in normal mammals (*1,20-22*). We distinguish, at the same time, organs where Crn is or is not distributed easily (*1,20,21*). We also illustrate that •OH accumulates in organs of mammals with CRF (*1,20,21*): chronic kidney disease (CKD) stages 3-5 (GFR: glomerular filtration rate: 60 mL/min/1.73 m^2) (*1,23*). We hope that this mini-review will clarify how much •OH might be produced daily, at the very least in mammals.

2. Detection of •OH

Because the •OH radical is so reactive, it is difficult to monitor directly. Therefore, an indirect monitoring method using a biomarker of •OH would be useful for patients with various diseases. In order to estimate the amount of •OH produced daily, the •OH-adducts and/or reactive products with •OH have to be measured as •OH biomarkers. Daily urinary excretions of such products have been used to estimate the amount of •OH produced daily. The molar ratio, (such metabolites)/Crn, in spot urine and 24-h urine has also been used in •OH-monitoring.

3. Daily amount of •OH scavenged by deoxyguanosine (dG) and Crn

We use •OH adducts of metabolites as markers of •OH.

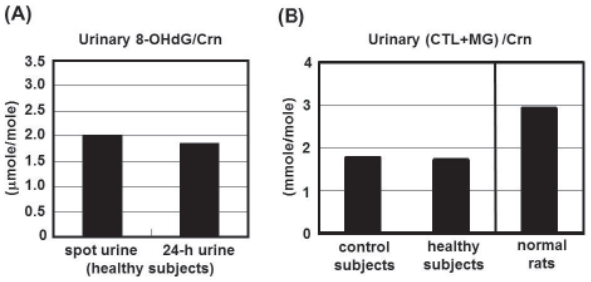

Figure 2. How much •OH is scavenged daily? Comparison of urinary levels of 8-OHdG/Crn (A) and (CTL + MG)/Crn (B).

One of the most frequently used biomarkers for •OH is 8-OHdG (*15-17*). However, their use should be limited, because only the amount of •OH formed inside nuclei and mitochondria can be shown (*1,17*). Furthermore, before the determination of 8-OHdG in urine, there are several reaction-steps from 8-OHdG-containing nucleotides. Therefore, we quantitatively compare the daily scavenged •OH in urine by dG with that by Crn. Since Crn is distributed throughout the whole body (*20*), its metabolites (especially CTL and MG) in urine can be monitored easily. In fact, the reported daily level of 8-OHdG excretion in urine of healthy subjects is fairly low; 8-OHdG/Crn: 2.03 ± 1.21 and 1.86 ± 1.09 μmole/mole (spot urine and 24-h urine, respectively: n = 67) (Figure 2A) (*17*); urinary 8-OHdG amount was also reported *circa* (ca.) 2.2 (1.7-2.8) and 6.05 (3.12-15.38) nmole/24 h, for control subjects (n = 85) and patients (n = 222), respectively (*18*). This means that dG scavenges 2-16 nmoles of •OH daily. Therefore, our estimation (see below) that the daily scavenged amount of •OH by Crn is ca. 50-500 μmoles in healthy subjects and severe CKD patients is very high. Daily

scavenging capacity with Crn might be at least 10^4-fold more than dG.

4. One of the best indices of *in vivo* •OH, the molar sum, CTL + MG, or its ratio to Crn, (CTL + MG)/Crn

Both the molar ratio of (CTL + MG)/Crn in 24-h urine and the urinary mole of (CTL + MG) against human subjects were reproducible to be 2.0 mmole/mole and ca. 20 μmole, respectively (Figure 2B) (*6,14*). Each one mole-detection of CTL or MG means that one mole of •OH, reacted with Crn, has been scavenged. Since both CTL and MG are directly detectable in the urine of mammals but not the serum of healthy individuals or normal mammals (*1,3,22*), we used urinary values.

Because Crn is distributed not only in nuclei and mitochondria but also in the cytosol and outside cells, the amounts of CTL and MG show how much •OH is scavenged by Crn therein. Theoretically, the molar sum, CTL + MG, and its ratio to Crn, (CTL + MG)/Crn, may be the best indices for the precise •OH level *in vivo*. We show that up to ca. 3% of Crn is used daily in order to scavenge •OH.

5. Increase in CTL and MG in patients with CKD

In patients with CKD at stages 3-5 (GFR < 60 mL/min/1.73 m^2), CTL and MG levels increase markedly (Figure 3) and •OH also increases in proportion to the severity of CKD. Both CTL and MG in serum and urine were initially indicated to be markers for CRF (*1,12-14*). However, both were later recognized to be markers of •OH (*1-3,5-9*).

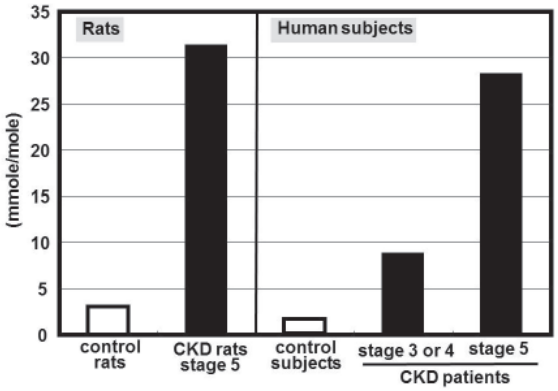

Figure 3. Amounts of •OH scavenged by Crn in mammals (rats and human subjects) in the presence of CRF.

6. Increase in •OH in patients with CKD

Healthy subjects and normal rats were indicated to use ca. 0.2 and 0.3% of Crn in order to scavenge •OH, respectively, because corresponding ratios, scavenged •OH/Crn, were 2.2 and 3.0 mmole/mole (24-h urine) (Crn scavenged ca. 25 μmole and ca. 200 pmole of •OH in healthy subjects and normal rats, respectively) (Figures 2 and 3). However, the production of •OH is increased in proportion to the severity of CKD, as shown in Figure 3. Because CTL/Crn and MG/Crn (mole/mole), for CKD patients in comparison with control subjects (eGFR > 60 mL/min/1.73 m^2), had been reported (*1*), we calculated their sum, (CTL + MG)/Crn (Figures 2 and 3), after CKD patients were classified into corresponding stages (Table 1).

Since MG levels in 48-h urine together with measured GFR values with time following adenine loading of rats had been reported (*22*), we assigned a stage of CKD for each sample and then mole of MG and the molar ratio of MG/Crn, and illustrated these in the previous review (*1*). From the molar ratio X (mmole/mmole), (CTL + MG)/Crn (Figure 3), the percentage of Crn used to scavenge •OH could be calculated as 10X %.

7. Prediction of sites where Crn scavenges •OH in mammals

Based on the reported Crn levels in rat organs (Figure 4) (*7*), and the reported MG levels in rat organs induced by Crn injection (*21,24*) and autoradiogram ^{14}C-Crn (*20*), sites and organs where Crn scavenges •OH in mammals was presumed (Figure 5). One-way flow of Crn from muscles and the brain, where Crn is synthesized and its concentration remains at a high level, into blood vessels was observed. In contrast, although Crn could be detectable in other organs (*21*) such as the kidney, liver, and heart, where it could not be synthesized, both in- and out-flows of Crn were observed. Sites where •OH is reacted with Crn to be scavenged, are ones

Table 1. Stages of CKD in mammals

Stage	Description*	Clinical GFR*, ** (mL/min/1.73 m^2)	Relative GFR (GFR/GFR_0)	Rat GFR******* (mL/min/kg)
1	Kidney damage with normal or GFR↑	> 90		15-17
2	Kidney damage with mild GFR↓	60-89		18-21
3	Moderate GFR↓	30-59	0.30-0.59	0.17-0.33
4	Severe GFR↓	15-29	0.15-0.29	0.08-0.16
5	Kidney failure	< 15 (or dialysis)	< 0.15	< 0.08

* The National Kidney Foundation K/DQI Clinical practice guidelines on CKD (2002).
** Clinical GFR_0, GFR of normal subjects, has been reported to be about 100 mL/min/1.73 m^2.
*** Rat GFR_0, GFR of normal rats, has been reported to be about ca. 0.55 mL/min/kg.
**** We classified CKD stages of rats based on rat GFR (Ienaga & Yokozawa, 2010).

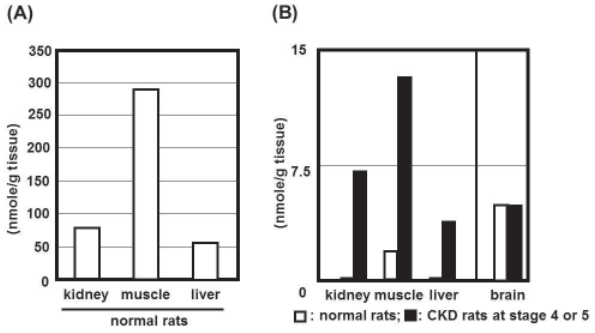

Figure 4. Crn and its •OH-product, MG, in organs of normal rats (A) and rats with CKD at stage 4 or 5 (B).

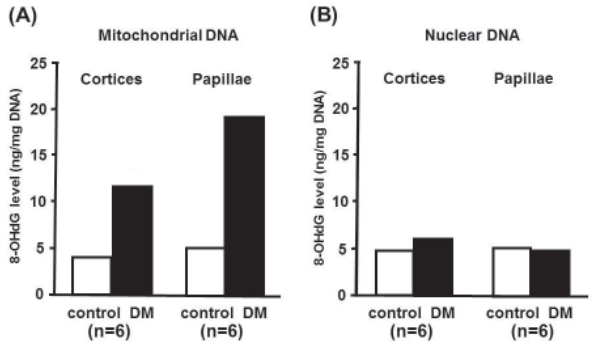

Figure 6. Changes in •OH inside mitochondria (A) and/or the nucleus (B) can be shown as changes in 8-OHdG levels.

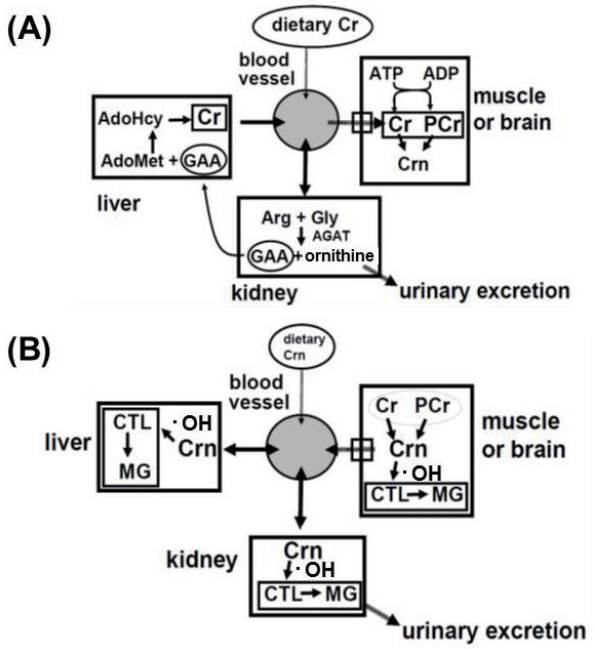

Figure 5. Flow of creatine (Cr) (A) and Crn (B) in mammals and organs where •OH is detectable.

where CTL, MG *etc*. are detected. In normal rats, only muscles and the brain had detectable levels of CTL and MG. However, in all organs in rats with CKD at stage 4 or 5, CTL and MG could be detected (Figure 4) (*21*).

8. Merits of measurement of 8-OHdG compared with Crn-related markers such as CTL + MG/Crn in mammals

If we want to know the total amount of •OH in mammals, Crn-related markers are likely to be more reliable than 8-OHdG for urinalysis. Absolute amounts of the former are ~10^4-fold higher than in the latter, and the former markers are determined directly without any further degradation process, whereas the latter are indirect, requiring not only degradation from the nucleotide chain but also excretion from the nucleus or mitochondria from cells into the urine *via* the cytoplasm and blood. However, for the estimation of DNA damage by •OH inside the nucleus or mitochondria, 8-OHdG is likely to be the better marker. Using a specific antiserum against 8-OHdG, we could show the difference in DNA damage between the nucleus and mitochondria. For example, at 8 weeks after the onset of diabetes, levels of 8-OHdG were significantly increased in DNA of mitochondria from the kidney of diabetic rats but not in nuclear DNA, suggesting the predominant damage of mitochondrial DNA (Figure 6) (*19*). If we want to further clarify the •OH levels scavenged by Crn in the nucleus and mitochondria, as well as in organs, we need a specific antibody against CTL and/or MG.

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