Electrolyzed Reduced Water Scavenges Active Oxygen Species and Protects DNA from Oxidative Damage

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Received March 21, 1997

Active oxygen species or free radicals are considered to cause extensive oxidative damage to biological macromolecules, which brings about a variety of diseases as well as aging. The ideal scavenger for active oxygen should be ‘active hydrogen’. ‘Active hydrogen’ can be produced in reduced water near the cathode during electrolysis of water. Reduced water exhibits high pH, low dissolved oxygen (DO), extremely high dissolved molecular hydrogen (DH), and extremely negative redox potential (RP) values. Strongly electrolyzed reduced water, as well as ascorbic acid, (+)-catechin and tannic acid, completely scavenged O2·− produced by the hypoxanthine-xanthine oxidase (HX-XOD) system in sodium phosphate buffer (pH 7.0). The superoxide dismutase (SOD)-like activity of reduced water is stable at 4°C for over a month and was not lost even after neutralization, repeated freezing and melting, deflation with sonicaton, vigorous mixing, boiling, repeated filtration, or closed autoclaving, but was lost by opened autoclaving or by closed autoclaving in the presence of tungsten trioxide which efficiently adsorbs active atomic hydrogen. Water bubbled with hydrogen gas exhibited low DO, extremely high DH and extremely low RP values, as does reduced water, but it has no SOD-like activity. These results suggest that the SOD-like activity of reduced water is not due to the dissolved molecular hydrogen but due to the dissolved atomic hydrogen (active hydrogen). Although SOD accumulated H2O2 when added to the HX-XOD system, reduced water decreased the amount of H2O2 produced by XOD. Reduced water, as well as catalase and ascorbic acid, could directly scavenge H2O2. Reduced water suppresses single-strand breakage of DNA by active oxygen species produced by the Cu(II)-catalyzed oxidation of ascorbic acid in a dose-dependent manner, suggesting that reduced water can scavenge not only O2·− and H2O2, but also 1O2 and ·OH.

Academic Press

MATERIALS AND METHODS

Electrolysis of water. Ultrapure water produced by an ultrapure system (ULTRAPUR LV-10T, TORAY, Tokyo) was added 0.1 g/l NaCl.
The principle of electrolysis was founded by Michael Faraday (1791-1867). In this process, reduction occurs at the cathode and oxidation at the anode. Dissociation of \( \text{H}_2\text{O} \) produces \( \text{H}^+ \) and \( \text{OH}^- \) ions. At the cathode, \( \text{H}^+ \) ions gain electrons to change into active atomic hydrogen (H). Active atomic hydrogen exhibits high reducing potential. It is then changed to hydrogen molecules \( \text{H}_2 \) which are chemically inert at room temperature. At the anode, \( \text{OH}^- \) ions lose electrons to form \( \text{OH} \), which results in the production of \( \text{O}_2 \) and \( \text{H}_2\text{O} \). Cathodic alkaline water (reduced water) is abundant in \( \text{DH} \), whereas anodic acidic water (oxidized water) is abundant in \( \text{DO} \). The relationships of RP with pH, DO, and DH in reduced water were shown in FIG. 1. Marked changes in these values occur in water after electrolysis. It should

RESULTS AND DISCUSSION

Characteristics of electrolyzed-reduced water. The principle of electrolysis was founded by Michael Faraday (1791-1867). In this process, reduction occurs at

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Inhibiting the attack by oxygen in solution and crystals be noticed that the DH value is higher in reduced water by collision (8). Atomic hydrogen can stably exist in any stable state, the extra energy is due to low DO and H2-water had no SOD-like activity. The SOD-like activity of reduced water is retained during storage, although the RP and DH values exhibit decay. These results clearly indicate that dissolved molecular hydrogen gas in reduced water was responsible for the negative RP value, but not for the SOD-like activity.

Stability of the SOD-like activity of reduced water. The SOD-like activity of reduced water was very stable in a closed glass bottle at 4°C for over a month. The activity was not lost even after neutralization, repeated freezing and melting, deflation with sonication for 10 minutes, repeated filtration with a 0.22-μm filter, boiling for 10 minutes, or autoclaving in a closed glass bottle at 121°C for 20 minutes (FIG. 2B). However, 90% the SOD-like activity was lost by autoclaving in an opened glass bottle, suggesting that the active substance in the reduced water is volatile. Atomic hydrogen is volatile and can reduce metallic oxide such as tungsten trioxide, though molecular hydrogen cannot easily do this (5). A sensitive detection method of atomic hydrogen is based on the color change of tungsten trioxide, though molecular hydrogen cannot reduce metallic oxide such as tungsten trioxide, though molecular hydrogen cannot easily do this (5). A sensitive detection method of atomic hydrogen is based on the color change of tungsten trioxide, though molecular hydrogen cannot easily do this (5). A sensitive detection method of atomic hydrogen is based on the color change of tungsten trioxide, though molecular hydrogen cannot easily do this (5).

When XOD solution was injected into the reaction mixture containing the diluted reduced water, strong CL was emitted immediately after injection (FIG. 2C), but the intensity of CL rapidly dropped below the control value. The initial strong transient emission of CL in diluted reduced water could be inhibited by SOD, indicating that a large amount of O2− was transiently generated just after the addition of XOD. AsA, (+)-

![Graph](image-url)
catechin and tannic acid, as well as SOD, did not show such a strong initial transient emission of CL (data not shown). Injection of the solvent without enzyme did not result in an emission of CL. More detailed experiments will be needed to clarify the mechanism of this phenomenon. The inhibitory effect of reduced water on the accumulation of O$_2^-$ was increased in a dose-dependent manner as shown in FIG. 2D, suggesting the stoichiometric action of active substance in reduced water.

Definition of the SOD-like activity of reduced water. Since this paper first reports the O$_2^-$ scavenging activity of electrolyzed-reduced water, the standardization of this activity is needed to compare the reducing potency of the reduced water prepared each time. In order to standardize the reducing potency of the reduced water, we defined an IC$_{50}$SO unit as a reducing potency of which reduced water can scavenge 50% of O$_2^-$ generated by the HX-XOD system under the conditions described in the MATERIALS AND METHODS section and an IC$_{50}$SO (%) as the concentration (%) of reduced water in which the 50% of O$_2^-$ generated by the HX-XOD system is scavenged. The IC$_{50}$SO values of SOD, AsA, (+)-catechin, and tannic acid were 0.05 U/ml, 3 μM (0.53 μg/ml), 130 μM (38 μg/ml), and 33 μg/ml in the HX-XOD system used here.

Catalase-like activity of reduced water. Although reduced water could scavenge O$_2^-$, there was a possibility that reduced water inhibited the enzyme activity of XOD or inhibited the reaction between O$_2^-$ and the luciferin analog reagent. To eliminate this possibility, the production of H$_2$O$_2$ was determined in the HX-XOD system. As shown in FIG. 3A, XOD produced not only O$_2^-$ but also H$_2$O$_2$ in this HX-XOD system. As shown in FIG. 3A, XOD produced H$_2$O$_2$ even in the presence of reduced water, demonstrating no inhibition of the enzyme activity by reduced water. As expected, the addition of SOD to the HX-XOD system resulted in the accumulation of H$_2$O$_2$, indicating that O$_2^-$ produced by XOD was changed to H$_2$O$_2$ by SOD. Reduced water first accumulated H$_2$O$_2$ and then gradually lowered the concentration of H$_2$O$_2$, suggesting that reduced water exhibits not only SOD-like activity but also catalase-like activity. The fact that reduced water stimulated the accumulation of H$_2$O$_2$ in the HX-XOD system in the first 5 minutes indicated that the decreased CL intensity in the presence of reduced water is not due to the inhibition of the reaction between O$_2^-$ and luciferin analog by reduced water, but due to the conversion of O$_2^-$ into H$_2$O$_2$ by reduced water. To demonstrate the catalase-like activity of reduced water, H$_2$O$_2$ was directly incubated with reduced water. As shown in FIG. 3B, reduced water scavenged H$_2$O$_2$ as well as AsA and catalase.

Suppressive effect of reduced water on the single-strand breakage of DNA caused by the Cu(II)-catalyzed oxidation of AsA. The DNA strand breakage is caused by the mixture of AsA and Cu(II) (10, 11). The Cu(II)-catalyzed oxidation of AsA is known to produce O$_2^-$ and H$_2$O$_2$ which react to produce 'OH (12). In order to demonstrate that reduced water can scavenge not only O$_2^-$ and H$_2$O$_2$ but also other active oxygen species, we examined the effect of reduced water on DNA breakage caused by the mixture of AsA and Cu(I). Super-coil plasmid DNA (Form I) changes to open-circular DNA (Form II) by a single-strand breakage. Open-circular DNA changes to linear DNA (Form III) by a double-strand DNA breakage. When plasmid DNA was incu-
bated with the mixture of AsA and Cu(II), the amount of super-coil DNA gradually decreased (FIG. 4). However, reduced water significantly inhibited the single-strand breakage by the mixture of AsA and Cu(II). As shown in Table 1, reduced water inhibited the DNA breaking reaction in a dose-dependent manner. Catalase also inhibited the breaking reaction but SOD did not, indicating that H₂O₂ participated in the breaking reaction, but O₂⁻ did not. Both radical scavengers specific to ·OH or O₂⁻ inhibited the breaking reaction. These results indicated that reduced water can prevent DNA damage caused by active oxygen species such as H₂O₂, ·OH, and O₂⁻ produced by the Cu(II)-catalyzed oxidation of AsA. Significant synergistic effect between reduced water and the radical scavengers used here was not observed (data not shown). It is noteworthy that reduced water can prevent DNA damage even in the presence of metallic ions which catalyze the autooxidation of most established antioxidants and reverse their protective effect against active oxygen species. However, the water must have a higher reducing ability for this purpose than scavenging O₂⁻.

Although aerobic organisms have evolved by acquisition of the ability to utilize oxygen, oxygen is principally a toxic substance. Recently biological activation of hydrogen by hydrogenases was reported (13). Hydrogenases, which are among the oldest enzymes (3.8 billion years old), can reversibly split molecular hydrogen to produce active atomic hydrogen. Active atomic hydrogen may have participated in the redox regulation of cellular functions. Water can permeate everywhere in the body and penetrates every membrane including the blood-brain barrier. To neutralize the toxic action of active oxygen species, electrolyzed-reduced water may be an ideal and very powerful antioxidant. Further intensive investigation on the effect of reduced water on cell biology, immunology and oncology should be promoted.

ACKNOWLEDGMENTS

We are grateful to Dr. David Barnes, Oregon State University, and Mr. Mark Selby for their critical reading of the manuscript.

**TABLE 1**

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<th>Addition</th>
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<th>Specificity</th>
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* AET = 2-(aminoethyl)isothiourium.
REFERENCES