ROS

RESEARCH ARTICLE

Oxidative Stress Caused by an SOD1 Deficiency Triggers the Accumulation of Oxidatively Modified Carbonic Anhydrase II in Erythrocytes

Takujiro Homma and Junichi Fujii

Department of Biochemistry and Molecular Biology, Graduate School of Medical Science, Yamagata University, 2-2-2 Iidanishi, Yamagata 990-9585, Japan

Correspondence: tkhomma@med.id.yamagata-u.ac.jp (T.H.), jfujii@med.id.yamagata-u.ac.jp (J.F.)

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ABSTRACT | Red blood cells (RBCs) are continuously exposed to reactive oxygen species (ROS) that are largely produced from intrinsic sources such as the oxidation of hemoglobin. Since superoxide dismutase 1 (SOD1) is the sole superoxide-scavenging enzyme in RBCs, a deficiency leads to the development of hemolytic anemia in mice, suggesting that the underlying mechanism involves a massive, oxidative stress-induced destruction of RBCs. We recently reported a decreased proteasomal function and the accumulation of ubiquitinated proteins in RBCs in SOD1-knockout (KO) mice. Because proteasomes are responsible for the degradation of both ubiquitinated proteins and oxidatively-modified (oxidized) proteins without ubiquitination, their malfunction results in the accumulation of these proteins. In the current study, we examined the issue of how elevated ROS are involved in the destruction of RBCs and the onset of anemia from the point of view of the accumulation of oxidized proteins. The findings indicate that carbonic anhydrase II (CAII) was the major protein within RBCs that was oxidized and that high levels had accumulated in SOD1-KO RBCs. Using purified CAII, we demonstrated that oxidative modification decreased its enzymatic activity in a ROS-dependent manner. In addition, the oxidized CAII molecules appeared to be degraded by proteasomes in RBCs. Based on these findings, we conclude that oxidative stress caused by an SOD1 deficiency disrupts the scavenging activity of proteasomes and accelerates the accumulation of oxidized CAII, leading to RBCs having a shortened life span.

KEYWORDS | Anemia; Carbonic anhydrase II; Oxidative stress; Proteasome; Superoxide dismutase

ABBREVIATIONS | AIHA, autoimmune hemolytic anemia; BH, biotin-hydrazide; CAII, carbonic anhydrase II; HNE, 4-hydroxy-2-nonenal; RBCs, red blood cells; ROS, reactive oxygen species; SOD1, superoxide dismutase 1

CONTENTS

1. INTRODUCTION

Reactive oxygen species (ROS) cause cellular damage by oxidizing lipids, proteins, and nucleic acids and are believed to be major contributors to aging and various diseases [1]. Red blood cells (RBCs) are continually exposed to oxidative damage by ROS owing to the mission, i.e., delivering oxygen molecules to the whole body [2]. While RBCs lack most of the usual cellular organelles, including mitochondria, relatively high amounts of ROS are produced as the result of the oxidation of hemoglobin. Hemoglobin constitutes the most abundant protein (5 mM) in RBCs and contains Fe(II)-heme as the oxygen carrier. Some of the hemoglobin is autoxidized to methemoglobin, which contains Fe(III)-heme and releases superoxide anion radicals [3, 4].

Because SOD1 is the sole superoxide-scavenging enzyme in RBCs, without SOD1, intracellular superoxide levels have been calculated to be 200-times higher than that in ordinary RBCs [4]. Indeed, elevated levels of ROS in SOD1-knockout (KO) C57BL/6 mice cause the lifespan of RBCs to be shortened by approximately 60–70% compared to RBCs of wild-type mice, resulting in the development of anemia [5]. This leads to autoimmune responses against RBCs in aged mice that are similar to those for autoimmune hemolytic anemia (AIHA) [5, 6]. The causal connection between oxidative stress and AIHA is further implicated by findings that elevated ROS levels in RBCs from AIHA-prone New Zealand Black (NZB) mice are accompanied by the accelerated onset of AIHA [7]. The reconstitution of SOD1 in erythroid cells improves anemic phenotypes not only in SOD1-KO NZB congenic mice, but also in mice possessing intrinsic SOD1 [8]. While a causal connection between oxidative stress and anemia is now becoming evident, the issue of how elevated ROS specifically lead to the destruction of RBCs remains unclear.

Proteins are oxidized during periods of oxidative stress, resulting in an acceleration in the production of oxidation products such as disulfide bond and carbonyl adducts [9]. The accumulation of these oxidatively modified (oxidized) proteins represents a hallmark of cellular aging and has been proposed to cause a disturbance in cellular homeostasis [10, 11]. Most nucleated cells are able to synthesize proteins to compensate for this via gene expression, but because RBCs are enucleated, they are unable to synthesize proteins. Proteasomes play a key role in maintaining cellular homeostasis by the proteolytic removal of oxidized proteins unless they are in heavily aggregated or cross-linked forms [12, 13]. Sustained oxidative stress accelerates the production of damaged proteins, which eventually exceeds the capacity for proteasomal removal, leading to their accumulation in the cells [14, 15]. We recently reported that the catalytic activities of proteasomes decrease due to increased levels of ROS, leading to the accumulation of ubiquitinated proteins in SOD1-KO RBCs [16]. Thus, oxidized proteins are not eliminated by proteasomes but accumulate, which triggers cellular dysfunction, premature aging, and, ultimately, the destruction of RBCs. However, the molecules that are targeted by superoxide that cause unfavorable reactions in anemia remain to be elucidated.

In the current study, we attempted to identify target proteins of ROS that are involved in the destruction of RBCs and the onset of anemia from the view point of the accumulation of oxidized proteins. The findings indicate that oxidative stress caused by an SOD1 deficiency induces the accumulation of oxidized carbonic anhydrase II (CAII), which appears to play a major role in the accelerated destruction of RBCs.

2. MATERIALS AND METHODS

2.1. Mice

C57BL/6J SOD1 hetero-knockout mice, originally established by Matzuk et al. [17], were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and backcrossed more than 10 times with C57BL/6N males. Genotypic analyses of the mice were performed using polymerase chain reaction (PCR) with specific primers, and wild-type (WT) and SOD1 homo-knockout (SOD1-KO) mice were used in this study. The animal room was maintained under specific pathogen-free conditions at a constant temperature of 20–22ºC with a 12 h alternating light-dark cycle. Animal experiments were performed in accordance with the Declaration of Helsinki under the protocol approved by the Animal Research Committee at Yamagata University.

2.2. Immunoblotting

RBCs collected from mice were washed three times with phosphate-buffered saline (PBS), and lysed in 20 mM Tris-HCl (pH 7.5). The lysate was centrifuged at 15,000 rpm for 10 min in a microcentrifuge. After centrifugation, the supernatant was collected and protein concentrations were determined using a Pierce® BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). The proteins were separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Chicago, IL, USA). The blots were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBST), and were then incubated overnight with an anti-CAII (sc-17244, Santa Cruz Biotechnology, Dallas, TX, USA) antibody diluted in TBST. After three washings with TBST, the blots were incubated with horseradish peroxidase-conjugated anti-goat IgG (sc-2020, Santa Cruz Biotechnology). After washing, the bands were detected using the Immobilon western chemiluminescent HRP substrate (Millipore, Burlington, MA, USA) on an image analyzer (ImageQuant LAS500, GE Healthcare).

2.3. Oxyblot Analysis

Protein oxidation was detected with an OxyBlot™ protein oxidation detection kit (Millipore). In typical experiments, RBCs were lysed in 20 mM Tris-HCl (pH 7.5) and total cellular proteins were denatured by adding 12% SDS to a volume equal to that for the cell lysate, which contained 20 μg of protein. The denatured proteins were then converted into 2,4-dinitrophenylhydrazone derivatives (DNP-hydrazone) by reaction with 2,4-dinitrophenylhydrazine (DNPH) for 15 min at room temperature. The derivatized proteins were then separated by 15% SDS-PAGE, detected with an anti-DNP antibody followed by a secondary antibody, according to the manufacturer's instructions.

2.4. Pull-Down Assay with Biotin-Hydrazide-Labeled Proteins

Lysates from WT RBCs (200 μg) or CAII from bovine RBCs (C2522, Sigma-Aldrich, St. Louis, MO, USA) were incubated with 5 mM biotin-hydrazide (BH, 347-06401, DOJINDO, Kumamoto, Japan) for 2 h at room temperature. Biotinylated proteins were purified by incubation with streptavidin agarose beads (N-1000, Solulink, San Diego, CA, USA) overnight at 4°C with gentle agitation followed by five washings with 1% NP40 in PBS. The supernatant and the resin-bound complex were boiled, separated by SDS-PAGE, and subjected to immunoblotting.

2.5. CAII Activity Assay

CAII activity assays were performed as described by Ho and co-workers with minor modifications [18]. Briefly, bovine CAII was incubated at 4°C for 1 h in 100 mM HEPES buffer (pH 7.4), in the presence of 2 mM $FeSO_4$, 10 mM sodium ascorbate, and 1 mM hydrogen peroxide (H_2O_2). The enzymatic reaction was then carried out in a total volume of 0.1 ml, containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 3 mM *p*-nitrophenyl acetate (N8130, Sigma-Aldrich) and 6 μg CAII at 37°C. The increase in the amount of p-nitrophenol produced was measured using a microplate reader (Varioskan Flash, Thermo Fisher Scientific) at an absorbance of 405 nm.

2.6. Culture of RBCs

RBCs were collected from mice through the tail vein, washed three times with PBS, and suspended in Dulbecco's modified Eagle medium (DMEM) (044-29765, Wako, Osaka, Japan) supplemented with 110 mg/l sodium pyruvate, 0.1% bovine serum albumin,

100 U/ml penicillin, and 100 mg/ml streptomycin, and dispensed into 1.5 ml tubes at a density of 1.0×10^7 cells/50 µl; they were maintained in a CO_2 incubator (5% CO_2, 95% air, 37°C).

2.7. Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6 software (La Jolla, CA, USA).

3. RESULTS

3.1. Preferential Oxidative Modification of Carbonic Anhydrase II (CAII) in RBCs

To determine the levels of proteins that were oxidized, we isolated RBCs from the WT and SOD1-KO mice and measured the level of oxidized proteins in lysates by detecting the concentrations of carbonyl groups. Oxidation induces the formation of reactive aldehyde groups on proteins that can be converted into a DNP adduct and detected by an anti-DNP antibody [19, 20]. The results indicated that an approximately 25–30 kDa protein was the major oxidized protein in the WT RBC and its concentration was elevated in SOD1-KO RBCs (**Figure 1A**, Top).

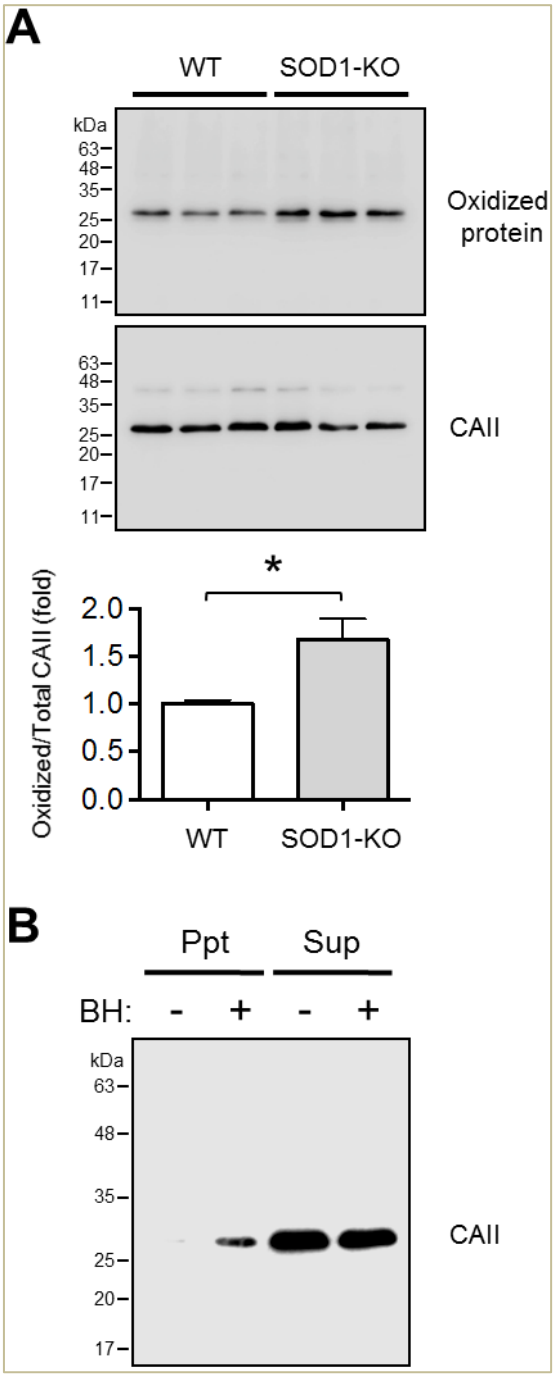
We then attempted to identify the 25–30 kDa protein that had been preferentially oxidized in RBCs. Carbonic anhydrase II (CAII) is a zinc metalloenzyme that catalyzes the reversible conversion of carbon dioxide and water into bicarbonate and protons [21]. Because CAII is the second most abundant protein in RBCs and has a similar molecular weight, we presumed that CAII was the likely candidate. The membrane was re-probed with an anti-CAII antibody, which resulted in positive bands at exactly the same molecular size (**Figure 1A**, Bottom). We next determined whether the amount of oxidized proteins changed in RBCs under conditions of culturing. We harvested RBCs from mice at 0, 24, and 48 h after incubation and examined the levels of oxidized proteins and total CAII by Oxyblot analysis and immunoblotting using an anti-CAII antibody, respectively. While the levels of oxidized proteins in RBCs from SOD1-KO mice were clearly higher compared to those from WT mice, the levels did not change during the incubation period (**Supplemental Figure 1**).

In order to confirm that the oxidized protein was CAII, we performed a pull-down assay of the oxidized proteins. An RBC lysate was incubated with biotin-hydrazide (BH), which reacts with reactive aldehyde moieties on proteins [22]. The proteins that had reacted with BH were pulled down by streptavidin-agarose followed by immunoblotting using the anti-CAII antibody. The findings indicated that the immunoreactive protein reacted with the anti-CAII antibody only in the precipitate (Ppt) from lysates that had been incubated with BH on the blot (**Figure 1B**). We therefore concluded that CAII was the very protein that we observed in the Oxyblot analysis. The ratio of oxidized CAII to total CAII was higher in SOD1-KO RBCs than in WT RBCs (**Figure 1A**, graph), suggesting that the elevated levels of oxidized CAII were due to an SOD1 deficiency.

3.2. Inactivation of CAII by ROS and Its Degradation by Proteasomes

Using purified bovine CAII, we examined the issue of whether the activity of CAII was affected by oxidative modification by ROS. We first incubated purified CAII with 1 mM H_2O_2 and 2 mM $FeSO_4$, which are the conditions used to produce hydroxyl radicals via the Fenton reaction, for 1 h and then subjected the products to a pull-down assay using the biotin-hydrazide and streptavidin-agarose methodology, as described above. As expected, the results revealed that CAII was oxidized by the hydroxyl radical produced via the Fenton reaction (**Figure 2A**). We next incubated purified CAII with 1 mM H_2O_2 and various concentrations of $FeSO_4$ for 1 h and then subjected the products to Oxyblot analysis as well as immunoblotting using the anti-CAII antibody. The results indicated that CAII was oxidized in a Fe(II) concentration-dependent manner (**Figure 2B**). We also observed that the esterase activity of CAII was decreased by hydroxyl radicals produced in the Fenton reaction and that this decrease was dose-dependent (**Figure 2C**). Thus, the oxidative modification of CAII was demonstrated to decrease the enzymatic activity of this protein.

Because oxidized proteins are known to be degraded by 20S proteasomes without ubiquitination [12], we examined the effects of an inhibitor of proteasomes (MG132) on cultured RBCs (10 µM, 24 h). The inhibition of proteasomal activity resulted in an increased level of oxidized CAII (**Supplemental Figure 2**), suggesting that the oxidized CAII is actually degraded by proteasomes in RBCs.



which is consistent with the fact that SOD1 plays an essential role in maintaining RBC homeostasis by protecting against oxidative stress [5, 6].

The considerable increase in ROS formation by mitochondrial dysfunction is a risk factor for developing neurodegenerative diseases such as Alzheimer's disease (AD) [23]. The oxidized form of CAII has been detected in the brains of AD and subjects with mild cognitive impairment (MCI), compared with age-matched controls [24–27]. Consistent with this, the activity of CAII is significantly lower (50% decrease) in brains of MCI subjects, compared with their age-matched controls. The carbonyl levels of CAII in MCI brains increase with decreasing CAII activity, suggesting that the oxidative inactivation of CAII may contribute to the progression of cognitive dysfunction [27].

Because CAII binds to hemoglobin and regulates its affinity for oxygen, the preferential oxidation of CAII might affect the delivery of oxygen by hemoglobin and cause the dysfunction of RBCs [28, 29]. Superoxide anions are continuously generated in huge amounts in RBCs via the auto-oxidation of hemoglobin [3, 4]. Under SOD1 deficient conditions, intracellular superoxide levels are about 200 times higher than those in ordinary RBCs [4]. Elevated superoxide

FIGURE 1. CAII undergoes preferential oxidative modification in RBCs. Panel A: a representative Oxyblot analysis of WT and SOD1-KO RBCs is shown (Top). The same blot was re-probed with an anti-CAII antibody to determine the total amount of CAII protein (Bottom). Graph depicting the quantification of oxidized proteins normalized to the corresponding total amount of CAII. Values are the mean ± SEM of mice (n = 3 for each group). *, $p < 0.05$; panel B: RBC lysates from WT mice were incubated with or without biotin-hydrazide (BH) and purified using streptavidin beads. The supernatant (Sup) and the precipitate (Ppt) were probed with anti-CAII antibody.

4. DISCUSSION

In the current study, we show, for the first time, that high levels of oxidized proteins, mainly CAII, accumulate in RBCs from SOD1-KO mice (**Figure 1**), then reacts with nitric oxide and forms peroxynitrite ($ONOO^-$), a strong, negatively charged oxidant [30]. The selective oxidative modification of CAII among RBC proteins may be based on its enzymatic characteristics; i.e., it contains Zn^{2+} at the catalytic center.

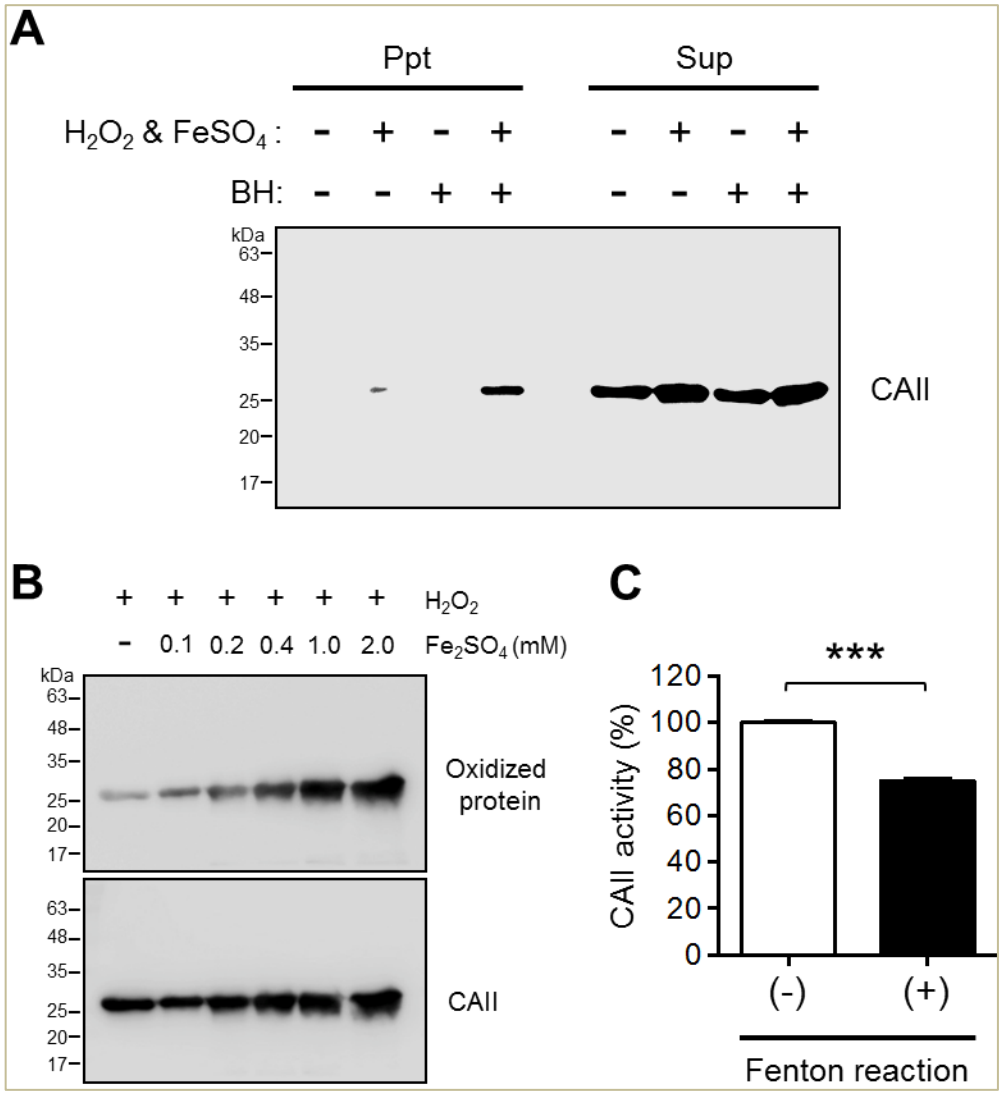


FIGURE 2. ROS induced oxidative modification of purified CAII, resulting in its decreased activity. Panel A: purified CAII was incubated with or without 1 mM H_2O_2 and 2 mM $FeSO_4$ (Fenton reaction). The sample was then incubated with or without biotin-hydrazide (BH) and purified using streptavidin beads. The supernatant (Sup) and the precipitate (Ppt) were probed with anti-CAII antibody; panel B: purified CAII was incubated with 1 mM H_2O_2 and various concentrations of $FeSO_4$ (0, 0.1, 0.2, 0.4, 1.0, and 2.0 mM). Oxidized CAII was detected by Oxyblot analysis (Top). The same blot was re-probed with an anti-CAII antibody to determine total CAII protein (Bottom); panel C: the enzyme activities in CAII with or without Fenton reaction (treated with 1 mM H_2O_2 and 2 mM $FeSO_4$) were measured (See Materials and Methods section for details). The data represent the mean ± SEM of at least three independent experiments (***, $p < 0.001$).

Anionic ROS, including superoxide and peroxynitrite, would be attracted by Zn^{2+} in the catalytic center. Moreover, because CAII catalyzes both the hydration of carbon dioxide to and the dehydration of bicarbonate ion, the catalytic center of CAII would be predicted to be structured so as to attract negatively

charged bicarbonate ions and hence may also attract negatively charged ROS, analogous to gathering superoxide by SOD1 [31]. In any case, the negatively charged ROS that are attracted to CAII may participate in the selective oxidation of amino acids on CAII. Reports that, in yeast in a stationary phase, even SOD1 is oxidized at its cysteine and histidine residues [32] appear to support this hypothetical mechanism for the oxidation of CAII.

The presence of relatively high levels of an autoantibody to CAII has been reported in several autoimmune diseases, including systemic lupus erythematosus (SLE) [33], Sjögren syndrome [33], Graves' disease [34], rheumatoid arthritis [35], and ulcerative colitis [36]. The development of sialoadenitis, a characteristic of the Sjögren syndrome, has been reported in mice that had been immunized with a CAII antigen [37]. It is plausible that the high immunogenicity of CAII reflects the fact that CAII is preferentially oxidized in preference to other proteins. Because oxidized RBCs are efficiently engulfed by macrophages, the resulting oxidized molecules would be recognized by the immune system as neoantigens [38, 39]. 4-Hydroxy-2-nonenal (HNE), a highly reactive lipid peroxidation product, reacts with and modifies proteins and alters their antigenic characteristics [40]. Considering the preferential binding of HNE to CAII [41], the HNE adduct of CAII may become a suitable antigen for the immune system. Consistent with this hypothesis, in a previous study, we reported that the levels of autoantibodies against CAII as well as those against lipid peroxidation products are elevated in SOD1-KO mice [5, 6].

It has been shown that moderately HNE-modified proteins are preferentially degraded by proteasomes, but that proteins that are more extensively modified undergo aggregation, which can inhibit proteasome function [42]. We recently reported on a decreased proteasomal function and increased accumulation of ubiquitinated proteins in SOD1-KO RBCs [16]. A significant decline in proteasome activity upon severe oxidative stress has also been reported in cultured cells [43, 44]. The level of oxidized CAII was increased after proteasomal inhibition (Supplementary Fig. 2), indicating that oxidized CAII in its non-ubiquitinated form undergoes proteasomal degradation. Another example of the proteasome-mediated degradation of oxidized protein in RBCs without ubiquitination is peroxiredoxin (PRDX) 2, the third most abundant protein in RBC, in which the cysteine sulfhydryl group is hyperoxidized (PRDX-$SO_{2/3}$). The amounts of PRDX-$SO_{2/3}$ show cyclic changes according to the circadian oscillation in RBCs under cultured conditions [45, 46]. Whereas ROS produced via the autoxidation of hemoglobin are involved in the hyperoxidation of PRDX, the removal of PRDX-$SO_{2/3}$ by proteasomal degradation appears to give rise to cyclic changes in PRDX-$SO_{2/3}$ in RBCs, even under cultured conditions [47]. In the case of SOD1-KO mice, however, PRDX-$SO_{2/3}$ in RBCs accumulates markedly with no associated cyclic changes [48], which can be rationally explained by the dysfunction of the proteasomal system by elevated ROS [16]. It is therefore likely that oxidative stress disturbs proteasome activity and, in turn, triggers the accumulation of both ubiquitinated proteins and non-ubiquitinated oxidized proteins, leading to the destruction of RBCs.

5. CONCLUSION

In conclusion, oxidative stress caused by an SOD1 deficiency induces the accumulation of oxidized CAII, leading to its inactivation in RBCs. Because oxidative stress induces a malfunction in the scavenging activity of proteasomes, such a dysfunction would result in the acceleration in the accumulation of damaged proteins. Thus, a fatal vicious cycle is triggered by an SOD1 deficiency that disturbs protein homeostasis, which would render RBCs being more susceptible to oxidative modification, shorten their lifespan, ultimately resulting in the development of anemia.

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