Erythropoietin Increases Expression and Function of Vascular Copper- and Zinc-Containing Superoxide Dismutase

Livius V. d’Uscio, Leslie A. Smith, Zvonimir S. Katusic

Abstract—Previous studies have shown that treatment with erythropoietin (EPO) exerts vascular protective effects. The exact mechanisms responsible for these effects are not completely understood. In the present study, we hypothesized that EPO stimulates expression and activity of copper- and zinc-containing superoxide dismutase (SOD1), thus protecting vascular tissue from oxidative stress induced by excessive concentrations of superoxide anions. EPO treatment of wild-type mice for 2 weeks (1000 U/kg, SC, biweekly) significantly increased aortic expression of SOD1. This effect resulted in a significant reduction of superoxide anion concentrations in aorta of treated mice. The ability of EPO to reduce vascular production of superoxide anions was abolished in SOD1-deficient mice. In a mouse model of wire-induced injury of the common carotid artery, treatment of wild-type mice with EPO prevented pathological remodeling, whereas the vascular effect of EPO was absent in SOD1-deficient mice. Our findings demonstrate that treatment with EPO increases vascular expression of SOD1. This effect appears to be an important molecular mechanism underlying vascular protection by EPO. (Hypertension. 2010;55:998-1004.)

Key Words: superoxide dismutase 1 ■ erythropoietin ■ superoxide anions ■ protein kinase B ■ vasculature ■ mice

Under physiological conditions, endothelium-derived NO exerts vascular protective effects as it dilates the vasculature, prevents adhesion of circulating blood cells, and inhibits vascular smooth muscle cell (VSMC) proliferation.1 The formation of superoxide anions, a major chemical inactivator of NO, is kept under tight control by endogenous superoxide dismutase (SOD) enzymes, which catalyze the conversion of superoxide anions to H2O2 and molecular oxygen.2 Three SOD isoforms are known to exist: constitutive copper- and zinc-containing SOD (SOD1), manganese-containing SOD (SOD2), and extracellular SOD (SOD3).2 In most tissues, SOD1 is expressed in the cytosol, whereas, in contrast, SOD2 is exclusively located in mitochondria. The third and the most recently discovered SOD3 is also a copper- and zinc-containing enzyme that, after secretion by VSMCs, becomes bound to the endothelial surface.3 The SOD1 isoform accounts for 50% to 80% of total SOD activity and is the predominant form of SOD in blood vessels.4–6 Most importantly, SOD1 protects intracellular NO bioavailability in endothelial cells by limiting the increase in superoxide anions, thereby preserving the normal intracellular concentration of NO.6,7

Erythropoietin (EPO) has been recognized as a “tissue-protective” cytokine.8 In the vasculature, protective effects of EPO are dependent on activation of endothelial NO synthase (eNOS) and biosynthesis of tetrahydrobiopterin.9–12 Furthermore, we have shown previously that endothelium-dependent relaxations were normalized in injured carotid arteries of wild-type mice treated with EPO.11 However, the local concentration of NO in the arterial wall is not only dependent on enzymatic activity of eNOS but is also critically affected by SOD activity and the concentration of superoxide anions. Indeed, high local concentrations of superoxide anions are considered a major mechanism of endothelial dysfunction after vascular injury.1–13 In the present study, we tested the hypothesis that EPO exerts an antioxidant effect in the arterial wall by increasing expression and activity of SOD1. To test this hypothesis, we used SOD1-deficient (SOD1−/−) mice and determined the effect of EPO on superoxide anion production in SOD1−/− mice arteries.

Materials and Methods

Experimental Animals and Model of Vascular Injury

Male SOD1−/− mice (B6;129S7-Sod1tm1Leb/J) and their wild-type littermates were obtained from Jackson Laboratory (Bar Harbor, ME) and were maintained on standard chow with free access to drinking water. Housing facilities and all of the experimental protocols were approved by the institutional animal care and use committee of the Mayo Clinic and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Wire-induced vascular injury was performed in the left common carotid artery as described previously.11 Mice were ran-
Body weight, biweekly, SC; Amgen). The dosage of EPO was randomly distributed to an injury group (PBS, Gibco) and an EPO group (recombinant human EPO—Ametasys). The dosage of EPO was selected on the basis of previous studies. After 14 days of treatment, the animal were euthanized (pentobarbital, 60 mg/kg of body weight, IP).

**Systolic Blood Pressure**
Systolic blood pressure (SBP) was recorded in quiescent mice by a tail-cuff method (Harvard Apparatus Ltd) before surgery and on day 14 of treatment.14

**Blood Cell Count**
Blood cell counts were performed with the ABAXIS VetScan HM2 Hematology System, as described previously.11

**Morphological Analysis of Carotid Arteries**
Morphological analyses were performed on perfused and fixed vessels in buffered formalin (10%). Each artery was embedded in paraffin, and cross-sections were continuously cut every 10 μm from one edge to the other edge of the carotid artery (8 sections). Corresponding sections of the contralateral artery were used as the control. Each section was mounted on slides and subjected to standard Verhoeff-Van Gieson staining.15 Adobe Photoshop software 6.0 was used to analyze the medial cross-sectional area (CSA).

**Western Blot Analysis**
Aortas and lungs were excised and homogenized in lysis buffer. Equal amounts of protein (50 μg) were separated by SDS-PAGE and transferred to nitrocellulose membrane (Amersham), after which the membranes were probed using primary antibodies against SOD1, SOD2, SOD3 (StressGen), Akt1, pAkt1(Ser473), 3-nitrotyrosine (Upstate), and catalase (Sigma). As a loading control, blots were rehybridized with anti–β-actin (Sigma).16

**Detection of Superoxide Anions**
Intracellular superoxide anions were quantified using a high-performance liquid chromatography/fluorescence assay that uses dihydroethidium as a probe.17 A stable fluorescent product, 2-hydroxyethidium, is formed from the reaction between dihydroethidium and superoxide anions. Aortas were opened longitudinally and incubated in Krebs-HEPES buffer containing 50 μmol/L of dihydroethidium (Molecular Probes) at 37°C for 15 minutes. The samples were washed to remove the free probe and incubated in Krebs-HEPES buffer for 1 additional hour at 37°C. The arteries were then homogenized in 4°C cold methanol and centrifuged at 12,000 rpm. The supernatant was analyzed by high-performance liquid chromatography/fluorescence (Beckman Coulter) in 37.0% acetonitrile in 0.1% trifluoroacetic acid aqueous solution. Data were quantified using an Amplex Red hydrogen peroxide (H2O2)/peroxidase assay kit (Invitrogen) and normalized against tissue protein levels.

**Measurement of Hydrogen Peroxide**
An Amplex Red hydrogen peroxide (H2O2)/peroxidase assay kit (Invitrogen) was used to perform the measurements of H2O2 release from mouse aorta as described previously.19

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**Figure 1.** A, Representative Western blot analysis for the expression of the SOD1 protein in wild-type (WT) mouse aortas after 14 days of treatment with EPO (top). The bar graph indicates the results of the relative densitometry as compared with β-actin (bottom). B, Quantitative analysis of production of superoxide anions, as detected by 2-hydroxyethidium, in the aorta of wild-type littermates. Data are shown as mean±SEM (n=6 to 8). *P<0.05 vs control wild-type mice (unpaired t test).

**Table 1.** Effect of 2 Weeks of Treatment With EPO on Blood Cell Profile in Wild-Type Littermates and SOD1−/− Mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WT</th>
<th>WT + EPO</th>
<th>SOD1−/−</th>
<th>SOD1−/− + EPO</th>
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</thead>
<tbody>
<tr>
<td>Hematocrit, %</td>
<td>45.8 ± 1.1</td>
<td>60.8 ± 1.2</td>
<td>42.2 ± 0.5</td>
<td>54.9 ± 0.6</td>
</tr>
<tr>
<td>Hemoglobin, g/dL</td>
<td>15.3 ± 0.4</td>
<td>20.8 ± 0.2</td>
<td>13.6 ± 0.2</td>
<td>18.1 ± 0.2</td>
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<td>White blood cells, 10⁶/mm³</td>
<td>7.1 ± 0.7</td>
<td>9.2 ± 0.6</td>
<td>5.2 ± 0.3</td>
<td>5.9 ± 0.4</td>
</tr>
<tr>
<td>Lymphocytes, 10⁶/mm³</td>
<td>5.6 ± 0.5</td>
<td>6.8 ± 0.5</td>
<td>4.1 ± 0.2</td>
<td>5.1 ± 0.3</td>
</tr>
<tr>
<td>Monocytes, 10⁶/mm³</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Granulocytes, 10⁶/mm³</td>
<td>1.1 ± 0.3</td>
<td>2.0 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>Platelets, 10⁹/mm³</td>
<td>682 ± 52</td>
<td>715 ± 22</td>
<td>681 ± 38</td>
<td>768 ± 70</td>
</tr>
</tbody>
</table>

WT indicates wild-type littermates. Data are mean±SEM (n=6 to 8).

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**Figure 2.** Effect of 2 weeks of treatment with EPO on protein expressions of SOD2 and SOD3 in the aorta of wild-type and SOD1−/− mice. A, Representative Western blot analysis for expressions of SOD2 and SOD3 proteins. The bar graphs indicate the results of the relative densitometry as compared with β-actin (B and C). Data are shown as mean±SEM (n=3 to 5).
Table 2. SBP of Wild-Type and SOD1−/− Mice and Those Treated With EPO for 14 Days

<table>
<thead>
<tr>
<th>Week</th>
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<th>WT + EPO</th>
<th>SOD1−/−</th>
<th>SOD1−/− + EPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>115±2</td>
<td>118±1</td>
<td>106±2*</td>
<td>106±2*</td>
</tr>
<tr>
<td>2</td>
<td>119±1</td>
<td>117±1</td>
<td>105±2*</td>
<td>109±1*</td>
</tr>
</tbody>
</table>

WT indicates wild-type littermates. Data are in millimeters of mercury expressed as mean±SEM of 5 to 7 mice. *P < 0.05 vs WT mice (ANOVA + Bonferroni).

Calculations and Statistical Analysis
All of the results are expressed as mean±SEM, and “n” indicates the number of animals from which tissues were harvested. Single values were compared by 1-way ANOVA with Bonferroni correction for multiple comparisons. When simple comparisons were made between 2 groups, where appropriate an unpaired Student t test was used. A value of P < 0.05 was considered significant.

Results

SOD1 Protein Expression
Treatment with EPO for 14 days increased protein expression of SOD1 in the aorta of wild-type mice (Figure 1A). In tandem, basal superoxide anion levels decreased (Figure 1B). In contrast, treatment with EPO did not increase the expression of SOD2 and SOD3 isoform proteins in wild-type mice (Figure 2). Likewise, EPO had no effect on SOD2 and SOD3 protein expressions in SOD1−/− mice (Figure 2).

Characteristics of SOD1−/− Mice
Red blood cell number, hematocrit, and hemoglobin were significantly reduced in SOD1−/− mice when compared with wild-type littermates (P < 0.05; Table 1). The white blood cell count was unaltered (Table 1). Treatment with EPO for 14 days selectively increased the red blood cell profile to a similar degree in both wild-type and SOD1−/− mice (P < 0.05; Table 1).

In agreement with a previous report, red blood cell number, hematocrit, and hemoglobin were significantly reduced in SOD1−/− mice when compared with wild-type littermates (P < 0.05; Table 1). The white blood cell count was unaltered (Table 1). Treatment with EPO for 14 days selectively increased the red blood cell profile to a similar degree in both wild-type and SOD1−/− mice (P < 0.05; Table 1). Administration of EPO for 14 days did not affect SBP in wild-type or SOD1−/− mice (Table 2).

Effects of EPO on Superoxide Anions Production
Genetic deletion of SOD1 increased Mn(III) tetra(4-benzoic acid) porphyrin chloride–inhibitable superoxide anions levels in SOD1−/− mice aortas (P < 0.05; Figure 3A). Superoxide anion production was quantitatively the same in a comparison of in vitro incubation of wild-type mouse aorta with the Cu²⁺–chelator diethyldithiocarbamic acid, an agent that inhibits both SOD1 and SOD3, indicating that intracellular superoxide anion regulation depends on activity of SOD1 (data not shown). Fourteen days of treatment with EPO did not prevent elevation of superoxide anions levels in SOD1−/− mice (Figure 3A).

Western blot analysis with a 3-nitrotyrosine antibody revealed a significant increase in 3-nitrotyrosine abundance in the lungs of SOD1−/− mice (P < 0.05; Figure 3B). EPO treatment for 2 weeks did not affect increased levels of 3-nitrotyrosine–positive proteins in SOD1−/− mice (Figure 3C).

Morphology of Injured Carotid Artery
Under basal conditions, medial thickness was significantly smaller in SOD1−/− mice as compared with their wild-type littermates (P < 0.05; Figure 4). Fourteen days after carotid artery mechanical injury, medial CSA significantly increased in both wild-type and SOD1−/− mice as compared with corresponding uninjured arteries (P < 0.05; Figure 4). Surprisingly, the injury-induced increase in medial thickness was significantly smaller in SOD1−/− mice as compared with wild-type littermates (P < 0.05; Figure 4E). Treatment with EPO for 14 days significantly decreased the medial CSA of injured carotid arteries in wild-type mice, whereas EPO treatment did not significantly affect wall thickness of injured carotid arteries in SOD1−/− mice (Figure 5).

Akt Activation in SOD1−/− Mice
Protein kinase B (or Akt) is a critical component of major signaling pathways involved in cellular proliferation, migration, and survival, events that contribute to vascular hyperplasia and restenosis. In the current study, Western blot analysis showed a significant decrease in Akt1 phosphorylation.
In the present study, we report several novel findings. First, treatment with EPO for 2 weeks increased protein expression of SOD1 and decreased superoxide anions concentrations in the aorta of wild-type mice. Second, EPO did not affect increased superoxide anions levels in SOD1−/− mice. Third, EPO treatment significantly prevented aberrant remodeling of injured carotid arteries in wild-type mice but not in SOD1−/− mice. Thus, our results suggest that augmenting expression and activity of SOD1 by EPO is an important mechanism responsible for the prevention of oxidative stress.

EPO is widely used in clinical practice to correct anemia. Most recently, several studies have shown that EPO has vasoprotective effects, an action that is independent of erythropoiesis.9–11 Impaired NO-mediated, endothelium-dependent relaxations and increased medial thickness after injury were normalized in wild-type mice treated with EPO.11

Discussion

In the present study, we report several novel findings. First, treatment with EPO for 2 weeks increased protein expression at Ser473 in SOD1−/− mice aortas as compared with wild-type littermates (P<0.05; Figure 6). In these wild-type littermates, treatment with EPO for 2 weeks significantly increased Akt phosphorylation at Ser473 (P<0.05; Figure 6). In contrast, EPO failed to activate Akt phosphorylation in SOD1−/− mice (P<0.05 versus EPO-treated wild-type littermates; Figure 6). Akt1 protein (total) remained constant and did not differ between wild-type and SOD1−/− mice.

**H2O2 Release and Catalase Expression in SOD1−/− Mice**

H2O2 release from the aorta was significantly decreased in SOD1−/− mice as compared with wild-type mice (P<0.05; Figure 7A). Furthermore, protein expression of catalase was significantly increased in the aorta of SOD1−/− mice (P<0.05; Figure 7B and 7C).

**Figure 5.** Effect of EPO on morphological changes of injured carotid arteries in wild-type and SOD1−/− mice. Results are mean±SEM (n=7 to 15) and expressed as percentile changes from uninjured carotid arteries. *P<0.05 vs PBS-treated wild-type mice; n.s. indicates not statistically different (ANOVA with Bonferroni).

**Figure 6.** Effect of 2 weeks of treatment with EPO on protein expression of Ser473-phosphorylated Akt1 in the aorta of wild-type and SOD1−/− mice. A, Representative Western blot analysis for expression of Ser473-pAkt1 and Akt1 proteins. B, Bar graph indicates the results of the relative densitometry compared with Akt1. Data are shown as mean±SEM (n=5 to 8 independent experiments). *P<0.05 vs wild-type mice; †P<0.05 vs EPO-treated wild-type mice (ANOVA with Bonferroni).
Interestingly, our present study showed that treatment with EPO selectively increased vascular SOD1 protein expression and decreased superoxide anion levels in wild-type mice but did not affect superoxide anion in SOD1−/− mice. We regard this observation as an important one, because the majority of the SOD in vascular wall is attributed to SOD1.6 This is in line with the previous observation showing that superoxide anion levels are decreased in the aorta of SOD1 transgenic mice.21,22 Most importantly, the stimulatory effect of EPO on the expression of SOD1 has important therapeutic implications. In our previous studies we have shown that EPO increases the expression of phosphorylated eNOS in wild-type mice.11 Moreover, the local concentrations of NO in the arterial wall are not only dependent on enzymatic activity of eNOS but are also determined by concentrations of superoxide anions.1 Indeed, increased local production of superoxide anions appears to be an important component of the vessel response to injury. Consistent with this concept, previous studies have indicated that reactive oxygen species are important mediators of smooth muscle cell proliferation and migration.23 Likewise, superoxide production is increased not only as an early response of the vessel wall to injury but also 2 weeks after injury.13,24–26 On the basis of these observations, the current results suggest that the vascular protective effects of EPO in wild-type mice may be mediated at least in part via increased vascular wall SOD1, thereby protecting NO from inactivation by superoxide anions. The exact molecular mechanism responsible for activation of SOD1 by EPO remains to be determined.

To determine whether deletion of the SOD1 gene may abolish vascular protective effects of EPO, vascular structures in injured carotid arteries of SOD1−/− mice were studied. Quite unexpectedly, we found that, under basal conditions, medial CSA of the carotid artery was significantly reduced in SOD1−/− mice. This is at variance with the results reported by Baumbach et al,27 showing that vascular hypertrophy is present in cerebral arterioles of SOD1−/− mice. The cause of discrepancy is unclear at the present time but could be related to the anatomic origin and functional differences between conduit arteries and cerebral arterioles. However, our findings also showed that vascular remodeling after wire-induced injury was less pronounced in SOD1−/− mice as compared with wild-type mice. This result suggests that genetic inactivation of SOD1 may have a previously unrecognized inhibitory effect on pathological remodeling after injury. Inactivation of SOD1 is known to increase intracellular concentrations of superoxide anions and peroxynitrite.6 In addition, we also showed that inactivation of SOD1 decreased the release of H2O2 from the aorta. Such a decrease is relevant for interpretation of our findings, specifically because H2O2 plays an important role in the regulation of cell growth, proliferation, and development, as well as in the progression of atherosclerosis.58 Most importantly, H2O2 (but not the superoxide anion) enhances VSMC proliferation and growth.29–31 Therefore, the reduced arterial wall thickness in SOD1−/− mice could be explained by the absence of superoxide anion dismutation, and significantly reduced the vascular concentration of H2O2.52 In addition, we observed an increased protein expression of catalase in the arterial wall of SOD1−/− mice. This observation is in agreement with our suggestion that local concentration H2O2 is low in arteries of SOD1−/− mice. Consistent with this interpretation of our findings, a recent study by Zhang et al19 demonstrated that overexpression of catalase decreases the hypertrophic effect of angiotensin II–induced hypertension, whereas overexpression of SOD1 was ineffective,21 thereby reinforcing the important role of H2O2 in the control of vascular wall thickness in vivo.

Akt is one of the most important molecular targets activated by physiological concentrations of H2O2,33,34 It is also an important mediator in cell growth and survival.35 Interestingly, phosphorylation of Akt1 at Ser473 was reduced in the aorta of SOD1−/− mice, indicating that the observed reduction in medial thickness may be caused by the decreased activity of Akt1.36 Moreover, previous studies have demonstrated that arterial injury causes proliferation of VSMCs via phosphorylation of Akt at Ser473.37–40 The importance of Akt signaling is further emphasized by the results of in vivo studies demonstrating that transduction of the injured carotid artery with a dominant-negative Akt mutant or treatment of animals with phosphatidylinositol 3-kinase inhibitor wortmannin, results in reduced VSMC proliferation.36,37,40 Furthermore, physiological concentrations of H2O2 cause phosphorylation of Akt.41 Thus, it appears that reduced phosphorylation of Akt1 in SOD1−/− mice is a likely consequence of a low intracellular concentration of H2O2.

We also observed an increase of 3-nitrotyrosine-positive proteins in SOD1−/− mice, indicating an increased formation of the potent oxidant peroxynitrite by interaction of the superoxide anion with NO. An excessive formation of peroxynitrite represents an important mechanism contributing to cell death and dysfunction in cardiovascular disease.42 Several in vitro and in vivo studies reported inhibition of Akt activity and Akt phosphorylation by peroxynitrite through a mechanism involving nitration and inactivation of phosphatidylinositol 3-kinase.53–55 These mechanisms may also help
to explain the reduction of arterial wall thickness in SOD1\(^{-/-}\) mice.

Increased superoxide anions production contributes to endothelial dysfunction and pathogenesis of hypertension.\(^{46}\) However, arterial blood pressure was paradoxically reduced in SOD1\(^{-/-}\) mice, despite the presence of increased superoxide anions concentrations. The exact mechanism of hypotension induced by genetic inactivation of SOD1 is unclear. However, several studies suggest that a decreased concentration of \(\text{H}_2\text{O}_2\) may reduce SBP. Indeed, vascular specific overexpression of catalase resulted in a reduction of blood pressure in mice,\(^{47}\) indicating that increased elimination of \(\text{H}_2\text{O}_2\) in the vascular wall may have hypotensive effects. Moreover, the plasma concentration of \(\text{H}_2\text{O}_2\) is increased in patients with essential hypertension and is positively correlated with SBP.\(^{48}\) There is also evidence that superoxide anions can chemically inactivate cate cholamines, thus lowering SBP in vivo.\(^{49}\) Although the exact mechanism of hypotension induced by genetic inactivation of SOD1 remains to be determined, the results of the present study offer new insights into alterations of the arterial wall architecture in SOD1\(^{-/-}\) mice. We speculate that elevated concentrations of superoxide anions associated with a low concentration of \(\text{H}_2\text{O}_2\) and subsequent impairment of Akt signaling may help to explain the lower arterial blood pressure observed in SOD1\(^{-/-}\) mice. On the other hand, we cannot rule out the possibility that changes in vascular architecture in SOD1\(^{-/-}\) mice are secondary to a reduction in blood pressure caused by a mechanism that remains to be determined.

Traditionally, an elevated concentration of superoxide anion and subsequent chemical inactivation of NO are considered detrimental for the vascular function.\(^1\) Quite surprisingly, cardiovascular phenotypic characteristics of SOD1\(^{-/-}\) mice suggest that loss of SOD1 (and subsequent increase in superoxide anion) may result in paradoxical hypotension and reduced propensity toward medial thickening. Despite the fact that these findings introduced difficulties in the interpretation of our findings, it is important to re-emphasize that treatment with EPO significantly reduced the production of superoxide anion and medial thickening in wild-type mice. In contrast, elevated production of superoxide anion and significant thickening of the injured arterial wall were not affected in SOD1\(^{-/-}\) animals, thereby supporting the major hypothesis of the present study.

**Perspectives**

A growing body of evidence indicates that EPO has tissue-protective properties that are critically dependent both on increased eNOS and Akt activity and increased bioavailability of NO.\(^9\)\(^{-12}\)\(^{50}\) The present study showed that, in wild-type mice, treatment with EPO increased vascular SOD1 expression and effectively prevented vascular remodeling after carotid artery injury. In contrast, genetic inactivation of SOD1 abolished the ability of EPO to reduce concentrations of superoxide anions, thereby suggesting that EPO exerts an antioxidant effect in the blood vessel wall by regulating expression and activity of the SOD1 protein.

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**Disclosures**

None.

**References**


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