Ferric Human Neuroglobin Scavenges Superoxide to Form Oxy Adduct

Taku Yamashita, Leila Hafsi, Eri Masuda, Hirofumi Tsujino, and Tadayuki Uno

Graduate School of Pharmaceutical Sciences, Osaka University; 1–6 Yamadaoka, Suita, Osaka 565–0871, Japan.
Received February 25, 2014; accepted March 13, 2014

Neuroglobin (Ngb) is the third member of the vertebrate globin family, and the structure was solved as a typical globin fold with a b-type heme. Although it has been proposed that Ngb could be involved in neuroprotection against oxidative stress, the protective mechanism has not been fully identified yet. In order to clarify functions under hypoxic condition, in this study, we focused on the scavenger activity of human Ngb (hNgb) against superoxide. The activity of hNgb for superoxide was evaluated to be 7.4 µM for IC_{50}, the half maximal inhibitory concentration. The result indicates that hNgb can be an anti-oxidant, and the value was almost the same as that of ascorbic acid. In addition, we characterized oxidation states of a heme iron in superoxide-treated hNgb with spectroscopic measurements. Superoxide-treated hNgb in the ferric form was readily converted to the oxygenated ferrous form, and the result suggested that ferric hNgb could scavenge superoxide by change of an oxidation state in a heme iron. Moreover, mutational experiments were performed, and the each variant mutated at 46 and 55 positions suggested a disulfide bond between Cys46 and Cys55 could be essential to be sensors for oxidative stress with the direct binding of superoxide. As a consequence, we concluded that redox changes of the heme iron and the disulfide bond could regulate neuroprotective functions of hNgb, and it suggests that hNgb can afford protection against hypoxic and ischemic stress in the brain.

Key words neuroglobin; superoxide; disulfide bond; superoxide dismutase (SOD); oxidation state

Results and Discussion

To assess response to oxidative stress of hNgb, we prepared wild-type and three variants as described previously. Although IC_{50} of hNgb for superoxide was estimated previously with lucigenin chemiluminescence, we applied BESSo to avoid the effect of the presence of reductant and to evaluate the IC_{50} value for superoxide scavenger activity. The activities of superoxide dismutase (SOD) and an anti-oxidant ascorbic acid were also examined for comparison. As shown in Table 1, we could evaluate IC_{50} value for wild-type hNgb as 7.4 µM. Although SOD showed almost 40000 times higher activity than hNgb, ascorbic acid was evaluated to have almost equal activity to hNgb (IC_{50}=8.3 µM). Ascorbic acid is reported to have an activity against the coagulate necrosis, and hence hNgb can be proposed to express the neuroprotective function as well.

Since hNgb is revealed to scavenge superoxide, we subsequently investigated how hNgb interacted with superoxide. The purified hNgb is normally in the ferric state. We then introduced KO_{2} to the ferric hNgb to generate superoxide, since conventional hypoxanthine–xanthine oxidase system generated much less amount of superoxide and could not induce detectable spectral change. As shown in Fig. 1A, the ferric spectrum was obviously shifted by the addition of KO_{2}, and the original Q band at 533 nm was replaced by two peaks at 543 and 577 nm. These new peaks are reminiscent of oxygen-bound heme proteins, and hence we prepared oxy-hNgb for comparison. We photoreduced ferric hNgb with the NADH/FMN system under air. Although the oxygen-bound form showed increased baseline by added catalase, the spectrum revealed that almost 90% of hNgb is in the oxygen-bound state. Notably, the two peaks in the Q-band region coincided completely with those of the superoxide adduct of Ngb.

Table 1. The Scavenger Activity of hNgb against Superoxide

<table>
<thead>
<tr>
<th>Scavenger</th>
<th>IC_{50} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Ngb WT</td>
<td>7.4±0.7</td>
</tr>
<tr>
<td>Human Ngb C46S</td>
<td>8.2±1.0</td>
</tr>
<tr>
<td>Human Ngb C55S</td>
<td>3.2±0.5</td>
</tr>
<tr>
<td>Bovine SOD</td>
<td>(178±20)×10^{-6}</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>8.3±0.8</td>
</tr>
</tbody>
</table>

The activities were measured triplicate and IC_{50} values are shown with standard deviations.

The authors declare no conflict of interest.
# These authors contributed equally to this work.

* To whom correspondence should be addressed. e-mail: unot@phs.osaka-u.ac.jp © 2014 The Pharmaceutical Society of Japan
and C55S mutants almost lost the superoxide binding property, with much less spectral change by the KO
2 application. In addition, few grains of KO
2 (ca. 2 mg) were introduced into ferric hNgb (5 µM) in the same buffer containing catalase (130 U/mL) to eliminate the by-product H2O2. (B) Time-dependent decay of KO
2-treated form. The spectra were measured at 0, 3, 12, 30, 60, and 180 min after introducing KO
2, and the changes are expressed with arrows.

hNgb, and hence it is now clear that ferric hNgb interacts directly with superoxide and is converted to oxygen-bound form.

In order to clarify the iron oxidation state of KO
2-treated hNgb, we introduced two heme ligands, CN− and CO. These ligands are utilized frequently to study heme properties.11,12 Although CN− is a good ligand to the ferric heme, the spectrum of KO
2-treated hNgb was not disturbed by the addition of KCN. On the other hand, the spectrum was fully changed immediately when CO gas was introduced into the KO
2-treated ferric hNgb. These results establish unequivocally that the ferric heme in hNgb is converted to ferrous-oxy form by the direct binding of superoxide.

Since the oxy-hNgb has been reported to be unstable (half-life = ca. 1 min),13 we traced the decay of KO
2-treated hNgb as shown in Fig. 1B. The spectrum changed in a time-dependent manner, and finally the ferric spectrum was recovered. This indicates that the heme in hNgb was not damaged by superoxide and that oxy-hNgb is converted to the ferric state. The half-life was evaluated as 21.6 min, which is much longer than that for authentic oxy-hNgb.13

The longer half-life of our oxy-hNgb implies that amino acids are in different conditions relative to the authentic oxy-hNgb. In the physiological condition, reduction and oxidation will apply not only to the heme iron but also to cysteine residues. Actually, hNgb has three cysteines, two of which (Cys46 and Cys55) form a disulfide bond and affect the oxygen affinity.14,15 Therefore, we prepared three Cys mutants (C46S, C55S, and C120S) in order to evaluate contribution of the disulfide bond to the superoxide binding. Although the C120S mutant indicated two prominent Q-bands, the C46S and C55S mutants almost lost the superoxide binding property, with much less spectral change by the KO
2 application (Fig. 2). This mutation study revealed clearly that the disulfide bond is crucial for the superoxide binding to the ferric hNgb. This result is entirely consistent with the previous studies, in which breaking of the disulfide bond results in a low oxygen affinity conformation of hNgb.8,16 Therefore, the disulfide bond is strongly suggested to be essential in regulating oxygen binding property. These mutants were, however, revealed to have superoxide scavenger activity similar to that of WT hNgb (Table 1). This implies that the disulfide bond is not a prerequisite for eliminating superoxide, but plays a key role in stabilizing bound O2 with long half-life.

In summary, we could show that ferric hNgb can scavenge superoxide (Table 1) to give ferrous-oxy form (Fig. 1A). The resultant oxy-hNgb is long-lived enough (Fig. 1B), and the disulfide bond between Cys46 and Cys55 is essential to the direct binding of superoxide to retain it as safe O2 (Fig. 2). In this context, the Cys residues are sensors for oxidative stress in the cells. Although several functions for hNgb have been listed, we can propose a schematic model for redox cycle of hNgb (Fig. 3). When cell condition inclined to oxidative (e.g. reperfusion after ischemia), an intramolecular disulfide bond could be constructed by the oxidative stress (state A), and the disulfide bond facilitates the reaction with superoxide to give ferric-superoxy adduct (state B). This adduct must be converted immediately to ferrous-oxy complex (state C) by intramolecular electron transfer. The oxy-hNgb is stable enough to retain the bound oxygen molecule, and finally returns slowly to the original state A in the oxidative cycle. When the cell condition inclined to hypoxic (e.g. ischemia), on the other hand, the disulfide bond would be reduced to thiols by

Fig. 1. (A) Absorption Spectra of hNgb in the Ferric, Photoreduced Oxygen-Bound, and KO
2-Treated Forms Which Are Shown with Thick Gray, Thin, and Thick Black Lines, Respectively.

Fig. 2. The Absorption Spectra of WT (A), C46S (B), C55S (C), and C120S (D) in 10 mM Sodium Phosphate and 100 mM NaCl (pH 7.0)

The ferric and the KO
2-treated forms are indicated with thin and thick lines, respectively.

Fig. 3. Proposed Cycle of hNgb in Various Redox Conditions

The pair of cysteine residues, Cys46 and Cys55, is depicted as either “-SH” or “-S” in order to indicate the presence of the disulfide bond.
intracellular reductants (state D), and the heme iron would be reduced (state E). Oxygen may bind to the ferrous heme (state F) but will be auto-oxidized quickly to return to the initial state D in the reductive cycle. The two proposed cycles are supported by the distinct stabilities of the oxy-hNgb (states C and F). Although oxy-hNgb (state F) is unstable under hypoxic (reductive) condition, toxic superoxide will be retained as a safer oxygen molecule (state C) under oxidative condition in which the disulfide bond formation is facilitated. Therefore, we conclude that these redox changes of heme iron and disulfide bond would regulate neuroprotective functions of hNgb in the brain.

References