Pharmacological Induction of Neuroglobin Expression

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Key Words

Neuroglobin · Cinnamic acid · Valproic acid · Ischemia

Abstract

Neuroglobin (Ngb) is an intracellular, oxygen-binding neuronal protein with protective effects against ischemia and related pathological processes. To identify small molecules capable of inducing Ngb protein expression, which might have therapeutic benefit, we examined Ngb expression by Western blot in cultured HN33 (mouse hippocampal neuron × N18TG2 neuroblastoma) cells. In addition to deferoxamine, which was shown previously to enhance Ngb levels, Ngb expression was increased by the short-chain fatty acids cinnamic acid and valproic acid ($\geq 100 \mu\text{mol/l}$), but not by other short-chain fatty acids, histone deacetylase inhibitors, or anticonvulsants. Drugs that stimulate the expression of neuroprotective proteins like Ngb may have therapeutic potential in the treatment of stroke and other neurological disorders.

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Introduction

Neuroglobin (Ngb) is an oxygen-binding protein related to hemoglobin and myoglobin, but expressed predominantly in neurons and endocrine cells [1]. Its ex-

pression is transcriptionally induced by neuronal hypoxia and cerebral ischemia, against which it confers protection [2–5]. Overexpression of Ngb also protects neurons against β -amyloid [6, 7], nitric oxide [8], and hydrogen peroxide [9] toxicity in vitro and attenuates histopathological and behavioral features in a murine model of Alzheimer's disease in vivo [6]. Although the mechanisms involved in the neuroprotective effect of Ngb are unknown, these observations suggest that measures which increase Ngb levels might have therapeutic benefit in hypoxic or ischemic brain disorders, including stroke.

Because mammalian Ngb is an intracellular protein that does not cross cell membranes [10], direct administration of Ngb is not a feasible therapeutic strategy. However, a small number of chemical agents have been shown to induce Ngb expression in vitro. These include cobalt and the iron chelator deferoxamine (DFO) [4], which also enhance levels of the hypoxia-inducible factors HIF-1 α and HIF-2 α , and the heme (ferrous protoporphyrin IX) oxidation product hemin (ferric protoporphyrin IX) [11], which stimulates transcription, translation and assembly of hemoglobins [12]. Of interest, both DFO [13, 14] and hemin [15] improve outcome from focal cerebral ischemia in rats, although these effects have been attributed to the induction of HIF-1 α and heme oxygenase-1, respectively. DFO is used clinically in the treatment of iron intoxication related to thalassemia, but allergic reactions

and pulmonary, renal and neurological toxicity may occur [16]. Hemin is used to treat acute porphyrias, but is expensive and unstable, and may produce severe phlebitis [17].

As a starting point for investigating possible pharmacological regulators that might be used to enhance Ngb expression during brain hypoxia or ischemia, we examined the effects of short-chain fatty acids found previously to induce fetal (γ) globin expression [18].

Materials and Methods

To study the effects of potential Ngb inducers in vitro, we used HN33 (mouse hippocampal neuron $\times$ N18TG2 neuroblastoma) cells, which show induction of Ngb by hypoxia [4] and hemin [11], and protection from hypoxia by forced Ngb overexpression [4]. Cells were plated at 1×10^5 cells/well on uncoated, 24-well plastic dishes and maintained as described [4]. Drugs were purchased from Sigma (St. Louis, Mo., USA), except for vorinostat (SAHA) (Cayman Chemical, Ann Arbor, Mich., USA). Cultures were treated with various concentrations of drugs, or with DFO as a positive control [4], and Western blotting was performed 24 h later using a polyclonal anti-Ngb antibody (Sigma), as described previously [4]. Additional controls consisted of HN33 cells transfected with full-length mouse Ngb cDNA cloned into a pcDNA 3.1 plasmid with CMV promoter (induced Ngb control), or HN33 cells transfected with the pcDNA 3.1 vector alone (baseline Ngb control) [4].

Results

Figure 1 shows that, as expected, Ngb expression was low in control HN33 cells and high in HN33 cells transfected with pcDNA 3.1-Ngb or treated with DFO. Of the short-chain fatty acids tested, cinnamic and valproic acids showed prominent induction of Ngb, whereas butyric, levulinic and succinic acids had little or no effect.

Fig. 1. Pharmacologic induction of Ngb protein expression in HN33 cells. **a** Western blot showing basal Ngb protein expression in wild-type (left) and Ngb-overexpressing (right) HN33 cells. **b** Western blots showing Ngb protein expression in wild-type HN33 cells exposed for 24 h to short-chain fatty acids (butyric, cinnamic, levulinic, succinic or valproic) or to DFO (positive control) at the concentrations indicated. **c** Average fold control induction of Ngb protein expression, normalized to actin expression, in wild-type HN33 cells exposed for 24 h to the same compounds at the same concentrations ($n = 3$; * $p < 0.05$ compared to control). **d** Relationship of drug class to compounds that did (cinnamic and valproic acids) and did not significantly induce Ngb protein expression in HN33 cells.

Some of the compounds we studied (e.g. valproic acid) are histone deacetylase (HDAC) inhibitors, and HDAC inhibitors are transcriptional activators, some of which exhibit neuroprotective effects [19], including beneficial effects on outcome from experimental stroke [20]. Therefore, we next investigated whether more potent and specific HDAC inhibitors also stimulate Ngb expression. The compounds chosen were the pan-HDAC inhibitors trichostatin A and vorinostat (SAHA) and the HDAC2- and HDAC-3 selective inhibitor apicidin [21]. However, neither trichostatin A (0.5–30 $\mu\text{mol/l}$), SAHA (5–100 $\mu\text{mol/l}$), nor apicidin (1–30 $\mu\text{mol/l}$) increased Ngb levels detected by Western blot (not shown). Therefore, the Ngb-inducing effects of drugs like valproic acid are unlikely to be due to HDAC inhibition.

Considering that the anticonvulsant valproic acid was among the most effective inducers of Ngb that we observed, and since valproic acid [20, 22] and other anticonvulsants [23, 24] are reported to be neuroprotective in some models of stroke, we examined whether a broader range of anticonvulsants might have Ngb-inducing activity. Phenytoin (20 $\mu\text{g/ml}$), phenobarbital (40 $\mu\text{g/ml}$), carbamazepine (12 $\mu\text{g/ml}$) and gabapentin (10 $\mu\text{g/ml}$) were tested at concentrations typically found in the blood of patients treated for seizures. However, none increased Ngb levels on Western blots (not shown).

Discussion

Although several studies have shown a neuroprotective effect of Ngb against hypoxia, ischemia and other disorders, therapeutic application of this effect is problematic. Ngb is an intracellular protein that, except in zebrafish [10], is not known to cross cell membranes, making its direct administration unlikely to be effective. However, small molecules like DFO [4], hemin [11], and cinnamic and valproic acids (this report) appear capable of inducing Ngb protein expression in cultured neurons. Both DFO [13, 14, 25, 26] and valproic acid [20, 22] are protective in rodent models of cerebral ischemia, but whether induction of Ngb contributes to their protective actions is unclear. Cinnamic acid reduced glutamate toxicity in primary rat cortical neurons cultures [27], but did not protect GT1-7 (immortalized mouse hypothalamic) cells from oxygen and glucose deprivation [28] *in vitro*. Whether these or other drugs can induce Ngb expression *in vivo* at subtoxic doses, and whether the extent of Ngb induction achieved in this manner will be sufficient to afford protection are unclear, and will require further study.

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