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BIOCHEMISTRY, BIOPHYSICS, AND MOLECULAR BIOLOGY

Cognitive Enhancer Noopept Activates Transcription Factor HIF-1

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Abstract— The effect of noopept (*N*-phenylacetyl-*L*-prolyl-glycine ethyl ester) on the DNA-binding activity of HIF-1 in SH-SH5Y cells and the mechanisms of stabilization of this transcription factor were studied in vitro. Noopept was shown to increase both the basal DNA-binding activity of HIF-1 and the activity induced by various hypoxia mimetics. The mechanism of stabilization of the oxygen-sensitive HIF1α subunit by noopept involves the inhibition of HIF-1 prolyl hydroxylase, which is indirectly indicated by the data obtained using the ODD-Luc reporter, and the positive effect on the level of the HIF1α protein. It was revealed that the effect of noopept is accompanied by changes in gene expression, which belong to different metabolic pathways and are controlled by the transcription factor HIF-1.

Keywords: HIF-1, hypoxia, noopept, Pro-Gly dipeptides, HIF-1α stabilization, FIH

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INTRODUCTION

Hypoxia-inducible factor-1 (HIF-1) is a transcriptional activator that coordinates a conserved adaptive cell response to decreased oxygen by regulating the expression of genes involved in angiogenesis, proliferation, erythropoiesis, glucose metabolism, pH maintenance, apoptosis, and migration [1]. HIF-1 is a heterodimer consisting of two subunits— the oxygen-sensitive subunit HIF-1α and the constitutively expressed HIF-1β. Hypoxia promotes an increase in the level of HIF-1α, its dimerization with HIF-1β, mobilization of co-activators (p300/CBP), and the binding of this complex to the hypoxia-response element (HRE) sequence in the regulatory regions of target genes. Under normoxia, oxygen-dependent hydroxylation of proline residues (Pro402 and Pro564) of the HIF-1α subunit by prolyl hydroxylases (PHDs) is a prerequisite for binding by the von Hippel-Lindau (VHL) protein, a component of the E3 ubiquitin protein ligase complex. Ubiquitinylated HIF-1α becomes a target for degradation by 26S proteosomes. In addition, at normoxia, asparagine hydroxylase (FIH, HIF-1 inhibition factor) hydroxylates the asparagine residue (Asn803) of the C-terminal transactivation domain (C-TAD) of HIF-1α, which blocks its interaction with the p300/CBP transcription co-activator and decreases the transcriptional activity of HIF-1. Thus, under normoxia, PHD and FIH inactivate HIF-1α, suppressing the HIF-1-dependent expression of target genes. Under hypoxia, the activity of PHD and FIH decreases, leading to a decrease of HIF1α degradation and its stabilization and initiation of the transcription of dependent genes [1].

To date, the components of the HIF-1-dependent signaling pathway are considered as targets of pharmacotherapeutic action for the treatment of common diseases such as ischemic heart disease, atherosclerosis, and oncological and neurodegenerative diseases. It should be noted, however, that an increase in HIF1 activity in various pathological processes may have both positive and negative functional consequences. This fact is taken into account when developing pharmacological approaches to the correction of HIF-1 in a certain disease. In particular, inhibitors of the HIF-1-dependent signaling pathway are being intensively studied as anticancer agents and therapeutics for pulmonary hypertension and a number of nonspecific lung diseases, whereas the activation of the HIF-1-regulated pathway seems to be an effective neuroprotective strategy for the treatment and/or prevention of ischemic heart disease, anemia, ischemic strokes, and neurodegenerative diseases [2, 3].

Linear and cyclic proline-containing peptides are of interest as neuroprotective agents. Some of them are used in clinical practice (noopept [4]), and others (in particular, active noopept metabolite and endogenous regulator of anxiety and memory, cyclo-*L*-prolylglycine [5], a substituted Gly–Pro dipeptide) are cur-

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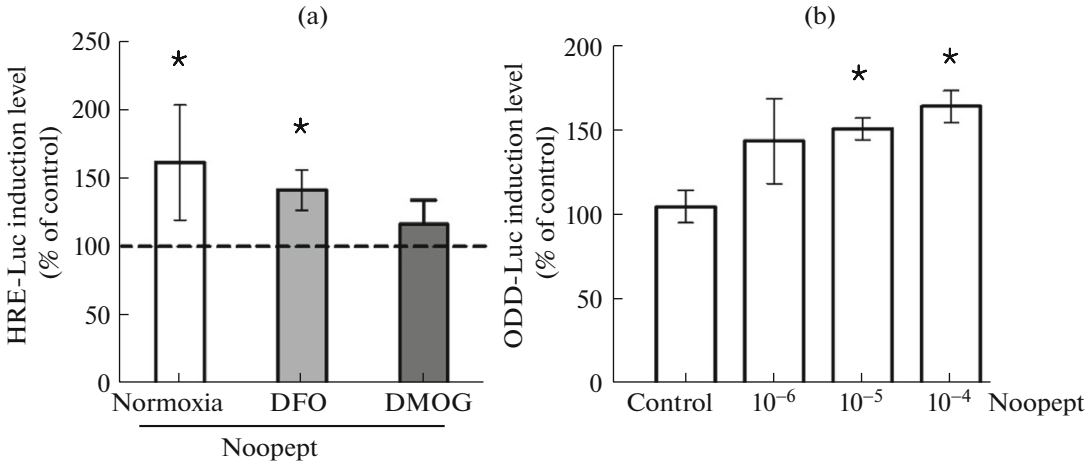

Fig. 1. Effect of noopept on the activity of HRE-Luc and HIF1-ODD-Luc reporter constructs in vitro. (a) SH-SY5Y cells (0.2×10^6 cells/mL) were transiently transfected with the HRE-Luc reporter construct using Lipofectamine 2000. Then, 6 h after transfection, noopept (10^{-4} M) was added, and the cells were incubated for 18 h, followed by DFO (10^{-4} M) or DMOG (10^{-3} M) co-incubation for 6 h. The luciferase activity was detected using Dual Luciferase Reporter Assay System. The values of the luminescent signal in the groups "normoxia," "DFO," and "DMOG" without noopept were taken as control. Data are presented as the mean value ± standard error of the mean. The experimental groups were compared using paired Student's *t* test for dependent samples ($N = 3$, $n = 9$; * $p < 0.05$ relative to the control). (b) SH-SY5Y cells were transiently transfected with the HIF1-ODD-Luc reporter construct. Then, 6 h after transfection, noopept was added (at concentrations of 10^{-6}–10^{-4} M), and the cells were incubated for 24 h. The values of the luminescent signal in the group without noopept were taken as a control. Data are presented as the mean value ± standard error of the mean. The experimental groups were compared using paired Student's *t* test for dependent samples ($N = 3$, $n = 9$; * $p < 0.05$ relative to the corresponding control).

rently being investigated. Recently, it was shown that noopept is able to increase both the basal and hypoxia mimetic-induced DNA-binding activity of HIF-1 [6]. Earlier, studies performed under supervision of R.U. Ostrovskaya on a hypobaric hypoxia model showed that preliminary administration of noopept (0.5 mg/kg) to mice increased the number of survived animals from 15 to 44% [7], which indicates that the drug has antihypoxic properties. In general, the available literature and our own experimental data suggest that noopept and other proline-containing compounds can increase the resistance to hypoxia due to their ability to affect the components of the HIF-1-dependent signaling pathway, thereby determining one of the mechanisms of neuroprotection. In this work, we studied the mechanisms of the HIF-1-positive effect of noopept.

Previously, the effect of noopept on the DNA-binding activity of HIF-1 was shown in HEK293 human embryonic kidney cells, with $CoCl_2$ being used as a pharmacological mimetic of hypoxia [6]. In this work, the study of the effect of noopept on the activity of the transcription factor HIF-1 was performed on SH-SY5Y neuron-like cells in the presence of pharmacological hypoxia mimetics of different mechanisms of action—deferoxamine (DFO) and dimethyloxalylglycine (DMOG). It is known that DFO is an iron chelator and inhibits prolyl hydroxylase, thereby stabilizing HIF-1α [8]. The analogue of 2-oxoglutarate DMOG inhibits the hydroxylation of HIF-1α due to competition with the endogenous cofactor of prolyl hydroxylase 2-oxoglutarate [9]. It is also known that DFO and DMOG inhibit the activity of FIH [8]. The luciferase construct for the analysis of HIF-1 activity contained four copies of the consensus sequence 5' ACGTG 3', which is the binding site for the HIF-1α (HRE-Luc construct; Panomics, United States) [10]. As follows from the data presented in Fig. 1a, noopept under basal conditions increased the HIF-1-dependent activity, on average, by 60%. Under mimicking of hypoxia by DFO, incubation of SH-SY5Y cells with noopept led to the induction of luciferase, on average, by 40% (in the case of using DMOG, by 16%). Thus, the results obtained confirm the ability of noopept to increase both the basal and DNA-binding activity of HIF-1 stimulated by hypoxia mimetics of different mechanisms of action.

To study the mechanisms of the HIF-1-positive effect of noopept, we used the HIF1-ODD-Luc reporter construct (Addgene, United States), which makes it possible to search for the prolyl hydroxylase (PHD2) inhibitors and HIF-1 stabilizers. This reporter is a fusion protein consisting of the luciferase protein and a C-terminal fragment of the oxygen-dependent degradation domain of HIF-1α (ODD HIF-1α; amino acid residues 530–653), which contains proline residues that are hydroxylated by prolyl hydroxylase. The principle of action of the test system is based on the induction of luminescence signal generation upon HIF1-ODD-Luc stabilization under the action of HIF prolyl hydroxylase inhibitors [11]. When assessing the effect of noopept on the activity of the

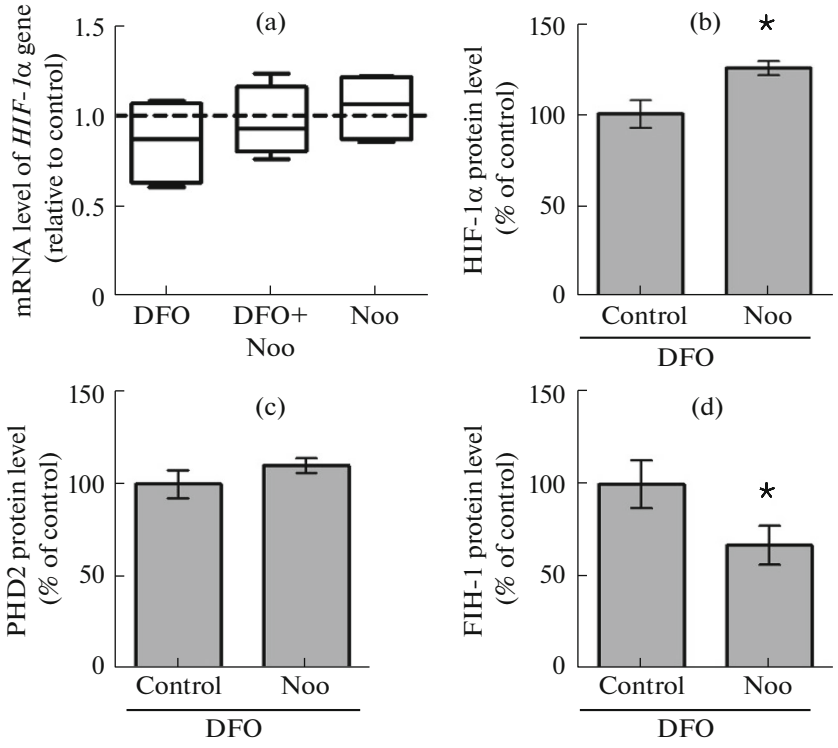

Fig. 2. (a) Effect of noopept on the mRNA level of the *HIF-1α* gene and the relative content of HIF-1α, PHD2, and FIH proteins. After 48 h of cultivation, SH-SY5Y cells were treated by DFO (10^{-4} M) and incubated for 6 h, after which the incubation medium was replaced with the medium containing noopept (10^{-4} M), and incubation was continued for 4 h. Then, mRNA was isolated. The mRNA level of the target gene was normalized to the mRNA level of the *HPRT* gene. Data are presented as a median with allowance of the 5th and 95th percentiles ($n = 3$, * $p < 0.05$ relative to the control, which was taken as unity). The experimental groups were compared using the Wilcoxon *t* test. (b–d) SH-SY5Y cells were treated by noopept (10^{-4} M), and the cells were incubated for 24 h. Then, 6 h before the end of the incubation with noopept, deferoxamine (10^{-4} M) was added to the cells, and co-incubation continued for 6 h, after which proteins were isolated. The target protein content values were normalized to the level of β-tubulin. Data are presented as the mean value ± standard error of the mean ($N = 3$, $n = 9$; *$p < 0.05$ relative to the control). The experimental groups was performed using paired Student's *t* test for dependent samples ($N = 3$, $n = 9$; * $p < 0.05$).

HIF1-ODD-Luc reporter, it was shown (Fig. 1b) that this compound increased the amplitude of the luminescent signal of the reporter in a concentration-dependent manner with the maximum effect (by 60%) at a concentration of 100 μM. The data obtained confirm the stabilization of HIF-1 by noopept and suggest the inhibitory activity of this compound on prolyl hydroxylase as a possible mechanism of action.

Next, we studied the effect of noopept on the mRNA level of the HIF-1α gene and protein and on the content of PHD2 (HIF-prolyl hydroxylase 2) and FIH (factor inhibiting HIF-1) proteins in an in vitro model of hypoxia induced by deferoxamine. It was found that the incubation with noopept in the presence of DFO did not change the level of mRNA of the *HIF-1A* gene as compared with the inducer effect (Fig. 2a). A slight, statistically insignificant increase in the content of *HIF-1A* mRNA was observed when cells were incubated with noopept. The effects of noopept on the content of HIF-1α, PHD2, and FIH proteins were analyzed by Western blotting. As follows from the data presented in Fig. 2b, under conditions of in vitro simulated hypoxia, noopept increased the level of the HIF-1α protein, on average, by 25%, whereas the effect of the drug on the PHD2 protein content was not detected (Fig. 2c). Under similar conditions, noopept decreased the relative content of the FIH protein, on average, by 33% (Fig. 2d).

To study the effect of noopept on the transcriptional activity of HIF-1, we analyzed the expression of individual genes (*VEGFA*, *PDK1*, and *BNIP3*), which are under the control of this transcription factor and are involved in ensuring cell adaptation under hypoxic conditions. Analysis of the data obtained showed that the pretreatment of cells with noopept (20 h) followed by incubation with DFO (4 h) led to an increase in the level of *VEGFA* and *PDK1* mRNA in comparison with the DFO group. In addition, a slight increase in the *BNIP3* mRNA level was observed; however, the differences were not statistically significant.

Thus, it was shown for the first time that the HIF-1–positive effect of the proline-containing dipeptide

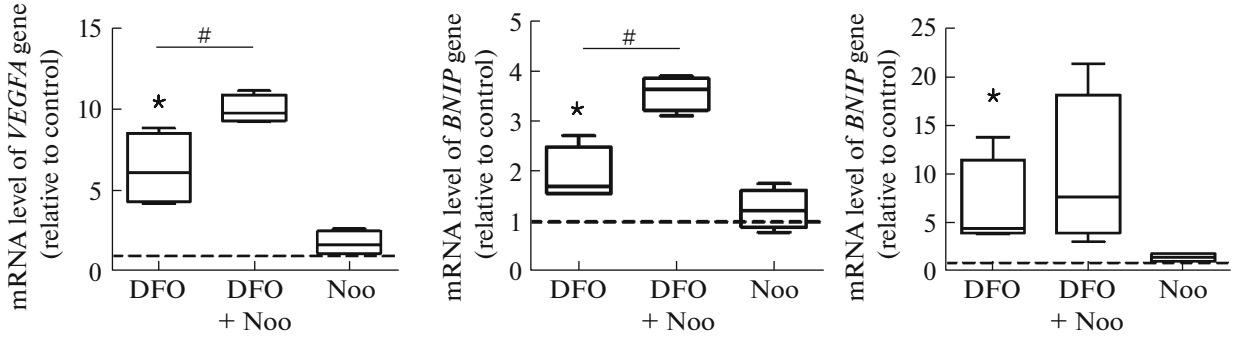

Fig. 3. Effect of noopept on the mRNA level of the target genes of the transcription factor HIF-1. SH-SY5Y cells were cultured in the presence of noopept (10^{-4} M) for 20 h, after which DFO (10^{-4} M) was added, and the suspension was incubated for 4 h. The mRNA level of the target genes was normalized to the mRNA level of the *HPRT* gene (real-time RT-PCR by the method of threshold cycle comparison [12] (REST Tool V. 2.0.7; Corbertt Research, United States). The data are presented as a median taking into account the 5th and 95th percentiles ($N = 2$, $n = 6$; $^{*}p < 0.05$ in relation to the control, which was taken as unity; $^{\#}p < 0.05$). The experimental groups were compared using the Wilcoxon *t* test.

noopept is realized due to the stabilization of the oxygen-regulated subunit of HIF-1α and an increase in the transcriptional activity of this transcription factor. The ability of noopept to stabilize HIF-1α is indicated by the increase in the content of the corresponding protein and the induction of the HIF1-ODD-Luc reporter. It is known that the stabilization of HIF-1α is insufficient for the transcriptional activation of the genes regulated by HIF-1. Asparagine hydroxylase FIH hydroxylates the Asn803 residue of HIF-1α, which blocks the binding of p300/CBP transcription coactivators to HIF-1α and prevents the formation of a functional transcriptional complex [13]. The data obtained indicate that noopept probably promotes the transactivation of HIF-1, because an increase in the mRNA level of the genes controlled by this transcription factor was established. This is indirectly indicated by the decrease in the level of the FIH protein, the inhibition of which is associated with the suppression of the transcriptional activity mediated by HIF-1 [14].

Stabilization of HIF-1α is a pathogenetically substantiated cytoprotection strategy. Today, there are published data about new PHD2 inhibitors and HIF-1 inducers with neuroprotective properties; however, most of them are not yet used as drugs [15]. Many of these compounds were shown to inhibit PHD2 and FIH, which results in the stabilization of HIF-1α and the triggering of HIF-1-dependent compensatory processes involved in neuroprotection mechanisms. The drug noopept, along with the nootropic properties, exhibits pronounced neuroprotective activity (which was established in different models of damage in vivo and in vitro and which is due to the combination of antioxidant and antiapoptotic properties), an inhibitory effect on voltage-gated calcium channels and glutamate release, as well as the ability to enhance the expression of neurotrophins [7]. The presence of the ability to stabilize HIF1-α in the mechanisms of the pharmacological action of noopept makes it possible to partially substantiate its nootropic, neuroprotective, and antidiabetic properties.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare no conflicts of interest. This article does not contain a description of research performed by the authors with the participation of humans or the use of animals as objects.

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