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Neuronal pentraxin 1 expression is regulated by hypoxia inducible factor-1α

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ABSTRACT

Neuronal pentraxins (NPs) are belong to sub family of long pentraxin proteins consist of neuronal pentraxin 1 (NP1), neuronal pentraxin 2 (NP2), and neuronal pentraxin receptor (NPR). Enhanced expression of NP1 in hypoxic conditions has shown to induce cell death in neuronal cells, however, the underlying mechanism of NP1 regulation by hypoxia remains elusive. To demonstrate that, we have cloned human NP1 gene promoter upstream of the luciferase gene and the activity of NP1 promoter was studied using HEK cell lines. Within the promoter region of the human NP1 gene, we identified six putative hypoxia inducible factor (HIF) responsive elements. By luciferase reporter assays we determined that the hypoxia inducible factor responsive element is located between −332 to −215 positions relative to the translation start site are essential for transcriptional activation of NP1 under hypoxic conditions. To further confirm the activity is solely due to hypoxia, we transiently transfected green fluorescent protein (EGFP) under transcriptional control of five copies of a hypoxia response element (HRE). The intensity of GFP was recorded at normal and hypoxic conditions. Taken together, our results demonstrate that NP1 gene is a target of as a hypoxia-inducible factor and it regulate NP1 expression by binding to hypoxia responsive elements (HREs) in its promoter region.

1. Introduction

Pentraxins (PTXs) are a super-family of multifunctional proteins that are characterized by the presence of a ~200 amino acid (aa)-long conserved domain in their carboxyl-terminal, called pentraxin domain and a variable N-terminal domain. All the members in this family share eight aa-long conserved sequence (HxCxS/TWxS, where x is any amino acid) in the pentraxin domain, called the pentraxin signature [1–3]. Based on the primary structure they have been divided into short C-reactive protein (CRP) and serum amyloid P component (SAP) and long (PTX3 and neuronal pentraxins) pentraxins [4]. Neuronal pentraxin (NP) family consists of neuronal pentraxin 1 (NPTX1 or NP1), neuronal pentraxin 2 (NPTX2 also called NP2, or NARP 3) and neuronal pentraxin receptor (NPTXR). Among all, NPTXR have been speculated that it could have been originated early at the divergence of vertebrates. Moreover, NPR is enriched at excitatory synapses where it associates with AMPA-type glutamate receptors (AMPAR) and enhances synaptogenesis [5]. Among all NPs, NP1 has shown to be exclusively expressed in central nervous system (CNS), whereas NP2 and NPR have shown to be expressed in divergent tissues (pancreas, liver, testis, heart and skeletal muscle) including brain. The NP family members are 50% identical each other and all have shown to participate in synaptic remodeling and neuronal plasticity [6–13]. The accumulated evidence suggests that the ischemia cascade leads to primary neuronal cell death via glutamate excitotoxicity [14,15]. Apart from neuro-developmental disorders, NP1 and NP2 were also shown to be associated with Type 2 diabetes and tumorigenesis of pancreatic cancer respectively [16,17].

In this study, we investigated whether expression of NP1 gene expression is up-regulated by HIF-1α in response to hypoxia in human embryonic kidney (HEK) cell lines. Our data provide an evidence that hypoxic induction of the NP1 gene is mediated by transactivation of NP1 promoter by HIF-1α through a consensus HRE binding site.

2. Materials and methods

2.1. Cell lines and culture conditions

Human embryonic kidney cell-line, HEK, were maintained in Growth Medium composed of DMEM (Mediatech, Inc., Herndon,

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VA) supplemented with 10% fetal bovine serum. Cells were grown on standard tissue-culture plastic ware in a 5% CO_2-humidified incubator at 37 °C. For hypoxia cells were treated with 200 mM cobalt chloride ($CoCl_2$, Sigma, St. Louis, Missouri) for 12 and 24 h. Control HEK cells were not exposed to cobalt chloride.

2.2. Cloning mouse NP1 promoter

The NP1 promoter region was PCR-amplified using 0.25 μM each of forward primer 5′-TGAGACCGGGATCGGGCTGG-3′ and reverse primer 5′ GGCCAGGAACTGCTTCGCGT-3′. PCR mixture contained 3 μM $MgCl_2$, 0.2 mM dNTPs, 2 μl RedTaq (Sigma), and 100 ng of genomic DNA. PCR was performed at 95 °C for 4 min, followed by 30 cycles at 95 °C for 30 s, 59.9 °C for 30 s, and 72 °C for 3 min using machine (BioRad). Amplified PCR product was purified and cloned into the TA cloning vector (Invitrogen, Carlsbad, CA). To construct the pGL4-NP1 plasmid for luciferase assays, TA-NP1 plasmid was digested with enzymes *Kpn*I and *Sac*I, and ligated into the pGL4.1 (luc2) vector (Promega). The deletion constructs (N1–N4) were created by digesting pGL4-Nptx1 with internal restriction enzymes followed by self-ligation.

2.3. Transfections and luciferase assays

Cells were plated 24 h before transfection at 1×10^5 cells per well in a twenty-four-well cell culture plate. In all, 1000 ng of various DNA constructs and 5 ng of Renilla construct (Promega) were mixed with 2 μl of Lipofectamin 2000 (Invitrogen) transfection reagent. The mixture was then incubated at room temperature for 20 min. After replacing fresh culture media the DNA-Lipofectamin 2000 mixture was transferred onto the cells and incubated for 8 h at 37 °C in a standard cell culture incubator. For oxygen deprivation the media were replaced with cobalt chloride containing media and incubated for 12 and 24 h. At the indicated times, the cells were washed twice with phosphate buffered saline and lysed with reporter lysis buffer (Promega). The enzymatic activity was measured for firefly and Renilla luciferase using Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Briefly, 50 μl of luciferase substrates were added to 40 μl of lysate and luciferase activity was measured using a Luminometer (Berthold Detection System, Oak Ridge, TN). Results were expressed as relative light units (RLUs) and that have been normalized with luciferase activity by Renilla activity. All experiments were performed in triplicate.

3. Results and discussion

3.1. Hypoxia regulates the expression of neuronal pentraxin 1 (NP1)

Hypoxia inducible factors, HIF-1α and 2α have shown to participate in beneficial and detrimental effects to neuronal cells by modulating the expression pattern of various genes in the ischemic environment [18,19]. Ischemic environment in the brain has shown to up regulate the expression of neuronal pentraxin 1 (NP1) and also has shown to induce neuronal cell death [20]. Therefore, it is essential to identify the role of hypoxia inducible factors in regulation of NP1 gene expression. To identify the correlation between hypoxia and NP1 regulation, the genomic sequence upstream of the mouse NP1 gene was retrieved from the NCBI database (Ac. No.: NT_165773.2) and cloned into luciferase reporter cassette (pGL4-NP1). To study the mechanism of NP1 gene expression, NP1 promoter is transiently transfected in human embryonic kidney (HEK) cell line. Following 12 h of the transfection, media was replaced with fresh media and media containing 200 mM cobalt chloride for an additional 12 and 24 h. As shown in Fig. 1, the NP1 promoter showed certain basal transcriptional activity under normal conditions; however, it showed threefold induction of Luc activity in hypoxia at 12 h as compared to pGL4 control vector. The reporter activity is slightly declined at 24 h as compared to 12 h and is higher than the basal transcriptional activity. Similar observation was detected in human DDX3 expression in breast cancer cell lines [21]. As a reference we have used VEGF promoter, a well known hypoxia regulatory gene. In order to determine if NP1 directly regulated HIF-1α at the transcriptional level, we carried out promoter analysis in HEK cells. We first determined the degree of induction of the full-length NP1 promoter by using increasing amounts of HIF-1α plasmid and found a dose-dependent effect (Fig. 1B).

3.2. Neuronal pentraxin 1 (NP1) is regulated by hypoxia

To test, whether the basal reporter activity is due to constitutive expression or stabilization of HIF-1α we co-transfected full length pGL4-NP1 promoter and green fluorescent protein (EGFP) under transcriptional control of five copies of a hypoxia response element (HRE) (Fig. 2A). Following 12 h of transfection, cells were exposed to hypoxic conditions and compared the intensity of green fluorescence using a 20× objective attached to a Zeiss fluorescence microscope, equipped with a filter set for 450–490 nm excitation and 500–550 nm emissions. EGFP signal intensity is detected in both normal and hypoxic conditions; however, more number of cells

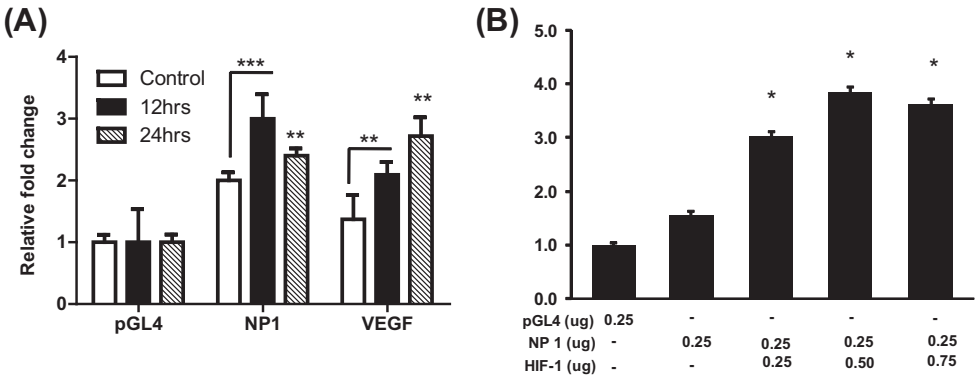

Fig. 1. Characterization of mouse NP1 promoter. (A) Luciferase (Luc)-based neuronal pentraxin 1 reporter activity in HEK cells. Relative luciferase activities are shown as bar graph indicates the fold of Luc activity. Error bars (standard deviation) from triplicate samples are shown. (B) Dose dependent activation of NP1 promoter activity in the presence of increasing amounts of HIF-1α in transient co-transfection assays.

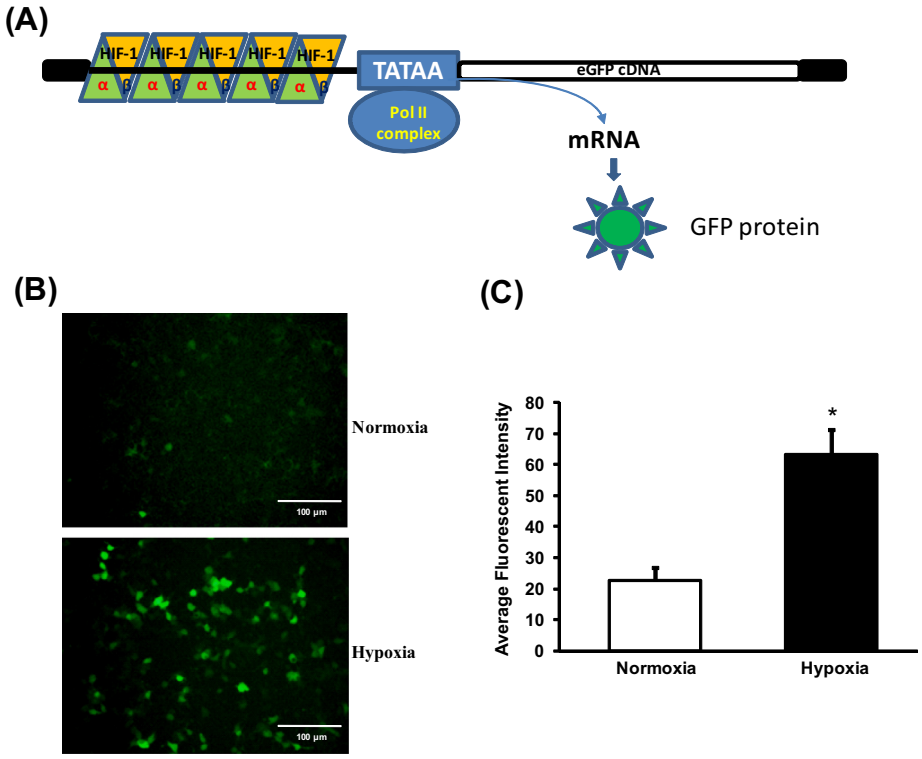

Fig. 2. A schematic diagram depicting the 5xHRE-EGFP construct. (A) Under hypoxic conditions, HIF-1α binds HREs in the 5xHRE-EGFP construct resulting in EGFP expression. (B) 5xHRE-EGFP is transiently transfected into HEK cells and the intensity of GFP expression were captured in normoxia (top) and hypoxia (bottom) conditions. (C) Bar graph showing quantification of the fluorescence intensity per cell.

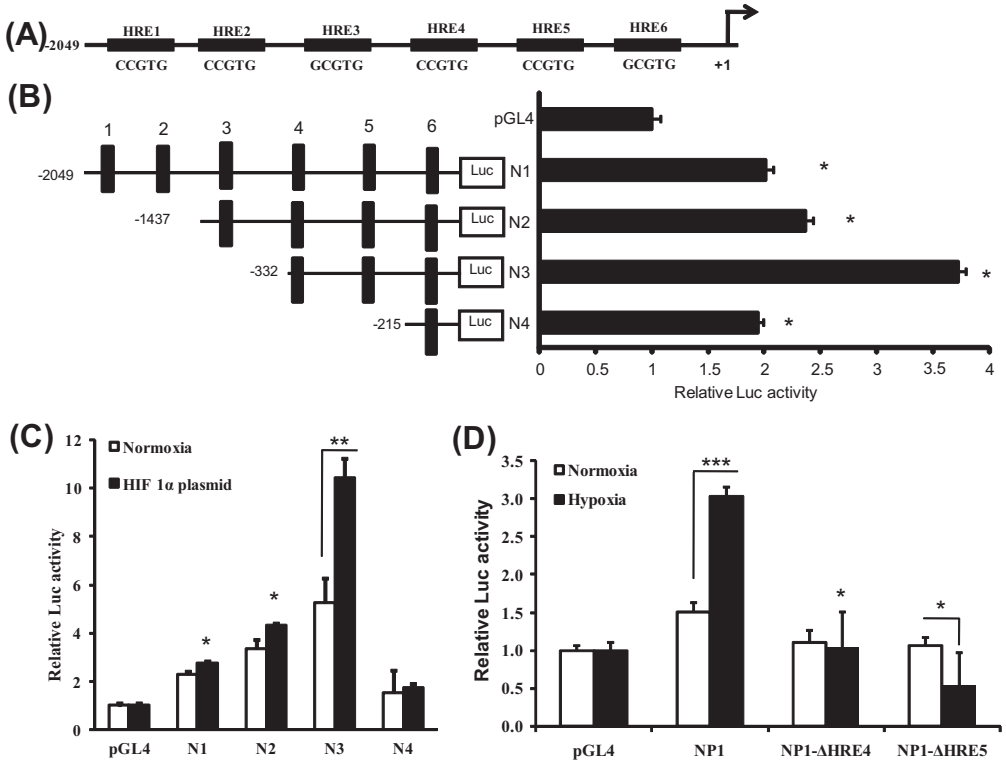

Fig. 3. Identification of functional HRE in the NP1 promoter (A) Schematic representation of putative core HRE sequences in the promoter region of the NP1 gene. (B) Linear representations of the NP1 promoter–reporter constructs and the relative firefly luciferase activity in HEK cells. (C) Effects of over-expression of HIF-1α on NP1 promoter in HEK cells under normoxia. (D) Histograms show relative firefly luciferase activity of mutated promoter–reporter constructs under hypoxia. Mean values from three independent transfections are shown. Error bars represent ±SD.

expressed EGFP in hypoxic conditions (Fig. 2B). From this we speculated that, the basal transcriptional activity of NP1 promoter in normal cell culture conditions (normoxia) may be due to HIF-1α stabilizing factors in HEK cells. CpG islands in the promoter have shown to regulate the expression of oxygen-regulated erythropoietin gene by stabilizing HIF-1α [22,23]. To identify the stabilizing factors we screened for CpG islands using the CpG island searcher software (http://www.ebi.ac.uk/Tools/emboss/cpgplot/). Three strong CpG islands at 47–442, 500–708 and 1627–2046 base pairs identified with 50% GC content. The observed CpG/expected CpG ratio in this region was greater than 0.60. This could play an important role in the regulation of NP1 under hypoxic environment.

3.3. Characterization of mouse NP1 promoter in HEK cell lines

To identify hypoxic responsive elements involved in the regulation of NP1 gene expression, we analyzed transcription factor analysis using MatInspector (Genomatix software) (http://www.genomatix.com). Genomatix suite predicted the presence of at least six hypoxia responsive elements (HREs; C/GCGTG) at −98, −327, −1035, −1457 and −1573 (Fig. 3A) and also several other transcription regulatory elements activator protein 2, E2F-myc activator/cell cycle regulator, and Nuclear factor kappa B/c-rel from translation start site. Next, we have generated different deletion luciferase constructs lacking HRE relative to the ATG translation start codon to identify the functional HRE in NP1 promoter. Fig. 3B, left panel, depicts a schematic representation of the different promoter–reporter deletion constructs (N1–N4) used for transfection experiments. Our results showed indicate that there may be a repressor(s) within the −332 to −1437 bp range (compare N3 expression to that of N2; Fig. 3B, Right panel). In addition, our N3 construct indicates that an enhancer(s) apparently is present within the −215 to −332 (compare N4 expression to that of N3; Fig. 3B, Right panel). Thus, under conditions used in HEK cells, the −332 bp promoter region was the most active of the reporter construct. To test whether exogenous HIF-1α over expression can cause a stimulatory effect on NP1 promoter activity in normoxic conditions, we co-transfected the 500 ng of NP1 promoter–reporter vector constructs with a vector that provides for constitutive expression of HIF-1α (500 ng). As shown in Fig. 3C, under these conditions the pattern of reporter activities mirrored that seen in Fig. 3B but the activities were increased by an order of magnitude. This data provides further direct evidence that HIF-1α is involved with the regulation of NP1 expression. To identify the importance of HRE4/HRE5, sequences were mutated and the mutant constructs were transfected into HEK cells. As shown in Fig. 3D, reporter activity levels decreased or lower than that of N1 promoter activity.

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