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Activation of Mammalian Tolloid-Like 1 Expression by Hypoxia in Human Neuroblastoma SH-SY5Y Cells

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Abstract

Mammalian Tolloid-like 1 (mTll-1) is an astacin metalloprotease that is a member of the Tolloid family of proteins. mTll-1 cleaves chordin, an inhibitor of bone morphogenetic proteins (BMPs) and potentiates activity of the BMPs. Prenatal stress and glucocorticoids decrease mTll-1 expression whereas voluntary exercise increase mTll-1 gene expression in the mouse hippocampus. Here, we studied the underlying molecular mechanisms by which hypoxia regulates human mTll-1 gene expression. When cells were subjected to hypoxia, the expression of endogenous mTll-1 was upregulated in SH-SY5Y human neuroblastoma cells. Dual-luciferase assay and site directed mutagenesis showed the presence of hypoxia responsive elements (HREs) at position 625 that was essential for activation of mTll-1 expression under hypoxic conditions. The binding of hypoxia inducible factor (HIF-1) protein to the HREs was confirmed by gel shift assay. These results indicate that the HRE motif is directly involved in the activation of the mTll-1 transcription under hypoxic conditions.

Keywords

Mammalian Tolloid-like 1 (mTll-1); Hypoxia; Hypoxia inducible factor (HIF-1); Hypoxia responsive element (HRE)

Introduction

It is well known that the susceptibility of the brain to hypoxia is greater than that of other organs, as it has a high oxygen demand and little energy is produced anaerobically [1]. Among the various organs of the human body, the brain consumes 20% of the oxygen intake [2]. Many of the adaptations to hypoxia are mediated by the activation of specific genes through the action of a hypoxia-inducible factor (HIF). The first HIF described (HIF-1) is a heterodimer made up of HIF-1 α and HIF-1 β subunits [3,4]. Under normoxic conditions, HIF-1 is hydroxylated by oxygen-dependent propyl hydroxylases and targeted for proteolytic destruction. In contrast, hypoxia leads to the accumulation of HIF-1 α and dimerization with HIF-1 β . Thus HIF-1 is stabilized, translocates into the nucleus and binds to specific nucleotide sequences called hypoxia-response elements (HREs) of target genes [5]. HIF-1 is one of the most important

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hypoxia-driven factors, playing a major role in coordinating of many adaptive responses to hypoxia in mammals [6]. More than two dozen HIF-1 target genes are known. One group of HIF-1 target genes involved in adaptive responses to oxygen deprivation facilitates oxygen delivery to deprived tissues. This group includes genes coding for erythropoietin (which stimulates production of erythrocytes), heme-oxygenase 1 (that mediates oxygen binding to heme), vascular endothelial growth factor (VEGF; triggers new vasculature formation), and inducible nitric oxide synthase which participates in local blood vessel dilation [7–9]. Another group of HIF-1-dependent genes act to compensate for the inhibition of oxidative phosphorylation that occurs when oxygen is lacking. This group includes genes coding for glycolytic enzymes (e.g., lactate dehydrogenase A, phosphofructokinase, L-phosphoglyceromutase) and for glucose transporters such as Glut1 [10,11].

Tolloid family proteins are pleiotropic, astacin-like metalloproteases [12]. There are four known mammalian Tolloid proteins: bone morphogenetic protein 1 (BMP-1), mammalian Tolloid (mTld), mammalian Tolloid-like 1 (mTll-1) and mammalian Tolloid-like 2 (mTll-2). BMP-1 and mTld are alternatively spliced variants from the same Tld gene. mTll-1 and mTll-2 are encoded by *Tll-1* and *Tll-2* genes, respectively. The family of Tolloid-like proteins plays important roles in various developmental events. It has been shown that mTll-1, for example, is expressed at high levels not only during development but also in the granular layer of the adult mouse cerebellum [13]. mTll-1 processes a variety of substrates including extracellular matrix proteins such as procollagen [14], laminin-5 [15], and proteoglycans such as perlecan [16]. Moreover, it has been shown that mTll-1 is negatively regulated by glucocorticoids [17] and activated by Nkx-2.5 homeobox proteins [18]. Two members of the Tolloid family, BMP-1 and mTll-1, cleave chordin *in vitro* and are thereby thought to potentiate the BMP signaling *in vivo* [14]. We have previously shown that mTll-1 is expressed in the hippocampus of both juvenile and adult mice [17]. Since the level of mTll-1 expression was increased in adult mice that participated in voluntary exercise, we hypothesized that intermittent hypoxia during exercise resulted in increased Tll-1 gene expression in neuronal cells. These studies suggested that mTll-1 gene transcription might be mediated by hypoxia. Here, we show that the promoter for mTll-1 contains a HIF-1 binding site in SH-SY5Y human neuroblastoma cells. We have performed functional studies to characterize the hypoxia-response element in the mTll-1 promoter. We also report that the hypoxia-response element motif at position 625 is essential for upregulation of mTll-1 expression under hypoxic conditions.

Methods

mTll-1 promoter cloning

To test the hypothesis that mTll-1 protein is a HIF-1-target gene and possibly involved in the adaptive response to hypoxia, the mouse Tll-1 promoter sequence was cloned by promoter walking using a mouse SspI genomic library (Clontech). The primers used for the promoter walking include two adapter primers, AP1 (5'-GTAATACGACTCACTATAGGGC-3') and AP2 (5'-CTATAGGGCACGCGTGGT-3') and two Tll-1 gene-specific primers Tll-1-GSP1 (5'-GTCTGCACATCAGCACATCTGAACT-3') and Tll-1-GSP2 (5'-ATTCTACGCCGCGCCAGACCTTAAAA-3'). The fragment was sequenced and the sequence was analyzed for potential transcription factor binding sites by MatInspector [19].

Cell culture and chronic hypoxia

The SH-SY5Y cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/l glucose and L-glutamine (Cambrex) containing 10% Fetal Bovine Serum (Invitrogen), 50 Units/ml penicillin and 50 µg/ml streptomycin. Hypoxia treatments were performed by transferring the cells into a humidified hypoxic chamber (Billups Rothenberg Inc.) containing 2.5% O₂, 5% CO₂ and 92.5% N₂ [20]. Corresponding control cells (normoxia condition) were

maintained in a 95% air/5% CO₂ incubator for the same period. To detect the presence of HIF-1 α protein, as marker of hypoxia, Western blotting was performed under hypoxia and normoxia in SH-SY5Y neuroblastoma cells (see supplementary material).

Luciferase reporter gene assay

For the luciferase reporter gene assay the TII-1/pGL-3 reporter construct carrying the putative mTII-1 promoter fragment upstream of the modified firefly luciferase reporter gene in a pGL-3 basic vector (Promega) was used [17]. The second construct had a mutated HIF-1 binding site. Mutation of the HIF-1 binding site was introduced by PCR based site-directed mutagenesis using the QuikChange Site directed mutagenesis kit (Stratagene) with the following primers: forward 5'-CAACTTCCCTTCCTGCGCCCGTACAAGCCCTAGCTCCAGCCC-3'; reverse 5'-GGGCTGGAGCTAGGGCTTGTACGGGCGCAGGAAGGGAAGTTG-3'. The native HIF-1 sequence ACGTG was changed to GTACA.

SH-SY5Y cells were seeded at a density of 3.5×10^4 cells per well in 96-well plates. After one day of incubation, the cells were transfected with 0.5 μ g/well of either an empty pGL3 promoter vector or vectors containing the wild-type of mTII-1 promoter or the mutant HIF-1-binding sequence and co-transfected with 0.5 μ g/well pRL-SV40 (Promega) encoding the Renilla luciferase gene. Transfection was performed using Lipofectin transfection reagent (Invitrogen). Serum-free medium used during transfection was replaced with serum-containing medium 6 h after the beginning of transfection. The cells were placed in the hypoxia chamber and exposed to hypoxic conditions for 24 h at 37°C. At the end of experimental treatments, luminescence assays were performed using the Dual-Glo Luciferase Assay System (Promega). Luminescence was measured with a Luminescence counter (Packard Bioscience). Promoter activities were determined as a ratio of firefly luciferase to Renilla luciferase luminescence in each well.

Nuclear extract preparation

SH-SY5Y cells were incubated for 24 hours under normoxia or hypoxia. To harvest, cells were washed twice with cold Dulbecco's phosphate-buffered saline (PBS), scraped into 5 ml of PBS, pelleted by centrifugation and resuspended in three packed-cell volumes of buffer A (10 mM Tris-HCl [pH 7.8], 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.4 mM PMSF, 2 μ g of leupeptin per ml, 2 μ g of aprotinin per ml, and 1 mM sodium vanadate) and incubated on ice for 15 min. Cells were lysed by gently vortexing with 0.1% NP-40 and the nuclei were pelleted by centrifugation at 3,000 rpm for 5 min, resuspended in two packed-cell volumes of buffer C (0.42 M KCl, 20 mM Tris-HCl [pH 7.8], 1.5 mM MgCl₂, 20% glycerol, 0.5 mM DTT, 0.4 mM PMSF, 2 μ g of leupeptin per ml, 2 μ g of pepstatin per ml, 2 μ g of aprotinin per ml, and 1 mM sodium vanadate), and mixed on a rotator at 4°C for 30 min. Nuclear debris was pelleted by centrifugation for 30 min at 13,200 rpm and nuclear extract aliquots were frozen in liquid N₂ and stored at -80°C.

RNA isolation and real-time RT-PCR

Total RNA was isolated from SH-SY5Y cells using TRI Reagent (Molecular Research Center, Inc.) 6, 12, 24 and 48 hours after hypoxia. Real-time RT-PCR was performed using 50 ng total RNA per reaction. RNA was combined with primer/probe sets and TaqMan Gold RT-PCR Master Mix (Applied Biosystems, Inc.). Gene-specific primers and probes were created for human TII-1 and glyceraldehyde phosphate dehydrogenase (GAPDH) using the Primer Express Software (Applied Biosystems, Inc.) and these are shown in Table 1. Real-time assays were run on an ABI PRISM 7000 Sequence Detector System (Applied Biosystems). The real-time PCR profile consisted of one cycle at 48°C for 30 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. All reactions were repeated twice. Samples were confirmed to be free of DNA contamination by performing reactions without reverse

transcriptase. The results of real-time PCR were normalized to GAPDH and analyzed by the $\Delta\Delta C_t$ method [21].

Electrophoretic mobility shift assays (EMSA).

To confirm specific binding of HIF-1 protein with the HIF-1 response element of the Tll-1 promoter EMSA was performed. Oligonucleotides corresponding to the HIF-1 response element in the Tll-1 promoter were designed and synthesized (Integrated DNA Technologies, Coralville, IA). The oligonucleotide sequences were: Tll-1 HRE forward 5'-TTCCCTTCCTGCGCCACGTGAGCCCTAGC-3'; Tll-1 HRE reverse 5'-GCTAGGGCTCACGTGGGCGCAGGAAGGGA-3'; HRE mutant forward 5'-TCCCTTCCTGCGCCCGTACAAGCCCTAGCTCC-3'; HRE mutant reverse 5'-GGAGCTAGGGCTTGACGGGCGCAGGAAGGGA-3'; nonsense forward 5'-GTCGGCACCGTCCCCTCGTCACGCTCTACT-3'; nonsense reverse 5'-AGTAGAGCGTGACGAGGGGACGGTGCCGAC-3'. Oligonucleotide pairs were hybridized to generate double-stranded DNA, end-labeled with $\gamma^{32}P$ ATP (MP Biologicals) and T4 polynucleotide kinase (NEB), and purified by polyacrylamide gel. Then the labeled probes were eluted from the gel with elution buffer (0.5M Ammonium acetate, 10mM magnesium acetate, 1mM EDTA [pH 8.0], 0.1% SDS). Labeled double-stranded oligonucleotides were incubated with 5 μ g of nuclear extract from SH-SY5Y cells grown under hypoxic and normoxic conditions in the presence of 200 ng poly dIdC. Additionally, competitor DNAs were preincubated with nuclear extract for 5 min prior to addition of labelled probe. For the supershift assay, 2 μ g rabbit anti-HIF-1 α antibody (Santa Cruz Biotechnology) was added to the reaction. The reactions were incubated on ice for 30 min followed by electrophoresis on a 6% nondenaturing polyacrylamide gel. Electrophoresis was performed at 200 V in 0.5x TBE (45 mM Tris-HCl, 45 mM boric acid, and 2.5 mM EDTA) at 4°C. Following electrophoresis, the gel was visualized on a Typhoon Phosphorimager (Amersham Biosciences).

Statistical analysis

Each assay had at least two replicates and each experiment or assay was performed at least three times and representative examples are shown. Data are reported as means $\pm$ SD, analyzed by a one-way ANOVA and $P < 0.05$ is considered significant.

Results

mTll-1 promoter contains HIF-1 binding site

Analysis of the mTll-1 promoter sequence reveals the presence of several potential sites for transcriptional regulation. Previously, we identified glucocorticoid response elements (GREs) which negatively regulate mTll-1 expression [17] and the presence of three Nkx-2.5 binding sites which activate mTll-1 expression [18]. Moreover, we found an ACGTG motif located 625 nucleotides upstream of the transcription start site. The HIF-1 binding site consensus sequence was described as either 5'-(G/C/T)ACGTGC(G/C)-3' [7] or 5'-RCGTG-3' [22]. Thus we also identified the presence of a potential hypoxia inducible factor 1 (HIF-1) binding site in the mTll-1 promoter (Fig. 1).

Hypoxia increases mTll-1 promoter activity in SH SY5Y cells

To investigate whether mTll-1 gene expression is either positively or negatively regulated by hypoxia, the luciferase reporter gene assay was performed. SH-SY5Y cells were transfected with pGL3/mTll-1 or pGL3/mTll-1 with a mutated HIF-1 binding site, or empty pGL3-basic vector using Lipofectamine LTX reagent. The transfected cells were placed under normoxic or hypoxic conditions for 24 hours and the luciferase assay was performed. Cells transfected with pGL3/mTll-1 and treated with hypoxia showed significantly higher luciferase activity

than cells transfected with pGL3/mTll1 under normoxia (Fig. 2; $P < 0.001$). There was no significant difference in luciferase activity under hypoxia in cells transfected with pGL3/mTll-1 with a mutated HIF-1 binding site compared to normoxic control (Fig. 2). These results indicate that hypoxia significantly increased the activity of the mTll-1 promoter in a neuronal cell line.

Expression of mTll-1 is increased in SH-SY5Y cells under hypoxia

Quantitative real-time RT-PCR was used to analyze the effect of hypoxia on mTll-1 mRNA expression in SH-SY5Y cells at different time points under hypoxic conditions. During hypoxia, the expression of mTll-1 was not increased significantly above controls during the first 6 h (Fig. 3). However, mTll-1 expression was significantly increased to 1.85-fold at 12 h under hypoxic conditions (Fig. 3; $P < 0.0001$) and remained upregulated to a maximum of 2.22-fold throughout the 48 h time period that was examined compared with controls ($P < 0.0001$).

HIF-1 directly binds to the mTll-1 promoter

The experiments above showed that hypoxia activates the mTll-1 promoter and that this activation appears to be through a direct interaction of HIF-1 protein with the mTll-1 promoter. To demonstrate direct binding of HIF-1 protein to the HIF-1 binding site within the mTll-1 promoter, we carried out an electrophoretic mobility shift assay. We found that nuclear proteins from SH-SY5Y subjected to hypoxia were able to bind to the labeled oligonucleotides containing the wild type HIF-1 binding motif of the mTll-1 promoter to form DNA-protein complexes (Fig. 4A). Competition assays showed that a molar excess of unlabeled wild type oligonucleotides (self competition) prevented the formation of DNA-protein complexes. No binding was observed under the normoxic condition (Fig. 4A). Mutant and nonsense oligonucleotides failed to form the DNA-protein complexes indicating that HIF-1 protein binds specifically to the HIF-1 binding site in the mTll-1 promoter (Fig. 4B). When antibody directed against HIF-1 α was added to the hypoxia-treated nuclear extract and the labeled HIF-1 oligonucleotides the DNA-protein complex was supershifted to a slower-migrating band (Fig. 4C). Competition assay and supershift assay further confirmed the specificity of the binding of HIF-1 protein to HRE motif.

Discussion

Upregulation of mTll-1 expression by hypoxia is mediated by HIF-1

Previous studies showed that at the protein level HIF-1 α is subjected to rapid degradation during normoxia by the pVHL-mediated ubiquitin-proteasome pathway [23]. Under hypoxic conditions, prolyl hydroxylation of HIF-1 α is blocked, which permits HIF-1 α protein to be stabilized so that HIF-1 α is free to bind with HIF-1 β to form the HIF-1 transcription complex. The HIF-1 heterodimer then binds to hypoxia responsive elements of the target gene. In this report we have identified a HIF-1-binding site in the promoter region of the mTll-1 gene (Fig. 1). The HIF-1 binding site is located 625 nucleotides upstream of the transcription start site. We have shown an increase in mTll-1 mRNA expression in response to hypoxia by real-time RT-PCR (Fig. 3). We have also demonstrated by reporter gene assay that the mTll-1 promoter is activated by hypoxia (Fig. 2) and individual mutations of the HIF-1 site in the mTll-1 promoter eliminated promoter activation by hypoxia. Furthermore, the HIF-1 protein complex activated by hypoxia binds to the HIF-1-binding DNA sequence as shown by EMSA (Fig. 4A–B) and confirmation that this transcription factor is HIF-1 was provided by the supershift assay (Fig. 4C). These results support our hypothesis that mTll-1 is a hypoxia-responsive gene and that HIF-1 protein is involved in transcriptional regulation of hypoxia-induced expression of mTll-1 in neuronal cells.

The physiological role of mTll-1 upregulation by hypoxia

It has been shown that mTll-1 cleaves chordin *in vitro* and potentiates TGF β -like growth factor signaling, such as the BMPs, *in vivo* [12,14]. Chordin binds TGF β -like ligands in the extracellular matrix and prevents them from interacting with their receptors. Cleavage of chordin by the tolloids releases TGF β -like ligands allowing them to function. The BMPs initiate signaling by interacting with serine/threonine kinase receptors that activate cytoplasmic Smad complexes which translocate to the nucleus and act as transcription factors [24]. Smads bind to DNA sequences directly, cooperate with other transcription factors, or bind and displace nuclear factors from their DNA binding sites to trigger genes involved in adaptive responses that facilitate oxygen delivery to oxygen-deprived tissues [25]. In addition, the BMPs stimulate angiogenesis through vascular endothelial growth factor (VEGF) [26]. Recent evidence indicates that VEGF may act directly on neurons to produce neurotrophic and neuroprotective effects under hypoxia. Sun and coauthors demonstrated that rats that underwent focal cerebral ischemia followed by intraventricular infusion of VEGF showed a reduction in the size of cerebral infarcts, cytopathological features of cell injury including cell shrinkage, DNA-strand breaks, and enhanced neurogenesis and cerebral angiogenesis, compared with rats that underwent focal cerebral ischemia followed by artificial cerebrospinal fluid infusion [27]. VEGF has also been implicated as a factor that promotes neurogenesis in the adult brain through the establishment of a vascular niche that favors the proliferation and differentiation of neuronal precursors [28]. Alternatively, VEGF may exercise a direct mitogenic effect on neuronal precursors [29], reduce hypoxic death of HN33 cells and cultured cerebral cortical neurons [30], and protect cultured hippocampal neurons from glutamate toxicity [31]. Together, these findings suggest that mTll-1, acting through activation of TGF β -like growth factors, is a hypoxia-sensitive gene that may play a role in the brain's response to ischemia and stroke.

Recent data suggest that mTll may act generally as a stress-response gene. Its exact response however, up or down regulation, appears to depend on the stimulus and the required adaptive response. For example, glucocorticoid response elements (GREs) were found in the promoter region of mTll-1 and glucocorticoid treatment resulted in decreased expression of mTll-1 in *in vitro* studies [17]. Moreover, in that study, mice subjected to prenatal restraint stress showed reduced mTll-1 mRNA expression. In another study, small birds (house sparrows) that overwinter in cold climates show significantly reduced levels of mTll-1 (but not mTll-2) mRNA expression, decreased levels of the muscle growth inhibitor myostatin, and greater muscle mass required for thermoregulation [32]. Myostatin is a TGF β family growth factor whose precursor is cleaved and activated by tolloid/BMP-1 metalloproteinases [33,34]. Therefore, a reduction in mTll-1 gene expression in birds induced by cold climates results in myostatin suppression and greater muscle mass needed for cold tolerance. The data shown in the present study are consistent with the regulation of mTll-1 by stressful stimuli, in this case hypoxia. However, here the response of mTll-1 to hypoxia results in increased mRNA expression through regulation by HIF-1. This response activates TGF β family growth factors that function in facilitating oxygen delivery, angiogenesis, and ultimately neuroprotection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Map of mammalian Tolloid-like 1 promoter. Potential transcriptional binding sites within the mouse promoter are shown. Abbreviations: Nkx-2.5, the heart homeobox transcription factor Nkx-2.5 binding site; GRE, glucocorticoid response elements; HIF-1, hypoxia inducible factor 1 binding site.

Figure 2.

Luciferase activity measured after transfection of SH-SY5Y cells with pGL3 containing the Tll-1 promoter (Tll-1), pGL3 containing the Tll-1 promoter with a mutated HIF-1 binding site (Mut Tll-1), or pGL3 basic vector as control. For all groups, cells were harvested after 24 hours hypoxic or normoxic treatment. Data are expressed as relative luciferase units (RLU) $\pm$ SEM. Asterisks indicate statistical significance using ANOVA. * $P < 0.001$; ** $P < 0.002$.

Figure 3. Real-time RT-PCR analysis of mTll-1 expression in SH SY5Y neuroblastoma cells under normoxia and 6, 12, 24 and 48 hours in hypoxic conditions. Asterisks indicate statistical significance by ANOVA. * $P < 0.0001$.

Figure 4.

EMSA analysis of the HIF-1 binding site of the mTll-1 promoter. (A) Lane 1, free sense probe without nuclear extract; lane 2, sense probe with nuclear extract from SH-SY5Y cells incubated under normoxia; lane 3, sense probe incubated with nuclear extract from SH-SY5Y cells exposed to hypoxia; lane 4, same as lane 3 using a 1-fold cold probe for “self” competition; lane 5, 10-fold cold “self” competition; lane 6, 100-fold cold “self” competition. (B) Lane 1, free sense probe without nuclear extract; lane 2, sense probe with nuclear extract from SH-SY5Y cells incubated under hypoxia; lane 3, nuclear extract from SH SY5Y cells exposed to hypoxia and incubated with a mutated HIF-1 probe; lane 4, nuclear extract from SH-SY5Y cells exposed to hypoxia and incubated with a nonsense HIF-1 probe. (C) Lane 1, free sense probe without nuclear extract; lane 2, nuclear extract from SH-SY5Y cells exposed to hypoxia with sense HIF-1 probe and 2 µg of HIF-1α antibody; lane 3, sense probe incubated with nuclear extract from SH-SY5Y cells exposed to hypoxia.

Table 1

Primers and probes used for the analysis of human Tll-1 by real-time PCR.

Target	Forward primer	Reverse primer	MGB probe
mTll-1	TGAGTGTGAACAGAAGATCCACAGT	CGCTGATTTCCTCAAGTGCAT	TGGCCAGACAAGTACCCAAGCAGGA
GAPDH	ACCCACTCCTCCACCTTTGA	CTGTTGCTGTAGCCAAATTCGT	ACTTTGTCAAGCTCATTCTGGTA