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ORIGINAL ARTICLE

Hypoxia-inducible factor-1α mediates up-regulation of neprilysin by histone deacetylase-1 under hypoxia condition in neuroblastoma cells

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Abstract

Hypoxia-inducible factor (HIF)-1 is the key transcriptional activator mediating both adaptive and pathological responses to hypoxia. The purpose of this study was to find the role of HIF-1 in regulating neprilysin (NEP) at the early stage of hypoxia and explore the underlying mechanism. In this study, we demonstrated that both NEP mRNA and protein levels in neuroblastoma cells were elevated in early stages of hypoxia. Over-expression of HIF-1α gene increased NEP mRNA/protein levels, as well as enzyme activity while knockdown of HIF-1α decreased them. Meanwhile, HIF-1α was shown to bind to histone deacetylase (HDAC)-1 and reduced the association of HDAC-1 with NEP promoter, thus activating NEP gene transcription in a de-repression way. In summary, our results indicated that hypoxia in the early stages would up-regulate NEP expression, in which interaction of HIF-1α and HDAC-1 may play a role. This study suggested that NEP up-regulation might be an adaptive response to hypoxia, which was mediated by HIF-1α binding to HDAC-1 at the early stage of hypoxia.

Keywords: Histone deacetylase-1, Hypoxia-inducible factor-1α, Neprilysin.

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Neprilysin (NEP) is a zinc-dependent metalloprotease enzyme that degrades a number of small secreted peptides, including β-amyloid (Aβ) (Iwata *et al.* 2000). NEP is also considered to be a biomarker of stoke and vascular dementia in which hypoxia occurs (Qian *et al.* 2012). Brain hypoxia affects both behavioral and cognitive abilities of individuals and leads to various neuropathology (Semenza 2000). Although prolonged hypoxia could cause cell death, hypoxia preconditioning itself could raise a protection against ischemic insults, which relies on transcriptional activity of hypoxia-inducible factor-1α (HIF-1α) (Bergeron *et al.* 2000; Sarkar *et al.* 2012; Semenza 2011). Previous research has found that chronic intermittent hypoxia directly and selectively increases levels of Aβ 42 in the Alzheimer's disease model (Shiota *et al.* 2013). Furthermore, several

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Abbreviations used: AD, Alzheimer's disease; AICD, amyloid intracellular domain; APP, amyloid precursor protein; Aβ, β-amyloid; BCA, bicinchoninic acid; ChIP, chromatin immunoprecipitation; Co-IP, co-immunoprecipitation; DMEM, Dulbecco's modified Eagle's medium; EGFP, enhanced Green Fluorescent Protein; FBS, fetal bovine serum; HDAC-1, histone deacetylase-1; HIF-1α, hypoxia-inducible factor-1α; HREs, hypoxia response elements; NEP, neprilysin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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studies have indicated that hypoxia reduces NEP expression and activity in neurons (Wang *et al.* 2011) and other cell types (Mitra *et al.* 2013). Another study has reported NEP activity is reduced but NEP mRNA level is unaltered in response to hypoxia (Oh-hashi *et al.* 2005). However, all these studies are conducted at a relatively late stage of hypoxia. It is unclear how NEP is regulated by HIF-1α at early stage of hypoxia.

Histone deacetylases (HDACs) are related to dementia. For example, histone acetylation and expression of genes important for learning and memory are reduced and this blockade is mediated by HDAC-2 (Graff *et al.* 2012). However, phylogenetic analysis indicates that the evolution of HDACs preceded the evolution of histones, suggesting that primary HDAC targets may not be histones (Tsankova *et al.* 2007). NEP gene expression is mediated by histone modification (Wang *et al.* 2011), but the relationship between NEP and HDACs at the early stage of hypoxia is not well-understood.

In the present study, we investigated the role of hypoxia and HIF-1α on NEP expression at an early stage of hypoxia. Our results demonstrated that both NEP mRNA and protein levels in neuroblastoma cells and primary glia cells were elevated within 60–240 min after acute hypoxia treatment. Over-expression or gene silencing of HIF-1α increases or decreases the expression of NEP, respectively. Further study indicated that interaction of HIF-1α with HDAC-1 may contribute to increased NEP transcription.

Materials and Methods

Cell culture and treatments

N2a neuroblastoma cells and WT7 cells (AβPPswe/PS1dE9 mutant knocked-in N_2a cell), which were kindly provided by Dr Sangram S. Sisodia and SeongHun Kim (University of Chicago), were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), at 37°C in a humidified atmosphere with 5% CO_2. Cells were passaged at a ratio of 1 : 6 when 90% confluence had been reached and discarded after 20 passages. Cells were used for treatment 24 h after splitting.

Primary cultures of cerebral cortical glia cells were prepared from newborn (within 24 h after birth) ICR mice of both sexes. The mice handlings in this study were raised and approved by Peking University Institutional Animal Care and Use Committee. Briefly, meninges-free cerebral cortices were cut into small cubes and suspended in DMEM. After mechanical dissociation, the cell suspension was sequentially passed through 70 and 10 μm nylon filters. 10% (v/v) FBS (Hyclone) was added to the medium containing filtered cell suspensions. Cells were seeded in 35 mm tissue culture dishes (Corning, corning, NY, USA). Cells were then incubated at 37°C in a humidified atmosphere with 5% CO_2. Culture medium was changed twice per week with DMEM containing 10% (v/v) FBS for the first 2 weeks and 7% (v/v) FBS afterward. Glia cells were used for experiments after four weeks.

For hypoxia treatment, N2a cells and glia cells were incubated in an anaerobic chamber (model 1029, Forma Scientific, Marietta, OH, USA) (Yu and Lau 2000), containing 85% N_2/10% H_2/5% CO_2, and incubated for 0, 30, 60, 120, 240 min, and then harvest for further experiments.

For transfection treatment, cells were transiently transfected with Enhanced Green Fluorescent Protein (EGFP)-HIF-1α cDNA plasmid or EGFP-N1 plasmid (as control) (Xiong *et al.* 2009) using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. For shRNA treatment, cells were transiently transfected with HIF-1α shRNA or scramble shRNA (Genechem, shanghai, China), using lipofectamine 2000 (Invitrogen). Sequence of shRNA is: 5′-AAGCATTTCTCT CATTTCCTCATGG-3′ (Ono *et al.* 2006). Cells were used for further experiments 48 h after transfection.

Analysis of gene expression

Total RNA was extracted using Trizol reagent (Invitrogen) and digested with RNase-free DNase for 30 min to exclude genomic DNA contamination. An equal amount of RNA from each sample was transcribed into cDNA by reverse kit (Takara, Dalian, China) according to the manufacturer's instructions. The abundance of transcripts in cDNA samples was measured by either real-time PCR or conventional PCR with primer as follows: HIF-1α forward primer 5′-GAGCTTGCTCATCAGTTGCC-3′ reverse primer, 5′-CTGTACTGTCCTGTGGTGAC-3′. Mice neprilysin forward primer, 5′-TTGCAGCCCCCATTCTTC-3′ reverse primer, 5′-GAT GCCCCCATAGTTCAATGA-3′; mice 18 s rRNA forward primer, 5′-AACGAGACTCTGGCATGCTAACT-3′, reverse primer, 5′-C GCCACTTGTCCCTCTAAGAA-3′. Regular PCR was performed on mycycler96 (Bio-Rad Laboratories, Hercules, CA, USA) as described previously with modifications (Guglielmotto *et al.* 2009; Li *et al.* 2010). The amplification program consisted of a denaturing step at 94°C for 30 s, an annealing step at 59°C for 30 s, and an extension step at 72°C for 30 s, 30 cycles for HIF-1α and 25 cycles for β-actin. Real-time PCR was performed on ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA) utilizing a SYBR Green PCR premix reagent (Takara). The levels of NEP or HIF-1α expression were normalized to β-actin or 18 S rRNA.

Western blot and Co-immunoprecipitation analysis

Cell sample preparation and western blot were performed as previously described (Xian *et al.* 2009) with little modifications. Briefly, after the indicated treatments, cells were harvested and lysed on ice in different buffer for different procedures:

For western blot, lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, 0.5% sodium deoxycholate, and Complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Cell lysates were collected, centrifuged at 12 000 *g* for 10 min, and quantified for total proteins by the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Samples containing equal amount of total proteins of samples were directly analyzed by 10% SDS–polyacrylamide gel electrophoresis.

For Co-immunoprecipitation procedures, IP buffer containing 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40 (NP-40), 2 mM EDTA. Cell lysates were collected,

centrifuged at 12 000 *g* for 10 min, and then quantified for total proteins by BCA protein assay kit. Equal amount of total proteins from different procedure groups were added into separate tubes, and each tube contained 20 μL Protein G Sepharose, and 1 μg antibody. After incubation at 4°C overnight with shaking, the beads were washed five times with TBST (0.1% tween-20). Equal volumes of sample were analyzed by 10% SDS–polyacrylamide gel electrophoresis.

For western blot detection, protein was transferred to 0.45 μm polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA, USA), blocked for 1 h in 5% (m/v) non-fat milk in Tris-buffered saline (TBS, pH 7.5) supplemented with 0.1% Tween-20. Antibodies and dilutions used in this study including Mouse anti-NEP (at 1 : 1000, product number AF1126; R & D Systems, Minneapolis, MN, USA), Mouse anti-HIF-1α (at 1 : 1000, product number sc-53546; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Mouse anti-HDAC-1 (at 1 : 3000, product number ab46985-100; Abcam, Cambridge, MA, USA), Mouse anti-β-actin (at 1 : 8000, product number MAB1501; Abcam). The membranes were incubated with these first antibodies overnight at 4°C followed by the appropriate horseradish peroxidase-conjugated secondary antibody (at 1 : 3000, product number 115-035-003; Jackson Immuno-Research, West Grove, PA, USA). The immunoblots were developed using the Electro-Chemi-Luminescence system (Pierce Biotechnology, Rockford, IL, USA). Quantitative densitometric analyses were performed on digitized images of immunoblots with Quantity One (Bio-Rad).

Neprilysin activity assay

NEP activity assay were conducted as previously described (Li *et al.* 2010), cells were harvested by MBS buffer [2-(*N*-morpholino) ethanesulfonic acid (MES) buffer containing 1% Triton X-100]. For each reaction, 30 μg of total protein were incubated with or without Thiorphan, a neprilysin inhibitor, in black-colored 96-well plates (Corning) with the complete protease inhibitor cocktail, 10 μM MG132 and 0.1 mM Succinyl-Ala-Ala-Phe-7AMC (Sigma, St Louis, MO, USA) in dimethyl sulfoxide. After incubating for 60 min at 37°C, 10 μM phosphoramidon and 5 μg/mL microsomal leucine aminopeptidase were added. After a second incubation for 30 min at 37°C, stop solution (0.5 M EDTA, pH 7.4) was added, and fluorescence absorbance was recorded using a FLUOStar fluorometer (BMG Lab Technologies, Offenburg, Germany) at an emission wavelength of 460 nm and an excitation wavelength of 390 nm.

Quantification of Aβ peptide levels

Aβ peptide levels were measured by sandwich enzyme-linked immunosorbent assay (ELISA). After the indicated treatments, cells were harvested and lysed on ice. Then Complete Protease Inhibitor cocktail and RIPAR buffer was added to the culture. Cell lysates were collected, centrifuged at 12 000 *g* for 10 min and quantified for total proteins by the BCA protein assay kit. All samples were assayed for Aβ 40 and Aβ 42 by ELISA according to the manufacturer's instructions (Biosource International, Camarillo, CA, USA). The detection limit for this assay was 5 pg/mL for Aβ 40 and 10 pg/mL for Aβ 42. The Aβ concentration was normalized to the control. All measurements were performed in duplicate.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation analysis was performed as described previously using N2a cells (Wang *et al*. 2011). Cells were washed twice with Phosphate Buffered Saline and fixed in 10% formaldehyde at 37°C for 10 min. After washed three times with cold Phosphate Buffered Saline, cells were harvested using 300 μL lysis buffers (1% SDS, 5 mM EDTA, 50 mM Tris.HCl, pH 8.1, protease inhibitor cocktail). Cell lysates were sonicated and then centrifuged at 14 000 *g* for 10 min at 4°C to collect the extracts in the supernatant. 20 μL supernatant was used as input, and the left supernatant was incubated with 1 μg primary antibodies over night and then incubated with 40 μL Protein G/A Sepharose mixture for another 1 h. Washing the complex for 10 min with each of the following buffers in sequence: 1 mL Tris-SDS-EDTA buffer I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris.HCl, pH 8.1, 150 mM NaCl), Tris-SDS-EDTA buffer II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris.HCl). And then DNA extraction was analyzed by conventional PCR and agarose gel electrophoresis. The DNA extraction was measured with primer as follows: Forward, 5′-TTCCCTGAAGTCAGGAGGTG-3′, reverse 5′-CCTCCCTCCT-TCGTTTTCTT-3′.

Quantitative and statistical analysis

Quantitative data were expressed as Mean ± SEM. Comparisons of the means among multiple groups were done by one-way ANOVA followed by Dunnett's or Tukey–Kramer's *post hoc* tests using a statistical software package (GraphPad Prism, version 4.0; GraphPad Software, San Diego CA, USA). The asterisks indicate significant differences versus control as follows: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Results

NEP mRNA and protein levels are increased at early stage of hypoxia

To investigate the effect of hypoxia treatment on NEP expression at an early stage, we examined NEP mRNA and protein levels in N2a cells and primary glia cells at 0, 30, 60, 120, 240 min after hypoxia treatment. As shown in Fig. 1a and b, NEP mRNA began to increase at 30 min and was significantly higher than control at 60–240 min after hypoxia. Consistently, NEP protein level showed significant elevation at 60–240 min (Fig. 1c–f). These results indicated that short term hypoxia could up-regulate NEP expression. NEP activity is associated with Aβ peptide levels. Then we tested the Aβ peptide levels in WT7 cell lines. The Aβ 40 and Aβ 42 peptide levels were increased at 30–60 min after hypoxia and then fell back to the control level at 120 min and even lower at 240 min (Fig. 1g and h). So in this study, no sustained elevation in Aβ level was seen at early stage of hypoxia, in contrast to the results reported at late stage of hypoxia (Shiota *et al.* 2013). The relatively low level of Aβ might attribute to the up-regulated NEP expression at early stage of hypoxia.

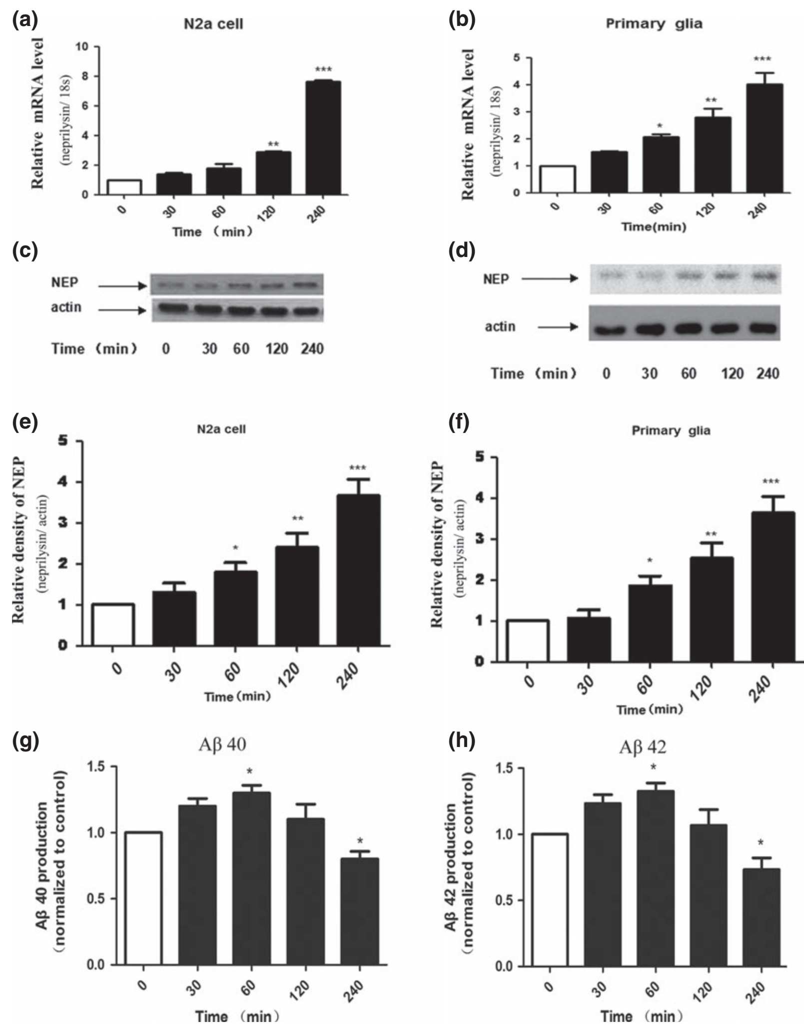

Fig. 1 Neprilysin (NEP) mRNA and protein levels are elevated in neuroblastoma cells at early stage of hypoxia. N2a neuroblastoma cells and primary glia cells were incubated under severe hypoxia conditions (85%N_2/10% H_2/5% CO_2) for 0, 30, 60,120, 240 min. NEP mRNA expression of N2a neuroblastoma cells (a) and primary glia cells (b) was determined by quantitative real-time PCR. NEP mRNA transcript levels were normalized against 18 S RNA. NEP protein levels of N2a neuroblastoma cells (c) and primary glia cells (d) were detected by western blot analysis. Representative blot of three independent experiments was shown. β-actin serves as protein loading control. (e, f) Average of densitometric analyses on western blot normalized to β-actin. The β-amyloid (Aβ) 40 (g) and Aβ 42 (h) peptide levels were detected by ELISA under the hypoxia condition. The data were shown as Means ± SEM from three independent experiments. $^{*}p < 0.05$, and $^{**}p < 0.01$, $^{***}p < 0.001$ versus control.

Expression level of HIF-1α affects mRNA/protein levels and enzyme activity of NEP

It is known that HIF-1α is stabilized and plays a key role in regulating gene expression in response to hypoxia. Our result did show that HIF-1α protein level was increased in response to hypoxia (data not shown). To investigate the relationship between HIF-1α level and NEP expression, we examined the effect of gene over-expression and knockdown of HIF-1α on NEP mRNA/protein levels and activity. Firstly, N2a cells were transfected with HIF-1α over-expressing plasmid or empty control plasmid under normoxia condition to detect whether increased HIF-1α level could elevate NEP protein level and enzyme activity. To ensure the success of transfection, we determined the mRNA levels of HIF-1α and its down-stream gene VEGF. As shown in Fig. 2a, both HIF-1α and VEGF mRNA levels were increased significantly in cells over-expressing HIF-1α in comparison with that in cells transfected with empty vector, indicating that HIF-1α transfection was successful. And the elevated HIF-1α mRNA expression led to increased HIF-1α protein level in HIF-1α-transfected cells compared with control cells (Fig. 2b). As expected, HIF-1α over-expression caused a dramatic elevation in NEP protein level (Fig. 2b and c), accompanied by increased NEP enzyme activity (Fig. 2d). To investigate whether HIF-1α knockdown could block NEP expression, N2a cells were transfected with HIF-1α or scrambled shRNA under both normoxia and hypoxia conditions. We first measured the efficiency of shRNA interference (RNAi). As shown in Fig. 3a, the basal level of HIF-1α mRNA was markedly decreased in HIF-1α siRNA-treated cells in comparison with control cells transfected with scramble shRNA. Western blot results revealed that NEP protein level was significantly decreased in HIF-1α knockdown cells under normoxia condition (Fig. 3b and c). Meanwhile, NEP enzyme activity decreased nearly 50% after HIF-1α knockdown (Fig. 3d). While under hypoxia condition, NEP protein level and (Fig. 3e and f) activity (Fig. 3g) in HIF-1α siRNA-treated cells were also lower than normal control, indicating that HIF-1α knockdown blocked both basal and induced NEP expression under hypoxia conditions. In addition to protein levels, NEP mRNA was also increased by HIF-1α over-expression and decreased by

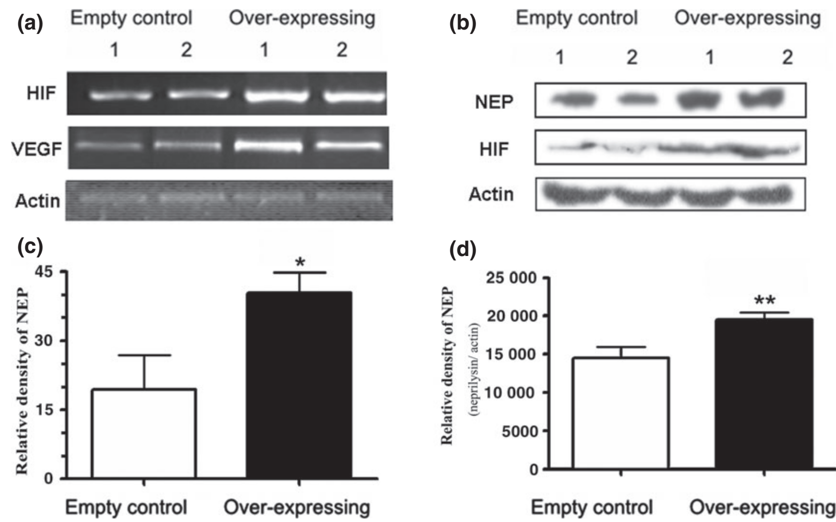

Fig. 2 Gene over-expression of hypoxia-inducible factor-1α (HIF-1α) increases neprilysin (NEP) protein level and enzyme activity. N2a cells were transfected with EGFP-HIF-1α plasmid or EGFP-N1 plasmid (empty control) for 48 h. (a) Same amount of total mRNA was used to determine the mRNA levels of HIF-1α and its downstream gene VEGF by conventional PCR. (b) NEP and HIF-1α protein levels were examined by western blot analysis. β-actin was used as protein loading control. (c) Average of densitometric analyses on western blot normalized to β-actin. The data were shown as Means ± SEM from three independent experiments. $^*p < 0.05$, versus control. (d) Same amount of protein (30 μg/reaction) was used for measurement of NEP enzyme activity. The data were shown as Means ± SEM for twelve independent experiments. $^{**}p < 0.01$ versus control.

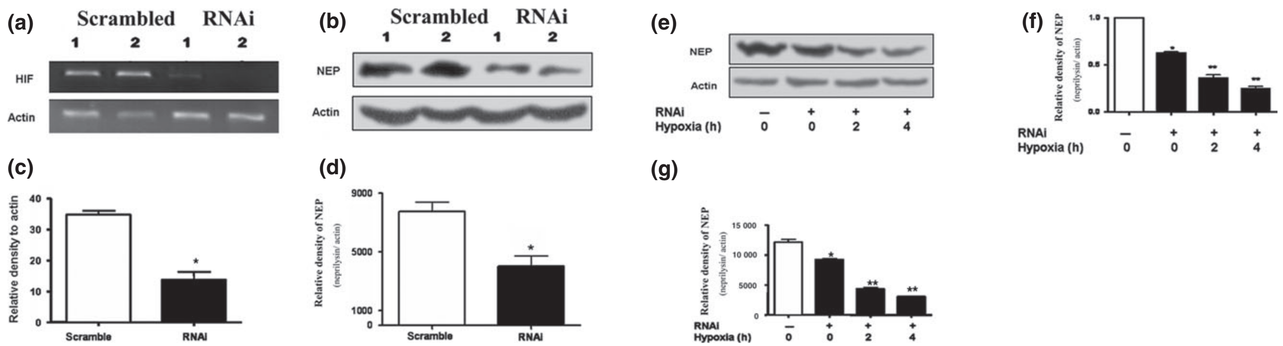

Fig. 3 Gene knockdown of hypoxia-inducible factor-1α (HIF-1α) decreased neprilysin (NEP) protein level and activity under normoxia and hypoxia conditions. N2a cells were transfected with HIF-1α shRNA plasmid (shRNA) or scrambled shRNA plasmid (scrambled) for 48 h. (a) HIF-1α mRNA was examined by conventional PCR and was found to be almost completely abolished after 48 h of treatment with HIF-1α shRNA. (b, c) NEP protein levels were examined by western blot analysis of ($n = 3$). β-actin was used as protein loading control. (d) Same amount of protein (30 μg/reaction) was used for measurement of NEP enzyme activity ($n = 12$). (e, f) After transfection with HIF-1α shRNA, cells were incubated under hypoxia conditions for 120 or 240 min and western blot was used to analyze NEP protein levels ($n = 3$). β-actin was used as protein loading control. (g) After transfection with HIF-1α shRNA, cells were incubated under hypoxia conditions for 120 or 240 min and same amount of protein (30 μg/reaction) was used for measurement of NEP enzyme activity ($n = 12$). The data were shown as Means ± SEM from three independent experiments. $^*p < 0.05$, and $^{**}p < 0.01$ versus scrambled control.

HIF-1α knockdown (Fig. 4), indicating that changes in NEP protein level might be because of regulation on NEP gene transcriptional level by HIF-1α (Fig. 4).

HIF-1α binds to HDAC-1 and reduces HDAC-1 association with NEP promoter

HIF-1α functions not only as a component of HIF-1 transcription factor, but also as a transcription factor-interacting protein to regulate the transcriptional activity of other transcription factors (Koshiji *et al.* 2005, 2004). A recent study has indicated that HDAC-1 can directly bind to NEP promoter region and suppress NEP expression (Belyaev *et al.* 2009). Moreover, it has been also reported that HIF-1α can directly interact with HDAC-1 (Yoo *et al.* 2006; Ellis *et al.* 2009) and dissociate HDAC-1 from certain gene promoter, up-regulating gene expression (Kato *et al.* 2008). Here we

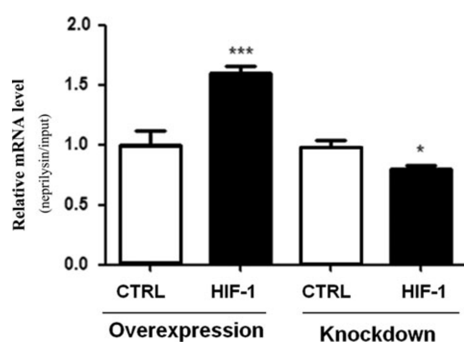

Fig. 4 Hypoxia-inducible factor-1α (HIF-1α) over-expression/knockdown increases/decreases neprilysin (NEP) mRNA level. N2a cells were either transfected with EGFP-HIF-1α plasmid (over-expression) or HIF-1α shRNA plasmid (knockdown) for 48 h, and then real-time PCR was performed to examine NEP mRNA levels. The data were shown as Means ± SEM from three independent experiments. $^{*}p < 0.05$, and $^{***}p < 0.001$ versus corresponding control.

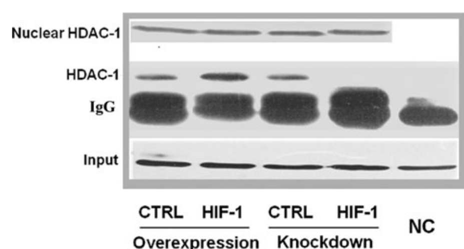

Fig. 5 Hypoxia-inducible factor-1α (HIF-1α) directly interacts with histone deacetylase-1 (HDAC-1). N2a cells were either transfected with EGFP-HIF-1α plasmid (over-expression) or HIF-1α shRNA plasmid (knockdown) for 48 h. Western blot and Co-IP procedures were carried out with anti-HDAC-1 antibody. First lane represented the total HDAC-1 level in the nucleus which remained unchanged. Second lane was the Co-IP result showing the amount of HDAC-1 binding to HIF-1α and IgG used in the Co-IP. Input was used as loading control. NC represented negative control.

investigated whether HIF-1α can affect association of HDAC-1 with NEP promoter.

Firstly, we performed the co-immunoprecipitation experiment using mouse anti-HIF-1α antibody and showed that significant amount of HDAC-1 was co-immunoprecipitated with HIF-1α in the control cells (transfected with empty vector or scrambled shRNA) (Fig. 5), confirming the protein interaction of HIF-1α with HDAC-1 that has been reported previously (Yoo *et al.* 2006). Moreover, the amount of HDAC-1 co-immunoprecipitated with HIF-1α was increased in the HIF-1α over-expressing cells and decreased in cells transfected with HIF-1α shRNA, with no change in the total level of HDAC-1 in nucleus (Fig. 5), indicating that the amount of HIF-1α protein correlates with the amount of HDAC-1 recruited by HIF-1α. Next, we examined whether HIF-1α level affected association of HDAC-1 with NEP promoter with chromatin immunoprecipitation assay. As shown in Fig. 6, HDAC-1 binding to

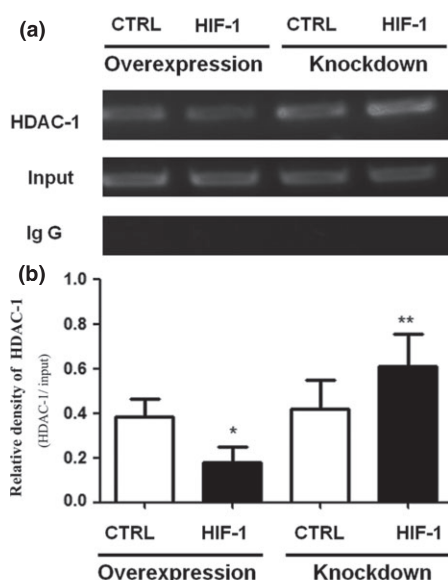

Fig. 6 Hypoxia-inducible factor-1α (HIF-1α) influences histone deacetylase-1 (HDAC-1) binding to neprilysin (NEP) promoter region. N2a cells were either transfected with EGFP-HIF-1α plasmid (over-expression) or HIF-1α shRNA plasmid (knockdown) for 48 h. Chromatin immunoprecipitation (ChIP) procedure was carried out with anti-HDAC-1 antibody and conventional PCR was performed to analyze HDAC-1 associated DNA with primers specific for mice NEP promoter 1. (a) Representative bands were shown for HDAC-1 associated NEP promoter DNA. Input was used as loading control and IgG was used as negative control. (b) Statistic analysis of ChIP result. The data were expressed as Means ± SEM from three independent experiments. $^{*}p < 0.05$, and $^{**}p < 0.01$ versus corresponding control.

NEP promoter negatively correlated with the levels of HIF-1α, indicating that protein interaction between HIF-1α and HDAC-1 affects binding of HDAC-1 to NEP promoter.

Discussion

Considerable evidence has pointed to important roles for chronic hypoxia in Aβ accumulation. For instance, intermittent hypoxia has been seen to directly and selectively increase levels of Aβ 42 (Shiota *et al.* 2013). This may result from increased production and reduced clearance of Aβ. It has been reported that prolonged hypoxia can up-regulate alzheimer's β secretase (BACE-1) (Zhang *et al.* 2007) which is a membrane-bound aspartic protease that cleaves the amyloid precursor protein at the β-secretase site and then participate in Aβ generation. Moreover, prolonged hypoxia is shown to decrease expression and activity of NEP in cultured neurons (Fisk *et al.* 2007; Wang *et al.* 2011). In this study, we found that, at early stage of hypoxia, NEP mRNA and protein levels were increased, which may conduct Aβ degradation. And this effect is mediated by HIF-1, a transcription factor that plays a key role in mediating both

physiological and pathological responses to reduced oxygen availability through regulating gene expression. The role of HIF-1α in neuronal survival is still a topic of debate. The adverse effects of HIF-1α may be dependent on the cell-specific responses to stresses (Vangeison *et al.* 2008), as well as the duration and the degree of severity of insults (Aminova *et al.* 2005). It is likely that HIF-1 mediates adaptive and pathological responses, respectively, at early and late stages of hypoxia/ischemia, respectively. The experimental model in our investigation was cell lines and primary glia cell which may not represent the real condition *in vivo*, leaving limitations for our finding in clinical application. However, our work suggests that under certain conditions, HIF-1 may play a role in the up-regulation of NEP expression.

HIF is known to bind to target genes through hypoxia response elements (Mitra *et al.* 2013). The proposed mechanism of NEP regulation in this study is that HIF-1α binds to HDAC-1 and reduces HDAC-1 association with NEP promoter. Consistently, it has been reported that HIF-1α can recruit HDAC-1 (Yoo *et al.* 2006) and disassociate HDAC-1 from promoter areas and activate certain gene expression during hypoxia (Kato *et al.* 2008). Prolonged hypoxic insult reduced NEP expression through histone modification where HDAC-1 is up-regulated and mediates suppression of NEP gene (Wang *et al.* 2011). It is possible that increased level of HDAC-1 may exceed the binding ability of HIF-1α, and the inhibitory effect of HDAC-1 on NEP transcription may no longer be counteracted by HIF-1α, leading to reduced NEP expression at late stage of hypoxia although high level of HIF-1α is still maintained.

Taken together, our results indicate that HIF-1α mediates up-regulation of NEP by HDAC-1 at an early stage of hypoxia. Our results may provide better understanding of the role of HIF-1α in regulation of protein expression in early stages of hypoxia.

Acknowledgments and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

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