Heat shock protein 90 is involved in regulation of hypoxia-driven proliferation of embryonic neural stem/progenitor cells

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Abstract Hypoxia may regulate the proliferation of diverse stem cells. Our previous study showed that hypoxia promoted the proliferation of embryonic neural stem/progenitor cells (NPCs) and that hypoxia inducible factor-1(HIF-1) was critical in this process. HIF-1 could be stabilized under hypoxic conditions, and heat shock protein 90 (HSP90) is an essential protein that controls the activity and stabilization of HIF-1 α . In the present work, we investigate whether HSP90 is involved in proliferation of NPCs under hypoxia by regulating HIF-1 α stabilization. Geldanamycin (GA), an HSP90 inhibitor, decreased the expression of HIF-1 α in NPCs during hypoxia-driven proliferation and reduced the expression level of HIF-1 α protein under hypoxia in a time-dependent manner. The proliferation of NPCs induced by hypoxia was inhibited after GA treatment for 24 h. Another HSP90 inhibitor, radicicol, had the same effect on NPCs as GA. Furthermore, the expression of erythropoietin (EPO) and vascular endothelial growth factor (VEGF) in NPCs under hypoxia was suppressed by GA. The above data indicated that HSP90 might be involved in regulation of hypoxia-driven proliferation.

Keywords Embryonic neural stem or progenitor cells · Geldanamycin · HIF-1 α · HSP90 · Hypoxia

Abbreviations

EPO erythropoietin
GA Geldanamycin
HIF-1 hypoxia inducible factor-1
NPCs neural stem/progenitor cells
VEGF vascular endothelial growth factor

Introduction

Hypoxia regulates the proliferation and differentiation of various stem cells both in vitro and in vivo (Zhu et al. 2005). Moderate hypoxia promotes the proliferation of neural stem/progenitor cells (NPCs) from the central nervous system or the peripheral nervous system in vitro (Morrison et al. 2000; Studer et al. 2000; Storch 2001; Acker and Acker 2003; Zhu et al. 2005; Park et al. 2006). Recently, our study demonstrated that hypoxia inducible factor-1(HIF-1) was critical in hypoxic-driven proliferation of NPCs. Overexpression of HIF-1 α significantly increased the proliferation of NPCs under normoxia and partially mimicked the effect of hypoxia on proliferation of NPCs in vitro. In contrast, knockdown of HIF-1 α expression decreased proliferation of NPCs induced by hypoxia. These observations suggest that HIF-1 plays an important role in hypoxia-induced proliferation of NPCs (Zhao et al. 2008).

HIF-1 is a transcriptional activator that functions as a master regulator of cellular and systemic oxygen homeostasis. HIF-1 mediates a variety of adaptive cellular and systemic responses to hypoxia by regulating the expression of more

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than 50 different genes to assist cells or animals in their adaptation and survival (Semenza 2000; Shi and Fang 2004). HIF-1 α activity is primarily regulated at posttranslational levels. It has been shown in extensive studies that HSP90 has an important effect on the stabilization and activity of HIF-1 α in various cell lines (Goetz et al. 2003; Bagatell and Whitesell 2004). HSP90 can be specifically inhibited by substances of the benzoquinone ansamycin antibiotic group such as geldanamycin (GA) and its derivatives (Janin 2005). The presence of active HSP90 is necessary for rapid HIF-1 α accumulation and HIF-1 activation under hypoxia (Minet et al. 1999; Hur et al. 2002; Han et al. 2005). HSP90 could directly interact with the PAS domain of HIF-1 α to make it keep stabilization (Katschinski et al. 2004). Suppression of HSP90 activity by GA could induce the degradation of HIF-1 α in an O₂ independent way and inhibit the angiogenesis and energy metabolism of tumors (Isaacs et al. 2002; Mabjeesh et al. 2002; Schwock et al. 2005; Alqawi et al. 2006; Lang et al. 2007).

HIF-1 α plays an important role in the proliferation of NPCs and in the survival of tumors under hypoxia, while HSP90 is required for tumor growth because of its effect on HIF-1 (Zhao et al. 2008; Carmeliet et al. 1998; Minet et al. 1999). Therefore, in the present study, we hypothesized that HSP90 may be involved in the regulation of hypoxia-driven proliferation of NPCs. First, the expression level of HSP90 and HIF-1 α under normoxia and hypoxia were detected. Then, we investigated the effect of the HSP90 inhibitor, GA, on expression of HIF-1 α and the growth of NPCs. Moreover, to determine whether suppression of HSP90 activity could disrupt the HIF-1 pathway, the transcriptional level of the target genes of HIF-1, for example erythropoietin (EPO) and vascular endothelial growth factor (VEGF), were detected after addition of the inhibitor.

Materials and methods

Animals

Wistar rats (13.5 days pregnant) were used. The Institutional Animal Care and Use Committee (IACUC) of the Academy of Military Medical Science approved all experiments involving rats.

Isolation and culture of NPCs

Cells derived from Wistar rat mesencephalon (E13.5) were mechanically dissociated, and grown in DMEM/F12 (1:1) medium containing 2 mM L-glutamine, 5 IU of penicillin, 5 μ g/ml streptomycin (BioWhittaker), 1% N2 (Life Technologies), 1% B27 (Life Technologies), 20 ng/ml epidermal growth factor (EGF; Sigma) and 20 ng/ml basic fibroblast

growth factor (bFGF; R&D Systems, Minneapolis, MN; Zhang et al. 2007). Cells were plated on plastic 35 mm dishes precoated with poly-L-lysine. After fixation with 4% paraformaldehyde at 4°C for 2 h, cells were identified by immunohistochemistry with a mouse monoclonal antibody (Molecular Probe) against nestin. The results showed that almost all of the cells were nestin positive. We could thus use them to perform the following experiments. The primary formed neural spheres were defined as passage zero (P₀) NPCs. The NPCs were subcultured two to five generations, and used in the following experiments.

Hypoxic condition

For decreased oxygen condition, an incubator chamber was used (Thermo 3111, Billups-Rothenberg, Del Mar, CA) in which oxygen concentration is adjustable. The incubator chamber was flushed with 5% CO₂/balance N₂. The actual concentrations of 20% O₂ and 3% O₂ inside the chamber were based on direct measurement with a microelectrode (Animus Corp., Malvern, PA). The time of hypoxia was calculated from the measurement that showed the desired oxygen concentration.

RNA extraction and reverse transcription-PCR

Cultures were washed once with PBS before solubilization in Trizol (Life Technologies) and then stored at -80°C. Total RNA extraction was performed according to the recommendations of the manufacturer (Life Technologies). The program for PCR was as follows: primers for HIF-1 α 5'-TGCTTGGTGCTGATTTGTGA-3', 5'-GGTCAGATGATCAGAGTCCA-3' were used to yield a 209-bp product for 30 cycles at 58°C; primers for HSP90 α 5'-AAGCACCTATGGCTGAC-3', 5'-GGTAGATGCGGTTGGAGT-3' were used to yield a 266-bp product for 28 cycles at 54°C; primers for HSP90 β 5'-TGTTGTGGATTCCGATGA-3', 5'-GTTTACCAAGCCGTGTTC-3' were used to yield a 221-bp product for 28 cycles at 50°C; primers for VEGF 5'-CCTCCGAAACCATGAACCTTCTGCTC-3', 5'-CAGCCTGGCTCACCGCCTTGGCTT-3' were used to yield a 593-bp product for 30 cycles at 55°C; primers for EPO 5'-CCCTGCTGCTTTACTATC-3', 5'-CCACCTTCATTCTTTTCC-3' were used to yield a 360-bp product for 30 cycles at 55°C. m18S rRNA primer: forward: 5'-TTATGGTTCCTTTGGTCGCT-3', reverse: 5'-ATGTGGTAGCCGTTTCTCAG-3' was used to yield a 355-bp product for 30 cycles at 56°C. The level of each mRNA expression was semi-quantified as relative to the endogenous expression level of 18S rRNA.

Protein extraction and Western blot

Cells were harvested quickly after either hypoxic or normoxic culture for the desired times and the total protein was extracted

with lyses buffer, which contained 100 mM Tris-HCl [pH 7.5], 300 mM NaCl, 2%(v/v) Tween-20, 0.4% NP-40, 20% glycerol, supplemented with protease inhibitors (1 µg/mL leupeptin and pepstatin, 2 µg/mL aprotinin and 1 mM PMSF) and phosphatase inhibitors (10 mM NaF and 1 mM Na₃VO₄). Western blot analyses were then carried out. Extracts were quantified by protein assay kit (Bio-Rad) and were fractionated by 8% polyacrylamide-SDS gel electrophoresis and transferred to a polyvinylpyrrolidone difluoride membrane (Immobilon-P, Millipore, Bedford, MA). The membrane was blocked with Tris-buffered saline (TBS-T) containing 5% dry milk at room temperature for 2 h. Membranes were incubated with mouse monoclonal antibody to HIF-1α (Sigma, H6536, dilution at 1:1,000), mouse monoclonal antibody to HSP90 (Stressgen, SPA-830, dilution at 1:1000), rabbit polyclonal antibody to HSP90α (Stressgen, SPS-771, dilution at 1:1,000; kindly provided by Prof. Yu-fei Shen), or mouse monoclonal antibody to HSP90β (Stressgen, SPA-843, dilution at 1:1,000; kindly provided by Prof. Yu-fei Shen) in TBS-T containing 5% nonfat dry milk. Membranes were treated with goat anti-mouse or goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (Santa Cruz; dilution at 1:1,000) in TBS-T containing 5% nonfat dry milk. Immune complexes on the membrane were visualized by using enhanced chemiluminescence's (ECL) detection system (Amersham Biosciences).

Cell cycle analysis

The neural spheres under hypoxic or normal condition were dissociated by using 0.25% Trypsin/EDTA. Single cell suspensions were obtained and washed three times with PBS. After fixation with 75% ethanol, cells were digested with DNase-free RNase in PBS containing 5 µg/ml propidium iodide for DNA staining (45 min at 37°C; Krishan 1975). The propidium iodide fluorescence and forward light scattering were detected via flow cytometry (FACS scan; Beckton, Dickinson) equipped with Cellquest software (Largo, FL).

Cells survival analysis

The neural stem/progenitor cells were cultivated in 96 Well Cell Culture Cluster (COSTAR) exposed to normoxia (20% O₂) or hypoxia (3% O₂) for 24 h after addition of GA (1 µM) or radicicol in 1 µM or 5 µM (Sigma; kindly provided by Prof. Xiao-dan Yu). We then added 15 µl Cell Counting kit-8 (CCK-8, Dojindo Laboratories, dilution at 1:10) into each well and settled the culture cluster in a 37°C incubator chamber to react for 4 h. The optical density (OD) was detected by Microplate Reader (RIO-RAD, Model 550).

Results

Effect of hypoxia on expression of HIF-1α and HSP90 in neural stem/progenitor cells

NPCs were obtained and identified as described in "Materials and methods". Our previous study showed that hypoxia promoted the proliferation of NPCs, and that NPCs could be expanded under hypoxia over a long period of time (Zhao et al. 2008). To study the molecular mechanism in neural stem/progenitor cells under hypoxia response, we examined the expression of HIF-1α and HSP90 under either normoxia (20%O₂) or hypoxia (3%O₂) for 1, 3, 6, 12, 24, 48, and 72 h. The whole cell lysates were extracted and tested by Western blot for HIF-1α and HSP90 expression, and β-actin was examined as a control. Figure 1a showed that expression of HIF-1α levels increased in neural stem/progenitor cells upon exposure to hypoxia for varying times. After culture under hypoxic condition for 6 h, the total amount of HIF-1α protein increased remarkably by 400% compared with control (Fig. 1b) and the band of HIF-1α expression became weaker during prolonged hypoxia. However, no obvious changes in HSP90 protein expression levels were detected by immunoblot analysis (Fig. 1c).

There are two major isoforms of HSP90, HSP90α (inducible form/major form) and HSP90β (constitutive form/minor form; Sreedhar et al. 2004). The isoform specificity extends to the functional role of HSP90 in cell growth and development. Here, we further examined the expression of HSP90α and HSP90β in NPCs under either normoxia (20%O₂) or hypoxia (3%O₂) for 1, 3, 6, 12, 24, 48, and 72 h. The whole cell lysates were extracted and tested by RT-PCR or Western blot. As shown in Fig. 2a, the expression of HSP90α and HSP90β mRNA was constitutively detectable in NPCs and they were unaltered after exposure to hypoxia. The protein expression levels of HSP90α and HSP90β in NPCs also had no obvious changes after exposure to hypoxia (Fig. 2b). Both mRNA and protein expression levels of HSP90β were lower than HSP90α, indicating that HSP90α might play a major role in growth of NPCs. This suggests that hypoxia did not significantly influence the expression of HSP90α and HSP90β.

Effect of geldanamycin on hypoxia-induced stabilization of HIF-1α

GA mediates the dissociation of HSP90 from client proteins and promotes their rapid degradation (Söti et al. 2005). It has been demonstrated that HSP90 interacts with HIF-1α, although a definitive biological role for this interaction has remained elusive (Katschinski et al. 2004). We therefore investigated whether HSP90 is involved in regulating HIF-1α protein stabilization in neural stem/progenitor cells. GA

Fig. 1 Hypoxia increased the expression of HIF-1 α but had no influence on expression of HSP90 in NPCs. Cells were exposed to 20%O₂ or 3%O₂ condition for different periods of time, and then collected for performing Western blot assay. **a** Representative photograph for HIF-1 α and HSP90 protein tested by Western blot (*N* normoxia; *H* hypoxia). **b** and **c** The

level of HIF-1 α and HSP90 protein expression measured by densitometry analysis. The HIF-1 α and HSP90 protein expression value was normalized to that of β -actin. Values are means $\pm$ SD from three independent experiments, * P <0.05 compared with control

(1 μ M) was added to the cell medium and cells were exposed to either normoxia (20%O₂) or hypoxia (3%O₂) conditions for 6, 12, and 24 h. As control, two groups of cells were exposed to normoxia or hypoxia for 24 h with DMSO (1 μ M). Figure 3a showed that HIF-1 α protein expression signal was detected weakly in NPCs under normoxia whether treated with HSP90 inhibitor or not. Under hypoxia, the stability of HIF-1 α increased slightly after treatment with GA at 6 h, but decreased dramatically over prolonged periods of time (Fig. 3B). The total amounts of HIF-1 α protein were reduced by 50% and 80% after treatment with GA for 12 and

24 h under hypoxia. These data showed that GA reduced expression of HIF-1 α protein in a time-dependent manner.

Our previous study compared the effect of HSP90 inhibitor on HIF-1 α stabilization in NPCs under either normoxia or hypoxia. As shown in Fig. 1a, we observed the down-regulation of HIF-1 α during prolonged hypoxia, and that the protein levels of HIF-1 α changed depending of the length of hypoxia treatment. This phenomenon will be explained in the discussion. To eliminate the interference of HIF-1 α down-regulation on the inhibiting effect of GA, and to examine the effect of GA on hypoxia-induced HIF-1 α

Fig. 2 Effect of hypoxia on the expression of HSP90 α and HSP90 β in NPCs. Cells were exposed to either 20% O₂ or 3% O₂ conditions for different periods of time and then collected for the performance of RT-PCR and western blot assay. Hypoxia did not significantly influence the expression of HSP90 α and HSP90 β . **a** Representative photograph for HSP90 α and HSP90 β mRNA tested by RT-PCR (*N* normoxia; *H* hypoxia). **b** Representative photograph for HSP90 α and HSP90 β protein tested by western blot (*N* normoxia; *H* hypoxia)

Fig. 3 GA repression of HIF-1 α expression is time-dependent in NPCs. Cells were exposed to 20% O₂ or 3% O₂ condition for 6, 12, and 24 h in the presence or absence of GA (1 μ M) and cell lysates were analyzed by Western blot. **a** and **c** Representative photograph for HIF-1 α protein tested by western blot (N normoxia; H hypoxia). **b** and

d The level of HIF-1 α protein expression measured by densitometry analysis. The HIF-1 α protein expression value was normalized to that of β -actin; Values are means $\pm$ SD from three independent experiments, * P <0.05; ** P <0.01 compared with control

expression over various time periods, NPCs were exposed to hypoxia for 3, 6, 12, and 24 h, and treated with GA (1 μ M) or DMSO as control. Figure 3c and d shows that GA decreased the protein level of HIF-1 α at each time point compared with the hypoxia control, and expression of HIF-1 α protein was nearly blocked after treatment with GA for 24 h. Hypoxia-induced expression of HIF-1 α was significantly delayed and inhibited by GA as compared with its control.

Effect of geldanamycin and radicicol on neural stem/progenitor cell viability and proliferation

The suppression of HSP90 activity by GA induced HIF-1 α protein degradation in a time-dependent manner and blocked the expression of HIF-1 α under hypoxia. Cells were exposed to normoxia or hypoxia with or without GA to test whether suppression of HSP90 activity could also influence the growth of neural stem/progenitor cells. After treatment with 1 μ M GA for 24 h, some single cells were dispersed from the neurospheres compared to the control group under either normoxia or hypoxia (Fig. 4a). Cell survival was then measured by CCK-8 spectrophotometric analysis after treatment of NPCs with GA in different doses from 10 nM to 1 μ M for 24 h. The data showed that the OD value of NPCs increased by 22% under hypoxia, indicating that hypoxia (3% O₂) promoted the survival of NPCs (Fig. 4b, DMSO control). The survival index of cells treated with GA of 10, 100, and

1,000 nM were reduced by 14%, 62%, and 68% under hypoxia and 7%, 56%, and 58% under normoxia, respectively. This inhibitory effect was dose dependent. This suggested that GA suppressed the growth of NPCs under hypoxia and normoxia.

We next investigated the proliferation index of NPCs by performing a flow cytometric measurement of DNA distributions of cells when treated with GA. Phase fractions calculated from such distributions are used to study the growth characteristics of NPCs. NPCs treated with 1 μ M GA under normoxia or hypoxia for 24 h were collected and processed with flow cytometry. Three cell subpopulations (G₁, S and G₂+M) were estimated. The proliferation index reflects the ratio of NPCs in the S phase and G₂/M phase of the cell cycle. Figure 5a and b showed that the proliferation index of NPCs increased by 17% after exposure to hypoxia and decreased by 49% after treatment with GA, indicating that 3% O₂ promoted the proliferation of NPCs and that GA inhibited the proliferation of NPCs under both hypoxia and normoxia. We also found that the number of S-phase cells under hypoxia increased by 60% compared to normoxia and decreased by 96% after treatment with GA (Fig. 5c). These results suggested that suppression of HSP90 activity by GA decreased the proliferation index of neural stem/progenitor cells while under hypoxia.

The HSP90 inhibitor, geldanamycin could lead to superoxide production which may interfere the effects we observed (Sreedhar et al. 2003). To clarify whether decrease in NPCs

Fig. 4 GA inhibits hypoxia-driven proliferation of NPCs.

a Photographs of NPCs treated with DMSO or GA (1 μ M) under normoxia or hypoxia; **b** Cells were exposed to 20% O₂ or 3% O₂ condition for 24 h in the presence or absence of 10, 100, and 1,000 nM GA. The survival index was measured by CCK-8 spectrophotometric analysis. Values are means $\pm$ SD from ten experiments, ** P < 0.01 compared with control

growth is caused only by inhibition of HSP90 or GA-induced superoxide production, another HSP90 inhibitor, radicicol, was used. Radicicol (1 or 5 μ M) was added to the cell medium and cells were exposed to hypoxia (3% O₂) conditions for 24 h. As control, two groups of cells were exposed to normoxia or hypoxia for 24 h with DMSO. The whole cell lysates were extracted and tested by Western blot for HIF-1 α expression, and β -actin was examined as a control. Figure 6a showed that HIF-1 α protein expression signal was detected weakly in NPCs under normoxia. The total amounts of HIF-1 α protein in NPCs was increased after exposure to hypoxia, but reduced after treatment with radicicol. This produced the same effect as GA. Cell survival was then measured by CCK-8 spectrophotometric analysis after treatment of NPCs with radicicol in varying doses from 10 nM to 5 μ M for 24 h. Radicicol of low concentration had no influence on cell growth, but the survival index of cells treated with radicicol of 1 and 5 μ M was reduced by 49% and 59% under hypoxia and 41% and 50% under normoxia, respectively (Fig. 6b). This inhibitory effect was dose dependent. Our results indicate that the HSP90 inhibitor, radicicol, which did

not induce the free radicals, can also decrease the HIF-1 α expression and suppress the growth of NPCs under hypoxia.

Geldanamycin decreased HIF-1 induced gene expression

The expression of vascular endothelial growth factor (VEGF) and erythropoietin (EPO), mainly regulated by HIF-1, was evaluated to further examine whether GA inhibited the transcriptional control of HIF-1 to its targeted genes. Neural stem/progenitor cells were incubated under normoxia (20%O₂) or hypoxia (3%O₂) in the presence or absence of GA (1 μ M). The mRNA levels of HIF-1 α , HSP90, VEGF and EPO were measured by RT-PCR as described in “Materials and methods”. Figure 7a showed that the mRNA levels of HIF-1 and HSP90 had no changes in NPCs exposed to normoxia or hypoxia, while the mRNA levels of VEGF and EPO increased by 43% and 120% under hypoxia compared to normoxia. After treatment with GA under hypoxia, the levels of VEGF and EPO mRNA decreased by 42% and 62% (Fig. 7b). Hence, inhibition of HIF-1 α expression by GA could consequently affect expression of its targeted genes such as VEGF and EPO.

Fig. 5 GA decreased the proliferation index of NPCs under hypoxia. **a** Flow cytometric analysis showed that hypoxia led to more NPCs in the S phase and G2/M phase of the cell cycle and that GA (1 μ M) reduced them significant; **b** Cartogram of flow cytometric analysis about

proliferation index. **c** Cartogram of flow cytometric analysis about number of S-phase cells. The data are the means $\pm$ SD ($n=3$). * $P<0.05$; ** $P<0.05$ compared with control

Discussion

Here, we investigated that the HSP90 inhibitors, geldanamycin (GA) suppressed HSP90 activity and decreased the

expression of HIF-1 α protein induced by hypoxia in a time-dependent manner. The expression of EPO and VEGF, which were mainly regulated by HIF-1, was also reduced by GA. Furthermore, hypoxia-driven proliferation of NPCs was inhibited after GA treatment for 24 h. Another HSP90 inhibitor, radicicol, had the same effect on NPCs growth as GA. These data indicated that HSP90 might be involved in regulation of hypoxia-driven proliferation of NPCs.

The regulation of HIF-1 α depends primarily on its protein stabilization, which is otherwise rapidly degraded by the ubiquitin–proteasome pathway in normoxic cells (Kallio et al. 1999; Maxwell et al. 1999). The HIF-1 α gene is continually transcribed, although its mRNA level shows some changes in different hypoxia conditions and cell types (Wiener et al. 1996). O₂-dependent degradation of HIF-1 α is mediated by prolyl hydroxylase (PHD), the von Hippel–Lindau (VHL)/E3 ubiquitin ligase complex, and the proteasome. When the oxygen concentration is reduced, due to a lack of the zymolyte, PHD losses its activity, allows HIF-1 α to escape the O₂-dependent degradation, maintain stabilization and make the nuclear translocation to produce an effect (Bruick 2003). It had been reported that HSP90 might regulate HIF-1 α activation in COS-7 cells (Minet et al. 1999), and modulate HIF-1 α activity by stabilization of the protein (Gradin et al. 1996). HIF-1 is a known downstream target of HSP90 inhibitors; GA, a HSP90 inhibitor, induces degradation of HIF-1 α protein through the proteosomal pathway. Hypoxia may regulate expression of HIF-1 α and HSP90. Katschinski reported that expression of both HSP90 and HIF-1 α protein

Fig. 6 Radicicol inhibits HIF-1 α expression and blocks hypoxia-driven proliferation of NPCs. **a** Cells were exposed to either 20% O₂ or 3% O₂ conditions for 24 h in the presence or absence of radicicol (1 or 5 μ M), and cell lysates were analyzed by western blot (N normoxia; H hypoxia). **b** Cells were exposed to 20% O₂ or 3% O₂ condition for 24 h in the presence or absence of 10 nM, 100 nM, 1 and 5 μ M radicicol. The survival index was measured by CCK-8 spectrophotometric analysis. Values are means $\pm$ SD from ten experiments, ** $P<0.01$ compared with control

Fig. 7 GA restrains HIF-1 target gene expression. Cells were exposed to either 20% O₂ or 3% O₂ conditions for 24 h in the presence or absence of GA (1 μM). **a** Expressions of HIF-1α, HSP90α, VEGF and EPO mRNA were analyzed by RT-PCR; **b** The level of HIF-1α,

HSP90α, VEGF, and EPO mRNA expression measured by densitometry analysis. The mRNA expression value was normalized to that of 18S; Values are means±SD from three experiments, **P*<0.05 compared with control

were up-regulated when the HepG2 cells were exposed to 3% O₂ (Katschinski et al. 2002). In the current study, we found the total amount of HIF-1α protein in neural stem/progenitor cells increased remarkably after treatment with hypoxia compared with normal condition. The expression level of HIF-1α decreased in NPCs exposed to hypoxia for 72 h. Kong X. demonstrated that the persistent down-regulation of HIF-1α during prolonged hypoxia was mainly caused by a decreased production of the protein without changing its degradation rate (Kong et al. 2007). Marxsen also demonstrated that HIF-1 could promote its degradation by induction of prolyl-4-hydroxylases to limit accumulation of HIF-1α in hypoxia (Marxsen et al. 2004). This regulation did not eliminate the function of HIF-1 in cell growth, however. In the study, we did not find the up-regulation of HSP90 level in NPCs under hypoxia. We also did not find the change in the alpha and beta isoforms of HSP90 in NPCs under hypoxia response. However, HIF-1α was degraded even under hypoxia after suppression of HSP90 activity. This indicated that HIF-1α was not only regulated by O₂-dependent degradation, but that HSP90 activity was also required for HIF-1α to escape this O₂-independent degradation.

HIF-1 plays an important role in tumor growth, so that treatment strategies that target HIF-1α may become an effective pathway in cancer therapy (Shi and Fang 2004; Vaupel 2004). HSP90 inhibitors are attractive substances because of their function in the response of tumor cells to hypoxia (Calderwood et al. 2006). In the past 10 years, extensive research has been performed and a wealth of literature has evolved around the relationship of HSP90 and HIF-1α. It has been reported that suppression of HSP90 activation could induce the degradation of HIF-1α in many kinds of cell lines, such as prostate cancer cells, hepatoma cell lines and human cervical carcinoma cells, but the concrete mechanism advanced only very slowly (Isaacs et al. 2002; Mabjeesh et al. 2002; Schwock et al. 2005; Alqawi et al. 2006; Lang et al. 2007). In 2007, Semenza identified the receptor of activated protein kinase C

(RACK1) as an HIF-1α-interacting protein that promotes PHD/VHL-independent proteasomal degradation of HIF-1α. RACK1 binds to Elongin-C and promotes ubiquitination of HIF-1α. Elongin-C-binding sites in RACK1 and VHL show significant similarity. HSP90 competes with RACK1 for binding to HIF-1α to maintain stabilization (Liu et al. 2007). We did not investigate this mechanism in depth, but we confirmed that the growth and proliferation of NPCs under physiological hypoxia need HSP90 activity as the same manner as tumor cells. Compared with β-actin we found that the amount of HIF-1α protein was markedly decreased after suppression of HSP90 activity, and that the HIF-1 pathway is important for NPCs proliferation.

GA was a widely applied drug in anticancer research which belongs to the benzoquinone ansamycin family. GA specifically inhibits HSP90 activity and influences tumor growth (Janin 2005). Although suppression of HSP90 activity by GA inhibited the proliferation of NPCs under hypoxia in our study, it had a similar effectiveness under normoxia. Figure 4b shows that proliferation of NPCs was repressed both under hypoxia and normoxia after treatment with GA. Compared with hypoxia, HIF-1α is expressed at low levels in NPCs under normoxia. This phenomenon could be explained by the need for HSP90 for proliferation of NPCs under normal conditions. It may be that another protein than HIF-1, is influenced by HSP90 and has an effect on cell growth. The cellular effects of GA and its derivatives such as 17AAG seem to be dependent on specific biological system studies. 17AAG has been shown to cause a morphological and functional differentiation in human breast cancer cell lines (Munster et al. 2001), whereas cytostasis and apoptosis in human colon adenocarcinoma cell lines were observed (Hostein et al. 2001). It is also evident that inhibition of HSP90 can sensitize cultured tumor cells and tumor xenografts to other forms of treatment (Bisht et al. 2003; Solit et al. 2003). Furthermore, our study shows that GA decreases the number of S-phase NPCs (Fig. 4D). de Carcer reported that HSP90 plays a major role in controlling

the metaphase-anaphase transition of HeLa cells through Polo-like kinase1 (de Carcer 2004). Geldanamycin treatment also led to superoxide production and a change in membrane fluidity (Sreedhar et al. 2003). However, in this study, the fact that demolition of the HIF-1 pathway had a negative effect on cell proliferation is positive. In our previous study, knockdown of HIF-1 α by RNAi decreased the proliferation of NPCs induced by hypoxia and overexpression of HIF-1 α significantly increased proliferation of NPCs under normoxia (Zhao et al. 2008). In fact, HIF-1 α remained at low level under normoxia. Mice that lacked the HIF-1 gene did not survive (Iyer et al. 1998), which indicated that HIF-1 may have an effect on cell growth under both normoxia and hypoxia. In addition, the proliferation of NPCs was promoted by hypoxia compared to normoxia because of the effect of HIF-1 and the expression of its target genes. Thus, the loss of the HIF-1 pathway after suppression of HSP90 activity is an important reason for the inhibition of NPCs proliferation under hypoxia. HIF-1 regulates the expression of more than 50 different genes to assist cells or animals in their adaptation and survival (Semenza 2000). In this study, we verified that reduction of the amount of HIF-1 α by GA could decrease expression of its target genes such as VEGF and EPO. Beside HIF-1 α , HSP90 activity was also important to other proteins which influence cell growth, and influenced the proliferation of NPCs under normoxia. Taken together, our study indicated that suppression of HSP90 activity could disrupt the HIF-1 signal pathway and inhibit the proliferation of NPCs under hypoxia. But the regulation of HSP90 to other proteins and their effect on cell growth needs further research.

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