Regional Immunoreactivity of Pax6 in the Neurogenic Zone After Chronic Prenatal Hypoxia

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Abstract. *Background/Aim: Neurogenesis is a complex process to generate new neurons from neural progenitor cells. Neural progenitor cells are observed in two principal neurogenic regions of the forebrain, the subventricular zone and the subgranular zone of the hippocampal dentate gyrus. The cerebral cortex also plays a role as the neurogenic zone under hypoxic conditions. Hypoxia has many effects on neurogenesis, but the effect of chronic prenatal hypoxia on paired box 6 (Pax6), a protein that plays an important role in neurogenesis, has not been studied in vivo. In the present study, we used a rat model to evaluate the effect of hypoxia on Pax6 immunoreactivity. Materials and Methods: Hypoxia status was induced by unilateral uterine-artery ligation in pregnant rats. The fetuses were obtained from the uterine horn on the twenty-first day of pregnancy and immunohistochemistry of the fetal brain was examined regarding anti-hypoxia-induced factor 1α and Pax6 antibody. Results: The density of HIF1α-IR cells in the hypoxia group was greater than the density of HIF1α-IR cells in the control group in the subventricular zone, subgranular zone, and cerebral cortex. The density of Pax6-IR cells in the hypoxic group was higher in both the subventricular zone and the subgranular zone than in the control group. However, the density of Pax6-IR cells in the cerebral cortex was lower in fetuses that experienced hypoxia than in control fetuses. Conclusion: These results suggest that Pax6 immunoreactivity showed diverse patterns in the neurogenic zone after prenatal hypoxia and Pax6 has important effects on neurogenesis.*

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Neurogenesis is a complex process by which new neurons are generated from neural progenitor cells. Neural progenitor cells exist in two principal neurogenic regions of the forebrain, the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampal dentate gyrus (1, 2). Multipotent neural progenitor cells in those regions can give rise to neurons, astrocytes, and oligodendrocytes (3). Progenitor cells in the SGZ of the dentate gyrus proliferate and migrate to the dentate gyrus to differentiate into neuron cells (4). Neural stem cells located in the SVZ move to the olfactory bulb and cortex via the rostral migratory stream (RMS) (5).

The protein paired box 6 (Pax6) plays an important role in neurogenesis, affecting cell proliferation, differentiation, and survival during the development of the central nervous system (6, 7). Pax6 is expressed in early progenitor cells and has a spatiotemporal pattern that is involved in brain patterning (8, 9). Pax6 is in the paired box family of proteins and is cloned on the basis of its homology to the Drosophila gene (10). Pax6 is a conserved transcription factor containing two DNA-binding domains, a paired domain and a paired-type homeodomain (11). Pax6 was found in the nucleus of the ventricular zone cells, which are most likely radial glial cells (12). Pax6 was also observed in the embryonic neuroepithelium in the adult brain, including the SGZ and the SVZ (13).

Hypoxia has many effects on neurogenesis. Intriguingly, it appears to promote rather than repress neurogenesis. For example, after transient hypoxic injury, neurogenesis was promoted in prenatal rat brain (14). After neonatal ischemic injury, neurogenesis was triggered in the SVZ (15). However, acute hypoxic injury caused cell death and apoptosis (16). In the SVZ, neural stem cells and oligodendrocyte progenitors are susceptible to hypoxia (17). Some studies have suggested that neuronal loss by

hypoxic insults was improved by the neurotrophic factor or erythropoietin (18, 19).

Some studies have reported a correlation between Pax6 expression and hypoxic conditions (20). However, the effect of chronic prenatal hypoxia on Pax6 *in vivo* has not been studied. The present study exposed a rat model to hypoxia to evaluate the effect of hypoxia on Pax6 immunoreactivity (IR).

Materials and Methods

Animals and surgery to induce hypoxia. Sprague-Dawley (SD) rats were supplied from a certified breeder (Damul Laboratory Animals, Daejeon, Republic of Korea) and were fed ad libitum. Rats were mated and confirmed pregnant by checking the vaginal plug. Hypoxic status was created by unilateral uterine-artery ligation in pregnant SD rats, as described in a previous study (21). Briefly, the animals were anesthetized with Zoletil (10 mg/kg; Virbac, Nice, France) and xylazine (0.15 mg/kg; Bayer, Leverkusen, Germany) on the sixteenth day of pregnancy. Each rat's lower abdomen was shaved and a midline incision was performed below the umbilicus, applying aseptic technique. Uterine arteries were located in the fat pad of the uterine horns. The ligation was performed with silk sutures (4/0) on one of the uterine arteries at the cervical end of the site. After ligation, the abdomen was sutured with nylon and disinfected using povidone–iodine solution. This protocol was demonstrated in a previous study to significantly decrease uterine blood flow and fetal body weight, inducing growth retardation in the fetal rat (22). All animal experiments were performed according to the guidelines of Chosun University Institutional Animal Care and Use Committee.

Tissue preparation. The animals were sacrificed on the twenty-first day of pregnancy. The fetuses were obtained from the uterine horn and post-fixed with 4% paraformaldehyde (PFA) solution. Fetuses removed from the artery ligation horn were categorized as the hypoxia group (n=10) and fetuses removed from opposite horn were categorized as control group (n=10). The fetal brains were separated from the bodies and kept in fresh 4% PFA at 4°C overnight. The cerebrums were washed with distilled water. Dehydration was performed with a series of ethanol solutions. The brains were embedded in paraffin. Sagittal sections of cerebrum were cut serially and three sections which were separated by 300 μm were selected from each animal. These sections were put on gelatin-coated slides (Fisher Scientific, USA).

Immunohistochemistry. Deparaffinized sections were washed with 0.1 M phosphate-buffer saline (PBS; pH 7.4). Slides were cooked in a microwave oven for 10 min and flooded in 0.01 M sodium citrate buffer (pH 6.0). The slides underwent a process blocking endogenous peroxidase activity with 0.3% hydrogen peroxide. After the sections were rinsed with PBS, the slides were incubated with primary antibodies overnight at 4°C: rabbit anti-hypoxia-induced factor 1α (HIF1α; 1:500, Abcam, Cambridge, UK) and rabbit polyclonal Pax6 (1:500, Abcam, Cambridge, UK). On the following day, appropriate secondary antibodies were used and the avidin-biotin-peroxidase (ABC) detection system (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA) was used to visualize immunoreactivity. Counterstain was achieved with thionin and the slides were mounted with PolyMount mounting medium (Polysciences, Warrington, PA, USA).

Quantification of IR cells. The sections were analyzed with the aid of a light microscope (BX41, Olympus) connected to a digital CCD camera. Each section was subdivided randomly into five areas in the cerebral cortex, SVZ and SGZ. The density of HIF1α- and Pax6- IR cells within a defined square region in each area were measured manually by two investigators who were blinded to the animal status.

Statistical analysis. We analyzed all data using the Statistical Package for Social Sciences (Information Analysis Systems, SPSS, USA). All measurements were compared between the hypoxia group and the control using Student's *t*-test. The level of statistical significance was set at $p<0.05$.

Results

HIF1α immunoreactivity. The density of HIF1α-IR cells in the cerebral cortex was significantly greater in the hypoxia group than in the control group (Figures 1 and 2). Similarly, the densities of HIF1α-IR cells in the SVZ and in the SGZ of the dentate gyrus, where the neurogenic zone is located, differed between the hypoxia and control groups (Figures 1 and 2).

Pax6 immunoreactivity. Interestingly, in the cerebral cortex, the density of Pax6-IR cells was significantly lower in fetuses that experienced hypoxia than in the control group (Figures 3 and 4). However, the density of Pax6-IR cells was greater in the SVZ in the hypoxia group than in the control group. The results for the SGZ and the SVZ were similar (Figures 3 and 4).

Discussion

The immunoreactivity of Pax6 was observed in three regions, the SVZ and the SGZ of the dentate gyrus and the cerebral cortex. Both the SVZ and the SGZ are neurogenic zones, as is the cerebral cortex, particularly under hypoxic conditions (23).

The density of HIF1α-IR cells was higher in all three zones, the SVZ, the SGZ, and the cerebral cortex, than the density of HIF1α-IR cells in the control. HIF1α consists of a nuclear protein complex and can bind with hypoxia-responsive enhancers (24). HIF1α exhibited striking expression during conditions of hypoxia-ischemia (25). However, HIF1α expression rapidly declined with return to normoxia (26). These findings suggested that the increasing density of HIF1α-IR cells was related to the hypoxic state of the experimental group.

In the SVZ, the density of Pax6-IR cells was greater in the hypoxic group than in the control group. One study observed that neurological loss after hypoxic injury was improved by the activation of the Wnt/β-catenin signaling pathway, which was mediated by Pax6 (27). Pax6 expression was observed in proliferating SVZ progenitors (28). Another study showed
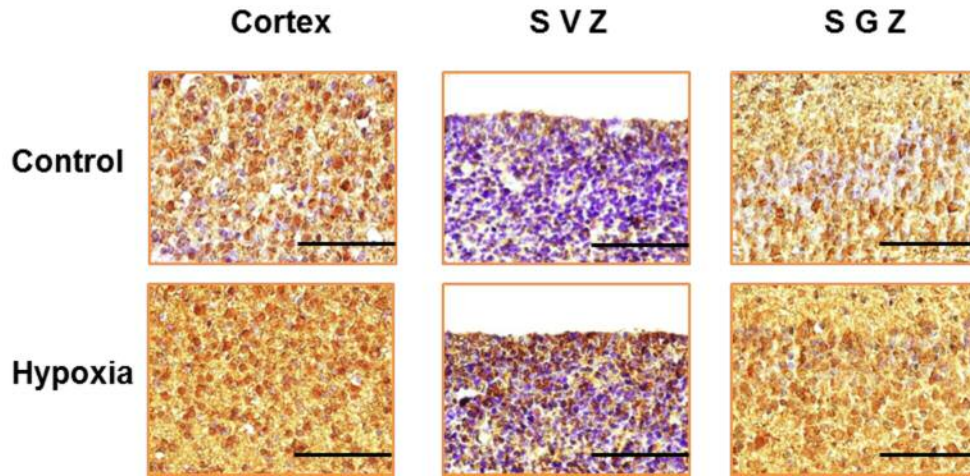
Figure 1. *HIF1α immunoreactivity (brown color) in the cerebral parietal cortex, SVZ, and SGZ. HIF1α-IR cells in the hypoxia group were more in density than in the control group. Scale bars=100 μm.*
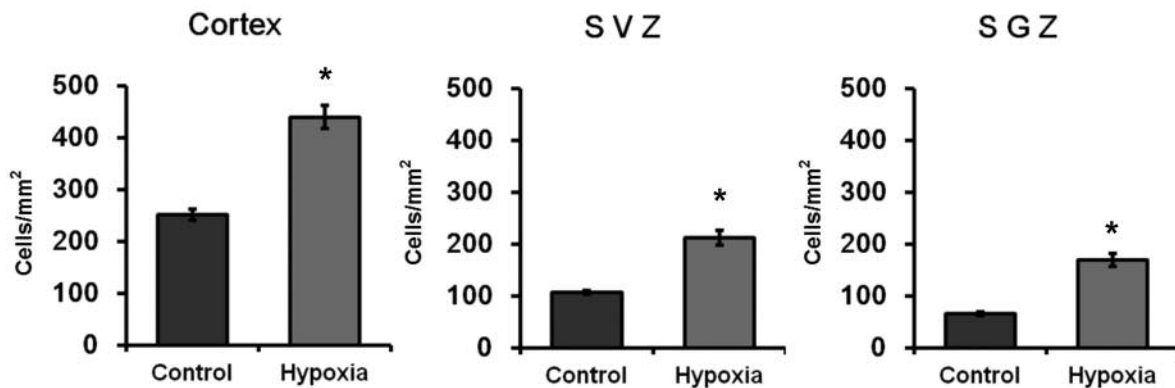

Figure 2. *Densities of HIF1α-IR cells in the cerebral cortex, SVZ, and SGZ in control and hypoxia fetuses. Density of HIF1α-IR cells was higher in hypoxia fetuses than in the controls in all three zones, the cerebral cortex, the SVZ, and the SGZ. *p<0.005.*

that hypoxic insult increased proliferation in SVZ-derived neural progenitor cell cultures and also increased Pax6 expression in SVZ tissue (29). These findings suggest that the increasing density of HIF1α-IR cells was correlated with the induction of neurogenesis after hypoxia.

In the SGZ, the density of Pax6-IR cells was also greater in the hypoxic group than in the control group. Some previous studies have determined that Pax6 plays a role in hippocampal neurogenesis. Pax6 is required to regulate the balance progenitor cell maintenance and neuronal progression in adult hippocampal neurogenesis (30). Overexpression of Pax6 led neuronal precursor cells to early maturation (31). However, there is insufficient evidence to explain the relationship between Pax6 and hypoxia in the SGZ.

In the cerebral cortex, the density of Pax6-IR cells was lower in the hypoxic group than in the control group. Low Pax6 expression was associated with disruption of cell migration, neuronal fate, and granule cell differentiation (32, 33). A previous study showed that chronic prenatal hypoxia caused cortical neuronal loss (34). Regarding migration of neural stem cells from the SVZ to the cerebral cortex, the alteration of Pax6 expression is related to survival of neuronal cells in the cerebral cortex.

Conclusion

Together, these results suggest that Pax6 immunoreactivity demonstrates diverse patterns in the neurogenic zone after
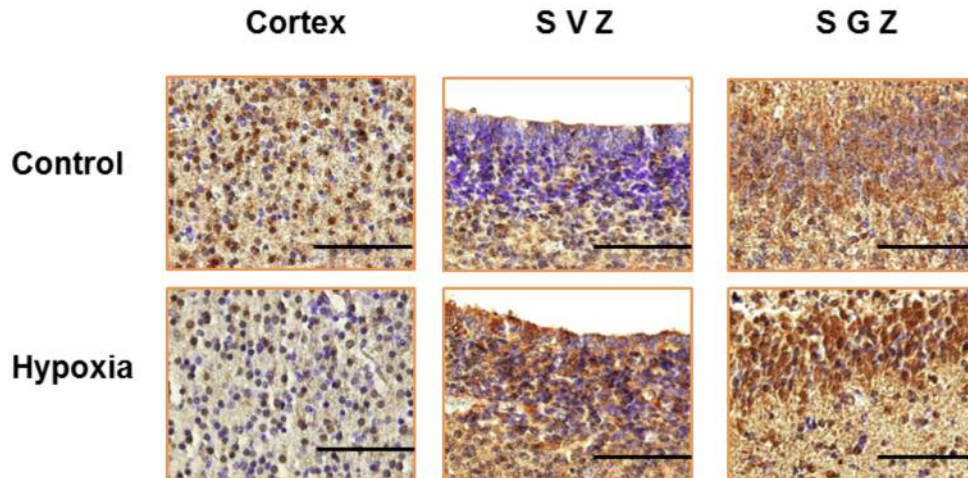
Figure 3. *Pax6 immunoreactivity (brown color) in the cerebral parietal cortex, SVZ, and SGZ. The cerebral cortex contained many Pax6-IR cells in the control group but not the hypoxia group. In the SVZ and SGZ, the Pax6-IR cells were fewer in the hypoxia group than in the control group. Scale bars=100 μm.*
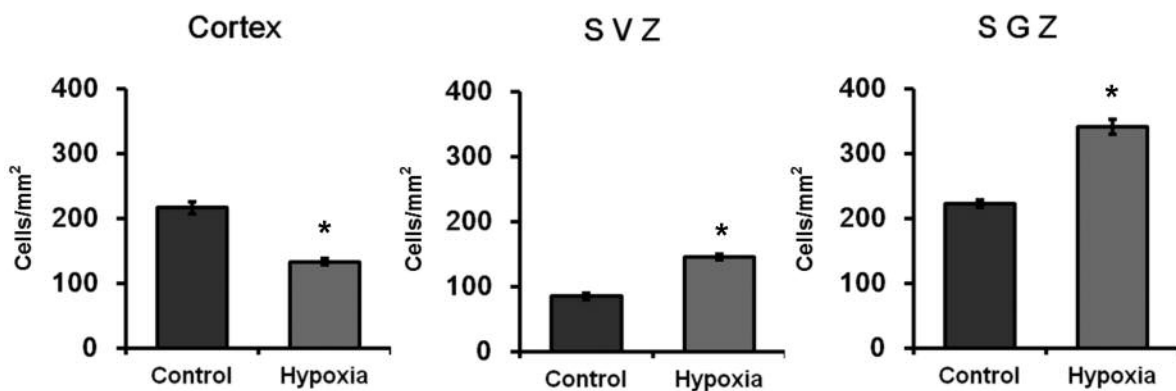

Figure 4. *Densities of Pax6-IR cells in the cerebral cortex, SVZ and SGZ. The density of Pax6-IR cells was lower in the hypoxic group than in the control group in the cerebral cortex. In contrast, in the SVZ and SGZ, the density of Pax6-IR cells was greater in the hypoxic group than in the control group. *p<0.005.*

prenatal hypoxia, due to the various effects of Pax6 on the diverse processes of neurogenesis, including cell survival.

Acknowledgements

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