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Perinatal Hypoxia-Ischemia Decreased Neuronal But Increased Cerebral Vascular Endothelial IGFBP3 Expression

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In adults, insulin-like growth factor binding protein 3 (IGFBP3) is the main carrier protein for circulating insulin-like growth factors (IGFs) (IGF-I and -II). While most IGFBP3 is synthesized in the liver, it is also expressed locally by many cell types including vascular endothelial cells. The regulation of this endothelial IGFBP3 expression, especially in response to hypoxic-ischemic injury, has not been investigated in vivo. Using *in situ* hybridization histochemistry, we studied the cellular distribution of IGFBP3 mRNA in rat brains following hypoxic-ischemic injury at 1, 5, 24, and 72 h of recovery. In normal P7 rat brain, IGFBP3 mRNA was found in neurons within the thalamus, hippocampus, and amygdaloid. Low levels of IGFBP3 mRNA were also detected in cerebral vascular endothelial cells. After the hypoxic-ischemic injury, the levels of neuronal IGFBP3 mRNA substantially decreased within 24 h in areas that were normally supplied by the middle cerebral artery. In the meantime, there was an immediate increase in IGFBP3 expression in vascular endothelial cells throughout the affected hemisphere. This vascular IGFBP3 expression was further enhanced with the highest level at 24 h of recovery whereas neuronal IGFBP3 expression was further decreased. By 72 h of recovery, IGFBP3 was no longer expressed in vascular endothelial cells. Taken together, the activation of IGFBP3 is a likely mechanism by which vascular endothelial cells respond to hypoxic-ischemic insult. In addition, increased endothelial IGFBP3 may modulate the interaction of IGFs with IGF-I receptors at the site of injury and/or act independently on endothelial cell growth.

Key Words: IGF; IGFBP3; endothelial cells; hypoxia-ischemia.

Introduction

In the circulation and extracellular compartment of adult animals, insulin-like growth factors (IGFs) are bound to a family of structurally and genetically related high-affinity IGF binding proteins (IGFBPs) *(1,2)*. These IGFBPs modulate the biological activity of IGFs by (1) regulating their renal clearance, (2) transporting them from the vascular compartment, and (3) modulating the interaction between IGFs and their cell-surface receptors. Among six IGFBPs identified so far, IGFBP3 is the main carrier protein for circulating IGFs *(1)*. Most IGFBP3 is produced in the liver, and like IGF-I, is regulated by nutrition and growth hormone (GH) at the level of posttranslational processes involving cell-surface interactions or proteolysis *(1)*. In addition, there is GH-independent, local production of IGFBP3 by cells of parenchymal and nonparenchymal origins *(3)*. Notably, IGFBP3 mRNA is expressed by vascular endothelial cells in the pulmonary and aortic artery *(4)*, liver *(5)*, kidney *(6)*, and reproductive system *(7–9)*.To date, the regulation of this endothelial IGFBP3 expression, especially in response to injury, has not been thoroughly investigated in vivo.

Endothelial cells are of critical importance in the overall response of an animal to hypoxic-ischemic injury because their integrity is essential for the passage of nutrients and oxygen, as well as growth mediators. This is especially true for the brain, an immune-privileged organ in which the tight junction of the vascular endothelial cells constitutes the blood-brain barrier. Compared to other cell types, endothelial cells are remarkably tolerant to hypoxia. Cultured vascular endothelial cells tolerate total anoxia (0%) for periods up to 5 d and severe hypoxia (3%) for periods up to several months *(10–15)*. Even under such a seemingly detrimental condition, endothelial cells continue to divide, and there is no evidence of significant cellular damage. The mechanisms underlying this hypoxia tolerance are unclear.

Previously, we investigated the response of IGF-system genes to hypoxia-ischemia using a well-established rat perinatal hypoxia-ischemia model *(16,17)*. We found a substantial decrease in the gene expression of IGF-I, IGFBP2, and IGFBP5 within the first 24 h following the hypoxic-

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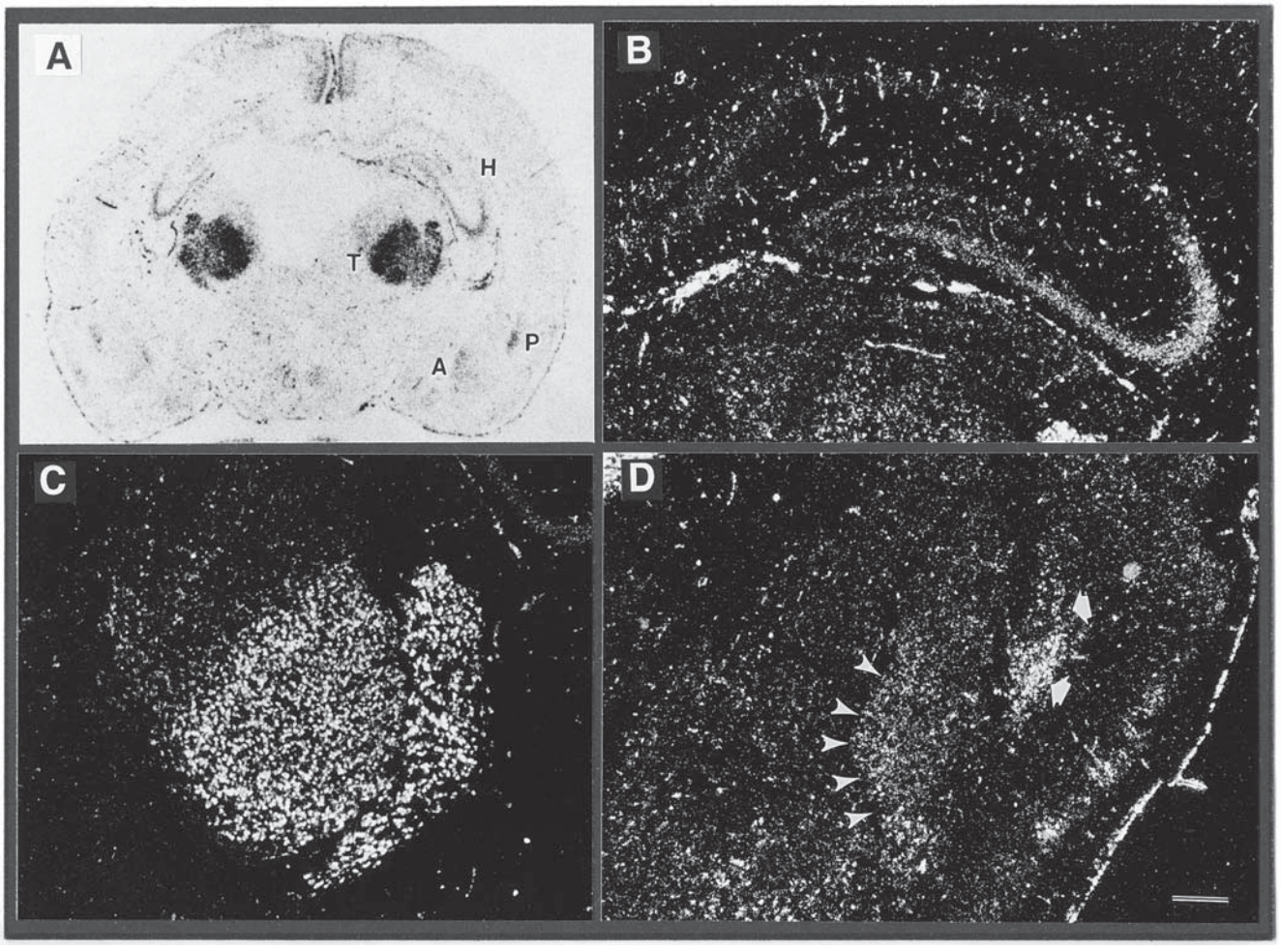

Fig. 1. Normal expression of IGFBP3 mRNA in P7 rat brain was shown by film autoradiograph **(A)**. IGFBP3 mRNA was distributed in neurons located in the thalamus (T), hippocampus (H), amygdala (A) and dorsal piriform nucleus (P). Cellular distribution of silver grain was shown by dark-field micrograph taken from the hippocampus **(B)**, thalamus **(C)**, amygdala (arrowheads in **[D]**), and dorsal piriform nucleus (arrows in **[D]**). The most abundant IGFBP3 mRNA was expressed in the thalamus **(A,C)**. In the hippocampus, IGFBP3 was distributed in every cellular layer, with the highest level found in the pyramidal cell layer and granular layer of the dentate gyrus **(B)**. IGFBP3 mRNA is also distributed in vascular endothelial cells, which is evident in dark-field (BV). Bar = 1.2 mm **(A)** and 0.28 mm **(B–D)**.

ischemic injury *(17)*. With increasing hypoxic time, the decrease in the levels of neuronal IGF-I mRNA correlated inversely with the increase in the number of apoptotic cells *(16)*. To understand the regulation of IGFBP3 gene expression in the brain, we first analyzed the cellular pattern of IGFBP3 mRNA distribution at postnatal d 7. We found that IGFBP3 was predominately expressed in specific neuronal populations that are anatomically associated with those expressing IGF-1 gene. In addition, there are low levels of IGFBP3 mRNA expression in cerebral vascular endothelial cells. We hypothesize that hypoxia-ischemia alters IGFBP3 expression, as occurs with other IGF-system genes. Accordingly, we examined the impact of the hypoxia-ischemia on neuronal and endothelial IGFBP3. To confirm the identity of vascular endothelial cells, we compared the anatomical organization of cells containing IGFBP3 mRNA with cells immunostained positively with factor VIII, a marker for vascular endothelial cells. Interestingly, we discovered that whereas hypoxia-ischemia resulted in a decrease in neuronal IGFBP3, endothelial IGFBP3 was increased. These results demonstrated a cell type–specific regulation of IGFBP3 expression by hypoxia-ischemia.

Results

Distribution of IGFBP3 mRNA in P7 Rat Brain

In normal P7 rat brain, cells containing high levels of IGFBP3 mRNA were distributed in selective areas and nuclei (Fig. 1A), including the thalamus (Fig. 1C), hippocampus (Fig. 1B), amygdala (Fig. 1D), and dorsal piriform nucleus (Fig. 1D). Based on their large size, pale cytoplasm, and distribution (Fig. 2A,C; Fig. 3D), cells that express IGFBP3 mRNA are most likely to be neurons. Since these nuclei also contain large projection neurons contain-

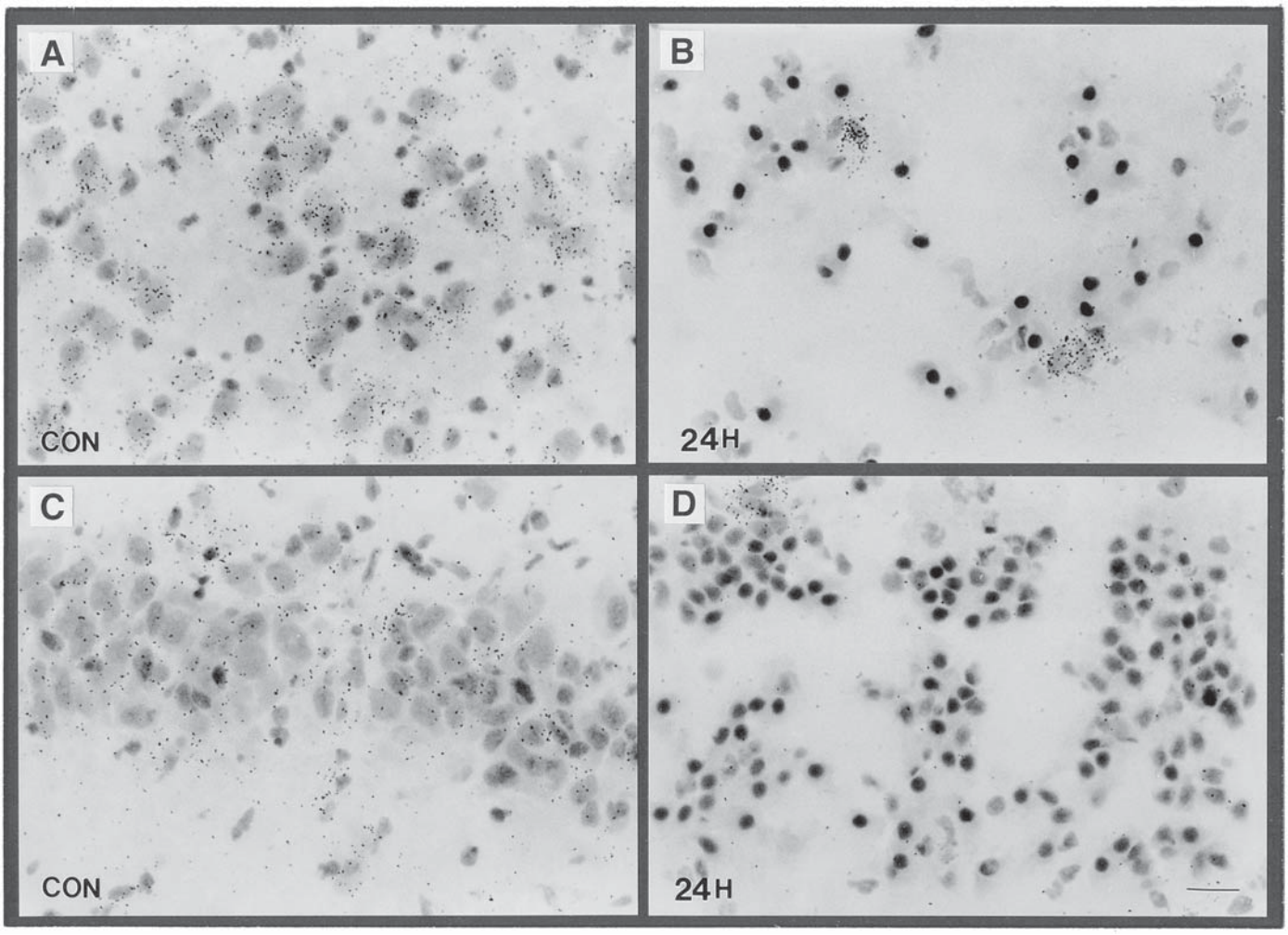

Fig. 2. Cellular distributions of IGFBP3 mRNA in the thalamus **(A,B)** and hippocampus **(C,D)** in the hemispheres either contralateral (Con) **(A,C)** or ipsilateral to the ligated carotid artery at 24 h after hypoxia-ischemia. The decrease of neuronal IGFBP3 mRNA level is most pronounced at 24 h, when cell death became most evident. Bar = 17 μm.

ing high levels of IGF-1 mRNA *(18)*, it is likely that IGFBP3 and IGF-I are coexpressed in the same group of neurons. In addition, low levels of IGFBP3 mRNA were found in vascular endothelial cells, which are evenly distributed in all brain regions with no association with specific nuclei (Figs. 1B and 3D).

Distribution of IGFBP3 mRNA in Response to Hypoxic-Ischemic Injury

Hypoxic-ischemic injury to P7 rat brain resulted in a decrease in neuronal IGFBP3 mRNA levels in the cerebral hemisphere ipsilateral to the ligated carotid artery (Fig. 2), as shown in the thalamus (Fig. 2A,B) and the hippocampus (Fig. 2C,D) at 24 h of recovery (*see* also Fig. 3B). The decrease in IGFBP3 expression persisted to at least 72 h of recovery (Fig. 3C), when the neuronal pattern completely disappeared in the ipsilateral hemisphere.

Following hypoxic-ischemic injury, the levels of IGFBP3 mRNA increased in vascular endothelial cells as early as at 1 h of recovery in the hemisphere ipsilateral to the ligated carotid artery (Fig. 3A,E). The vascular endothelial cells containing elevated IGFBP3 mRNA levels were not particularly associated with any nuclei normally containing IGFBP3-expressing neurons. Instead, abundant IGFBP3 mRNA was congregated in vascular endothelial cells within areas that were supplied by the middle cerebral artery. The identity of the vascular pattern of IGFBP3 mRNA distribution (Fig. 4A) was confirmed by comparing with the pattern of immunostaining of adjacent sections with antibody against factor VIII (Fig. 4B). This increase in vascular endothelial IGFBP3 was more pronounced by 24 h (Fig. 3B,F), but was resolved completely at 72 h (Fig. 3C,G).

Effect of the Severity of Hypoxia on IGFBP3 Expression

There was a positive correlation between the length of hypoxia and levels of IGFBP3 mRNA in vascular endothelial cells (Fig. 5). The longer the duration of hypoxia, the higher the IGFBP3 mRNA levels. The induction of IGFBP3 expression in vascular endothelial cells was already evident following 60 min of hypoxic-ischemic injury (Fig. 5A) and

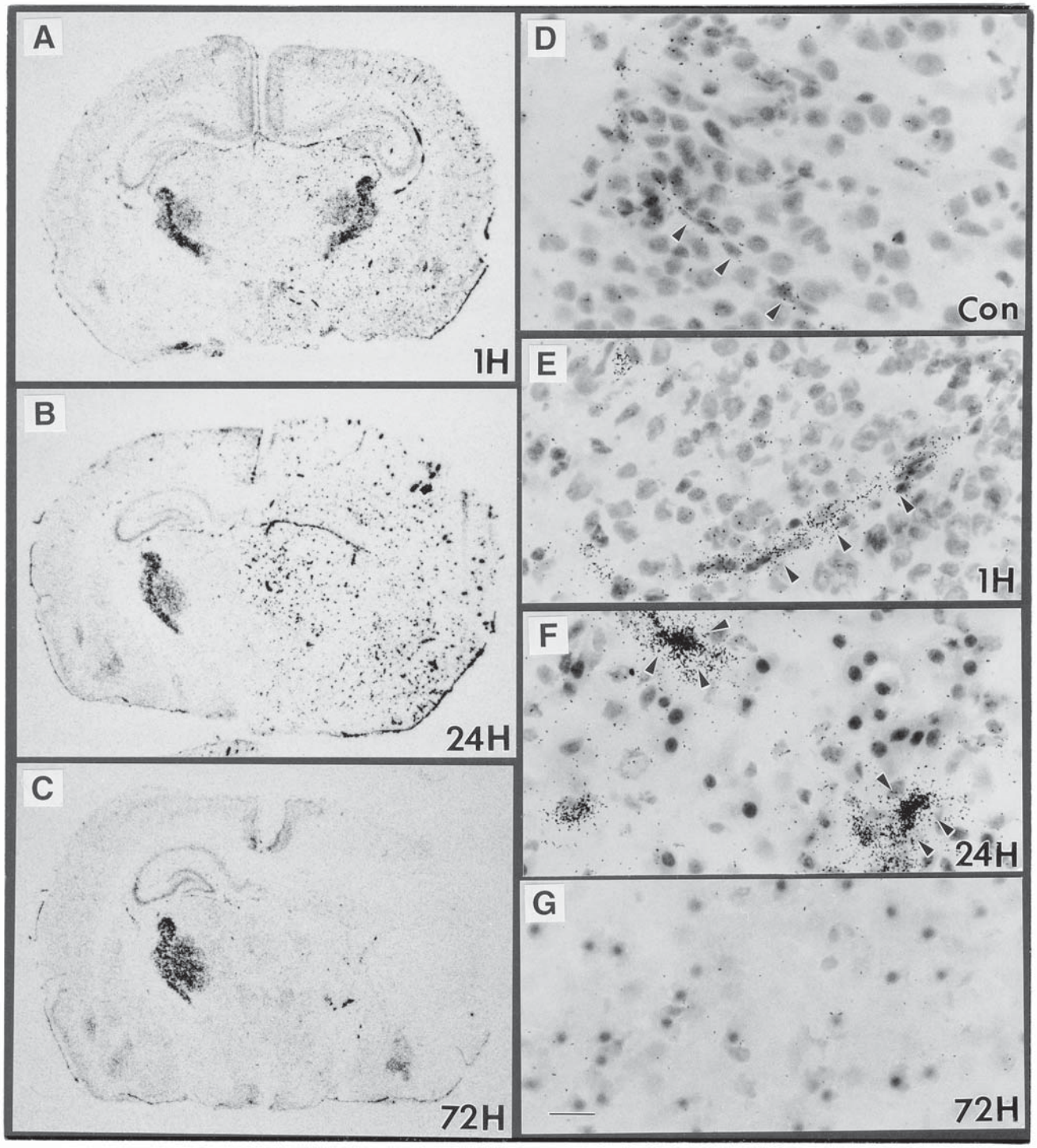

Fig. 3. The effects of hypoxia-ischemia (150 min) on the cellular distribution of IGFBP3 mRNA during the first 72-h of recovery. **(Left)** the autoradiographic changes of IGFBP3 expression at 1 **(A)**, 24 **(B)**, and 72 h **(C)**. At 1 h in the ipsilateral hemisphere, there were evenly distributed clusters of cells containing IGFBP3 mRNA. This pattern was most pronounced at 24 h (arrowheads in **[F]**) and was resolved at 72 h of recovery **(G)**. Bright-field micrograph shows that this immediate increase in IGFBP3 mRNA was mainly localized in vascular endothelial cells **(E)**. In contralateral (Con) cortex, IGFBP3 was expressed at a low level at 24-h **(D)**. Bar = 1.2 mm **(A–C)** and 17 μm **(D–G)**.

was more pronounced as the duration of hypoxia was increased up to 150 min (Fig. 5B–D). Besides vascular endothelial cells, IGFBP3 mRNA was not detected in other cell populations within the area normally supplied by the ligated middle cerebral artery at 24 h of recovery (Figs. 2B and 5D).

Discussion

The normal distribution of IGFBP3 mRNA has not been systematically investigated in relation to the development of rodent brain, nor in the context of the expression of other IGF-system genes. Both IGFs and IGFBP2–5 are expressed

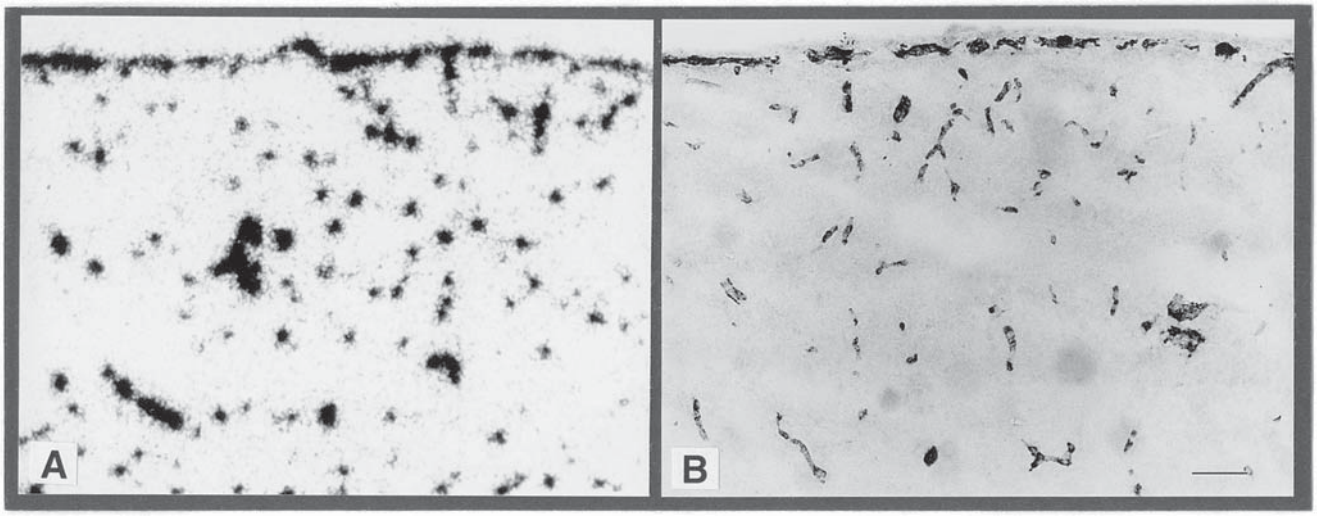

Fig. 4. Comparison of the cellular distribution of IGFBP3 mRNA and vascular endothelial cells. Immunocytochemical staining with an antibody against factor VIII was used to identify vascular endothelial cells in the cortex at 24 h after hypoxia-ischemia **(B)**. A similar cellular distribution of IGFBP3 mRNA on adjacent tissue sections is shown by film autoradiograph **(A)**. Bar = 70 μm.

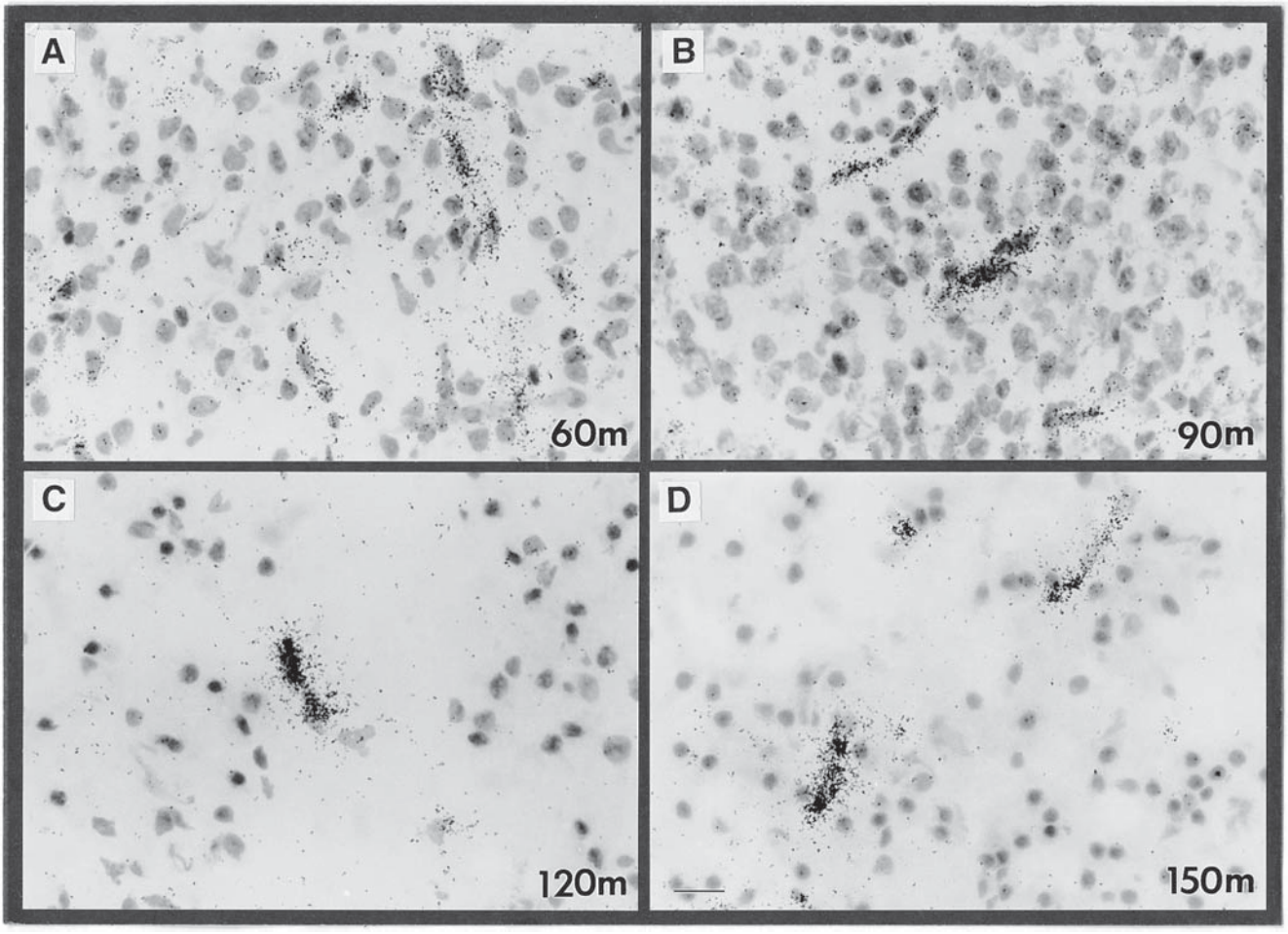

Fig. 5. Cellular distribution of IGFBP3 mRNA in ipsilateral cortex following 60 **(A)**, 90 **(B)**, 120 **(C)** or 150 **(D)** min of hypoxia at 24 h of recovery. After 60 min of hypoxia, there was an immediate increase in vascular IGFBP3 mRNA level. With the longer hypoxia time (90 and 120 min), IGFBP3 mRNA levels were further increased in vascular cells. Although 150 min of hypoxia did not further increase cellular IGFBP3 levels, there were more vascular cells expressing IGFBP3 mRNA (not shown). Bar = 17 μm.

in rodent brain. IGF-I is expressed in projection neurons of the somatosensory and cerebellar systems *(18)*, whereas IGF-II is expressed by choroid plexus, ependymal cells, and microglia *(9)*. In general, within those nuclei that contain IGF-I-expressing neurons, IGFBP2 is expressed in astrocytes *(20,21)*, and IGFBP 5 is expressed in the same neuronal population as that of IGF-I *(22)*. At postnatal d 7, we found high levels of IGFBP3 mRNA in selective nuclei such as the thalamus, hippocampus, amygdala, and dorsal piriform nucleus. These areas normally contain neurons expressing high levels of IGF-I *(18)*, IGFBP2 *(20,21)*, and IGFBP5 *(22)*. Based on morphological examination, IGFBP3 mRNA is present in large projection neurons, characteristic of those expressing IGF-I. The colocalization of IGFBP3 with other IGF-system genes, however, will need to be confirmed by double labeling of mRNA and/or proteins of IGFBP3 and IGF-I.

The neuronal localization of IGFBP3 has not been previously reported, nor has the regulation of neuronal IGFBP3 by hypoxia-ischemia. IGF-system genes are known to participate in the brain's response to hypoxic or ischemic insult in the adult *(19,23)* as well as in young animals *(17)*. However, the resulting expression of each IGF-system gene differs depending on the age of the animal and the model of the hypoxia-ischemia. At postnatal d 7, when the developing neurons are more susceptible to hypoxia-ischemia damage, neuronal IGF-I expression decreased immediately in affected areas *(17)*, whereas this decrease took much longer to detect when hypoxia-ischmia was given at postnatal d 21 *(23)*. This age difference in the response to hypoxia-ischemia was also observed in IGFBP3 gene expression. In the same model of perinatal hypoxia-ischemia, we found a decrease in neuronal IGFBP3 expression within 24 h of recovery in affected brain regions. This immediate decrease in neuronal IGFBP3 mRNA was not reported when hypoxia-ischemia was given to rats at postnatal d 21 *(23)*. Instead, an increase of IGFBP3 mRNA was described in cerebral cortex at 72 h of recovery *(23)*. Therefore, neuronal IGFBP3 is likely to respond to hypoxia-ischemia in the same fashion as IGF-I, IGFBP2, and IGFBP5 coordinately in an age- and cell type-specific manner.

Low levels of IGFBP3 were also found in vascular endothelial cells of normal d 7 rat brain, a cellular distribution consistent with that in many tissues (*see* Introduction). Most interestingly, the response of vascular endothelial IGFBP3 gene expression to hypoxia-ischemia is distinctively different from that of neuronal IGF-I. IGFBP3 mRNA levels were increased in cerebral vascular endothelial cells as early as 1 h after hypoxia-ischemia and reached the highest levels at 24 h of recovery. Apparently, this vascular IGFBP3 expression responded to lowered oxygen and glucose levels in the cerebral circulation. In cultured bovine pulmonary artery endothelial cells, but not aortic endothelial cells, persistent hypoxia increased the expression of IGFBP3, but not that of other IGFBPs *(24)*. This increase was seen at 24 h and reached maximum by 48 h after the onset of the hypoxia. In our model of perinatal hypoxia-ischemia, although hypoxia (8%) is also present, the level of hypoxia *(25)* differed from the persistent and complete hypoxia (0% oxygen) used in the cell culture study *(24)*. Since the vascular endothelial cells on the contralateral cerebral hemisphere did not demonstrate the upregulation of IGFBP3, partial hypoxia alone was not sufficient to activate the IGFBP3 transcription in vascular endothelial cells in vivo. In fact, ischemia alone is also insufficient to activate endothelial IGFBP3 expression (data not shown). Although a direct comparison between the cultured vascular endothelial cells and cerebral blood vessels is difficult, the combination of persistent ischemia and a relatively transient hypoxia is responsible for the immediate induction of IGFBP3 gene expression in vascular endothelial cells in vivo. Nevertheless, this immediate, age- and cell type–dependent induction of endothelial IGFBP3 is likely to be part of the emergency response to lowered oxygen and glucose levels in the cerebral circulation.

Whether hypoxia can directly activate IGFBP3 gene expression is not clear. In cell cultures, IGFBP3 expression can be induced by transforming growth factor-β (TGF-β) *(26)*, retinoic acid *(27)*, antiestrogens *(28)*, and the tumor suppressor p53 *(29)*. Interestingly, p53 accumulates in the nucleus during hypoxia and there is increased p53 transactivational activity *(30)*. This hypoxia-induced p53 can be stabilized by an interaction with hypoxia-inducible factor (HIF-1α), the only identified hypoxia-induced transcription factor *(31)*. It can be speculated that, in a young brain, hypoxia results in accumulation of HIF-1α, which, via stabilizing p53, activates the transcription of IGFBP3 in cerebral vascular cells. This possibility is now under investigation.

The consequence of endothelial IGFBP3 accumulation is also subject to speculation. Since neuronal, but not circulating, IGF-I decreased immediately following hypoxia-ischemia *(17)*, IGFBP3 on the surface of vascular endothelial cells may help attract IGF-I, which may stimulate angiogenesis *(32,33)*, a well-known endothelial response to hypoxia-ischemia *(34)*. On the other hand, IGFBP3 can be biologically functional independent of IGF-I. In cell cultures, IGFBP3 can inhibit growth without binding IGF-I and blocking its access to the IGF-I receptor, travel to the cell nucleus instead of remaining outside the cell, induce apoptosis, and mediate the potent growth inhibitory actions of TGF-β [Rechler, M. M. (1997) Editorial: Growth inhibition by insulin-like growth factor (IGF) binding protein-3-What's IGF got to do with it?]. How these IGF-I-independent activities relate to the function of IGFBP3 in the context of posthypoxic-ischemic response is unknown. However, results of this investigation have helped us gain new insights into the regulation and potential functions of IGFBP3 in cerebral vascular endothelial cells in combating perinatal hypoxia-ischemia.

Materials and Methods

Perinatal Hypoxia-Ischemia Model

Pregnant Wistar rats (Charles River) were housed in individual cages and fed standard laboratory chow ad libitum. Offspring were delivered vaginally, and litter size was adjusted to 10 pups/litter on the day of delivery and remained with the dam until the day of experimentation. Hypoxia-ischemia was induced in 7-d-old rat pups as previously described *(25)* with minor modifications. Twenty-eight rats were randomly divided into seven groups (four rats per group). Three groups were exposed to 60, 90, or 120 min of hypoxia, and their brains were obtained at 24 h of recovery. The other four groups were exposed to 150 min of hypoxia, and the brains in each group were obtained after either 1, 5, 24, or 72 h of recovery. Prior to exposure to hypoxia (8% O_2/92% N_2), pups were placed in a 37°C water bath for 20 min. Preliminary studies demonstrated that this period of prewarming produced a more uniform degree of damage in littermates but required a reduction in the hypoxic exposure to ensure survival. Unilateral common carotid artery ligation combined with 8% oxygen produces brain damage predominantly in the territory of the middle cerebral artery of the cerebral hemisphere ipsilateral to the carotid artery occlusion *(25)*. Damage does not usually occur in the contralateral cerebral hemisphere or in either hemisphere when the animal is exposed to arterial ligation or hypoxia alone. All rat pups were then returned to their dams for 1, 3, 5, 24, or 72 h, at which times they were killed by decapitation and the brains were removed and frozen in isopentane at –40°C and stored at –70°C until analyzed.

In Situ Hybridization

In situ hybridization histochemistry was used to examine the cellular distribution and levels of IGFBP3 gene. Rat IGFBP3 cDNA clones were used for the synthesis of cRNA probes *(35)*. cRNA probes were synthesized in a 10-μL reaction mixture containing 100 μCi each of ^{35}S-CTP and ^{35}S-UTP (Amersham, NJ); 10 m*M* NaCl; 6 m*M* $MgCl_2$; 40 m*M* Tris (pH 7.5); 2 m*M* spermidine; 10 m*M* dithiothreitol (DTT); 500 μ*M* each of unlabeled; 25 μ*M* each of unlabeled of UTP and CTP; 1 μg of linearized template; 15 U of the appropriate polymerase (Gibco, BRL, MD); and 15 U of RNasin (Promega, WI). The reaction was incubated at 42°C for 60 min, after which the DNA template was removed by digestion with DNase I. Average specific activity of probes generated by this protocol was 2 to 3 × 10^8 dpm/μg.

Prior to hybridization, tissue sections were warmed to 25°C, fixed in 4% formaldehyde, and acetylated in 0.25% acetic anhydride/0.1*M* triethanolamine hydrochloride/0.9% NaCl. Tissues were dehydrated through an ethanol gradient, delipidated in chloroform, rehydrated, and air-dried. ^{35}S-labeled cRNA probes were added to fresh hybridization buffer (10^7 cpm/mL), which was composed of 50% formamide; 0.3 *M* NaCl; 20 m*M* Tris-HCl (pH 8.0), 5 m*M* EDTA, 500 μg of tRNA/mL; 10% dextran sulfate; 10 m*M* DTT; and 0.02% each of bovine serum albumin, Ficoll, and polyvinylpyrolidone. Hybridization buffer was added to the sections, which were then covered with glass cover slips and placed in hu midified chambers overnight (14 h) at 55°C. Next slides were washed several times in 4X SS C (Sodium Citrate Buffer NaCl 15 m*M*, NaCitrate 15 m*M*) to remove cover slips and hybridization buffer. Then the slides were dehydrated and immersed in 0.3 *M* NaCl, 50% formamide, 20 m*M* Tris-HCl, and 1 m*M* EDTA at 60°C for 15 min. Sections were then treated with RNase A (20 μg/mL; Boehringer Mannheim, Indianapolis, IN) for 30 min at 37°C, and passed through graded salt solutions, followed by a 15-min wash in 0.1X SSC at 50°C. Slides were dehydrated and air-dried and apposed to Hyperfilm-beta Max (Amersham) for 2–5 d. Finally, they were dipped in Kodak NTB2 nuclear emulsion, stored with desiccant at 4°C for 6–15 d, and developed and stained with cresyl violet or hematoxylin and eosin for microscopic evaluation.

Immunocytochemistry

Immunocytochemistry was used to identify reactive astrocytes in the injured neonatal brains. A biotin-avidin-horseradish peroxidase method was used employing a monoclonal antibody against Von Willebrand Factor (Dako, Denmark), a specific marker for vascular endothelial cells. The immunocytochemistry was performed on fresh, frozen tissue sections (16 μm thick) adjacent to those used for *in situ* hybridization studies. After fixing in 4% buffered formalin and blocking in 10% normal goat serum, tissue sections were incubated with a 1:200 dilution of factor VIII antibody. Thereafter, tissue sections were treated with biotinylated horse antimouse serum (1:200) for 2 h followed by a 45-min incubation with streptavidin-peroxidase conjugate. The antigen-antibody complex was visualized by incubation with 3,3'-diaminobenzidine.

Data Analysis

In each rat brain, six representative coronal sections were taken at the following stererotaxic coordinates bregma 1.45, 2.25, 3.45, 4.85, or 9.40 mm *(36)*. *In situ* hybridization results in all brain sections were first analyzed by film autoradiograph. Since endothelial IGFBP3 mRNAs were increased in the entire ipsilateral hemisphere, we chose 4 microscopic fields from each of the five sections in both ipslateral and contralateral cortex to analyze the level of IGFBP3 mRNA (number of silver grains) in response to different lengths of hypoxia and at different time points of recovery. For the *in situ* hybridization experiment, a sense IGF-1 receptor cRNA probe was used as the control to hybridize the sections adjacent to those that were hybridized to IGFBP3, and no hybridization signals were detected.

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