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Research Article

Picroliv modulates the expression of insulin-like growth factor (IGF)-I, IGF-II and IGF-I receptor during hypoxia in rats

J. P. Gaddipati[a], H. Mani[a,b], K. K. Banaudha[a], S. K. Sharma[c], D. K. Kulshreshtha[a] and R. K. Maheshwari[a,*]

[a]Center for Combat Casualty Care and Life Sustainment Research, Department of Pathology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda (Maryland 20814, USA), Fax + 1 301 295 1640, e-mail: rmaheshwari@usuhs.mil
[b]Armed Forces Medical College, Pune (India)
[c]Central Drug Research Institute, Lucknow (India)

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Abstract. The insulin-like growth factors (IGFs), IGF-I and IGF-II, play important roles in normal growth and differentiation. In recent studies, IGFs have been implicated in tissue repair and regeneration after hypoxic-ischemic injury. The growth effects of these genes are exerted primarily through IGF-I receptor (IGF-IR). We have earlier shown that picroliv, obtained from the roots of *Picrorhiza kurrooa*, reduces cellular damage caused by hypoxia in vitro. We have now studied the modulation of IGF-I, IGF-II and IGF-IR in hypoxia and the ability of picroliv to modify their expression in vivo. Male Sprague-Dawley rats, placed in l0% oxygen for 4 days, were sacrificed, and the expression of IGF-I, IGF-II and IGF-IR was determined by immunohistochemistry, in situ hybridization and reverse transcriptase polymerase chain reaction (RT-PCR) in brain, liver and lung. One group of animals was pretreated with picroliv and the other served as control. IGF-I and IGF-IR were expressed in distinct regions of the brain but not in liver or lung. IGF-I was mainly expressed in the hippocampus and cerebellum, whereas IGF-IR expression was also observed in the cortex. A significant reduction in the messenger RNA (mRNA) level of these genes was observed in response to hypoxia. Pretreatment with picroliv not only prevented such downregulation but more importantly resulted in increased levels of IGF-I and IGF-IR. These observations correlated with reduced neuronal cell death observed in these animals. The mRNA of IGF-II was constitutively expressed and was not altered by hypoxia. Modulation of IGF-I and IGF-II expression by picroliv, a novel pharmacological agent, could benefit in similar clinical settings such as myocardial ischemia and certain cerebral injuries.

Key words. Picroliv; hypoxia; ischemia; antioxidant; insulin-like growth factor (IGF); IGF-binding protein (IGFBP).

Hypoxia and ischemia cause cell injury and lead to cell death. Several growth factors have been shown to be induced following ischemic injury of the brain, including transforming growth factor (TGF)-β1 [1], basic fibroblast growth factor (bFGF) [2], insulin like growth factor (IGF)-I [3] and members of IGF-binding proteins (IGFBP) [4]. These growth factors and others have been shown to protect neurons against excitotoxic and ischemic damage by autocrine or paracrine mechanisms [5]. Among these, IGF-I has elicited a lot of interest,

* Corresponding author.

since recombinant human IGF-1 (rhIGF-1) is now available, and it has been recently investigated as a neurotrophic factor in diseases such as traumatic brain injury, myotonic dystrophy and amyotrophic lateral sclerosis [6]. Furthermore, a role for IGF has been suggested in the rescue of CNS neurons following hypoxic-ischemic injury [3]. However, treatment with rhIGF-1 may result in various side effects, of which increased heart rate, weight gain, facial and generalized edema, papilledema, and ophthalmologic and intracranial hypertension are noteworthy [6]. Thus, there exists a need for novel therapeutic agent(s) that could enhance IGF levels without causing such significant side effects.

We have investigated one such possible agent, picroliv, a mixture of iridoid glucosides (kutkoside and picroside) obtained from the roots and rhizomes of the plant *Picrorhiza kurrooa*. Picroliv has been shown to possess antioxidant [7] and membrane-stabilizing properties [8]. We have previously shown that picroliv reduces cellular damage caused by hypoxia [9]. In the same study picroliv was also seen to enhance the expression of vascular endothelial growth factor (VEGF) in vitro in human umbilical vein endothelial cells (HUVEC) and Hep 3B cells. This compound has also been recently documented to possess protective activity in myocardial ischemia [10]. We have now analyzed the effect of picroliv on the expression of IGFs and their receptor IGF-IR in brains, lungs and livers of rats following hypoxia.

Materials and methods

Animals and treatment. The study was performed on 9–11-week-old male Sprague-Dawley rats weighing 300–350 g. Animal protocols were approved by the Laboratory Animal Review Board at the Uniformed Services University of the Health Sciences. Rats were randomly divided into two groups ($n = 12$ each). One group was pretreated with an aqueous solution of picroliv (12 mg/kg) once daily for 7 days by oral lavage following the protocol of Rastogi et al. [11] and Shukla et al. [12]. The other group served as controls and was similarly handled and fed an equivalent volume of water. Half the animals from each group were exposed to hypoxia by placing the animals in an environment of 10% O_2, 90% N_2 for a period of 4 days as per the experimental protocols described by Tozzi et al. [13] and Poiani et al. [14]. The other half were controls kept under normoxic conditions, and breathed room air.

Tissue collection and slide preparation. After the treatments, rats were sacrificed using pentobarbital anesthesia, and brain, liver and lungs were surgically excised. Immediately after excision of the organs, a portion was frozen at -80 °C and another portion was fixed in 10% buffered neutral formalin. The formalin-fixed specimens were embedded in paraffin, sectioned at 3–5 μM and mounted on aminoalkylsilane-coated slides (Sigma Diagnostics, St. Louis, MO, USA). Sections from each group were stained with hematoxylin and eosin for routine histologic evaluation.

Immunohistochemistry. Immunostaining for IGF was performed using goat polyclonal anti-IGF (Santa Crutz Biotechnology, Santa Crutz, CA) by an indirect avidin-biotin immunoperoxidase technique (Quick Universal Kit, Vector Laboratories, CA) as described earlier [15]. In short, tissue sections from brain, liver and lung on slides were deparaffinized, hydrated and treated with 3% hydrogen peroxide in methanol for 10 min to remove endogenous peroxidase activity. Nonspecific staining was blocked by using the blocking serum provided in the kit, and the sections were incubated with IGF antibody for 10–16 h at 4 °C. To ascertain that the reaction of antibody was specific, sections from each set were incubated with normal serum separately. Slides were washed with phosphate-buffered saline (PBS). Biotinylated secondary antibody IgG(H + L) was added for 1 h, followed by avidin-biotin-peroxidase complex for 30 min. Diaminobenzidine (DAB) was used as substrate for peroxidase, and slides were counterstained with Harris hematoxylin.

In situ hybridization. For in situ hybridization, oligonucleotide probes for IGF-I and IGF-IR were obtained from Calbiochem (Cambridge, MA), and IGF-II probe was purchased from Chemicon International Inc. (Temecula, CA). The oligonucleotides were 3′ end-labeled using [^{35}S]dATP (NEN Life Science Products, Boston, MA) and terminal transferase (Promega, Madison, WI). The labeled probes were purified through NAP-5 columns (Pharmacia Biotech, Uppsala, Sweden). In situ hybridizations were done as previously described [16]. Briefly, tissue sections were fixed in 4% paraformaldehyde in PBS, acetylated with 0.05 M triethanolamine containing 0.25% acetic anhydride, followed by an 0.1 × SSC wash and dehydration in ethanol series. The tissue sections were prehybridized at room temperature and then hybridized with the labeled probe (2×10^6 cpm) in 100 μl of hybridization solution (4 × SSC, 1 × Denhardt's solution, 1 mM EDTA, 100 μg/ml salmon sperm DNA, 100 μg/ml yeast transfer RNA (t-RNA), 10% dextran sulfate and 50% formamide) overnight at 37 °C. In each set, a few sections were pretreated with RNAase for 30 min at 37 °C before hybridization and served as controls. After the hybridization, sections were rinsed with 2 × SSC followed by a series of washes in SSC to a final stringency of 40 °C in 0.1 × SSC. After air drying, the sections were exposed to Kodak NTB2 autoradiography emulsion for 7–10 days at 4 °C and were then processed with Kodak D19 Developer and Kodak rapid fixer. After thorough rinsing in water, they were counterstained with Harris hematoxylin and were mounted with permount.

Messenger RNA analysis by RT-PCR. Total RNA was prepared from the frozen tissues using Trizol method (Life Technologies, Gaithersburg, MD). PCR primer oligonucleotides for IGF-1, IGF-II and IGF-IR were purchased from Chemicon International, Temecula, CA. Complementary DNA was synthesized using 1–2 µg of total RNA and Superscript II RNase H-reverse transcriptase (Life Technologies, Gaithersburg, MD) following the manufacturer's instructions. The PCR reaction mixture contained 10 pmol of each primer pair and 2.5 U of Taq DNA polymerase (Life Technologies, Gaithersburg, MD). Amplification reactions were carried out through 25–30 cycles (95 °C, 30 s; 55 °C, 45 s; 72 °C, 45 s), using 10% of cDNA except for IGF-II, which contained 35–40 cycles. PCR products were analyzed by electrophoresis on 2% agarose gels. Densitometric analysis of the PCR products (10 µl each) was performed with Instant Imager (Packard, Meriden, CT).

Results

We studied the expression and localization of IGF-I, IGF-II and IGF-IR in brains, livers and lungs of animals subjected to 4 days of normoxia or hypoxia with or without prior picroliv treatment. IGF-I and IGF-IR expression was detected in the brain but not in the liver or lung tissue samples. Hypoxia resulted in changes in the expression pattern of these genes. These changes were largely prevented by picroliv. Picroliv treatment per se did not cause any alterations in the animals not subjected to hypoxia (data not shown). IGF-II signal intensity on in situ hybridization was at a very low level in all the tissues. RNase-pretreated sections did not show any positive signal confirming the specificity of the probes.

Histologic evaluation. Evaluation of multiple H&E-stained sections from the various regions of the livers and lungs did not show any alterations following hypoxia. Brain sections showed significant neuronal loss in the form of necrosis in the hippocampus, most evident in the CA-1 sector. However, hippocampus in the picroliv-treated rats was well preserved following hypoxia (fig. 1).

Gene expression of IGF-I. In situ hybridization showed that IGF-I is expressed in distinct regions of the normoxic rat brain. The expression was detected mainly in the hippocampus and cerebellum (fig. 2). After 4 days of hypoxia, a significant decrease was observed in the IGF-I mRNA in these regions of the brain as compared with the normoxic controls. Picroliv treatment reduced this downregulation, and IGF-I mRNA levels were higher than the control animals. The protective effect of picroliv was greater in the cerebellum. Immunohistochemistry yielded similar results, thereby showing that the Purkinje cells from normoxic and picroliv-treated hypoxic animals have comparable levels of IGF-I protein, whereas in hypoxic animals it was not present at detectable levels (fig. 3). The mRNA expression patterns analyzed by RT-PCR are in agreement with the above observations (fig. 6). The quantitations from the densitometric analyses of PCR products indicate approximately 2- to 2.5-fold increase of IGF-I in the brain of picroliv-pretreated hypoxic animals compared with vehicle-treated hypoxic animals. At the same time, IGF-I levels did not significantly differ in picroliv-treated and control animals, not subjected to hypoxia.

IGF-II gene expression. Unlike IGF-I, IGF-II was constitutively expressed at uniform basal levels in all tissues without any specific regional variation, and either hypoxia or picroliv did not alter its expression. Both in situ hybridization and RT-PCR methods indicated a low level of IGF-II expression. RT-PCR products of IGF-II were visualized on agarose gels by ethidium bromide staining only after 35–40 amplification cycles (fig. 6), whereas 25–30 cycles were sufficient for visualizing amplification products for other genes.

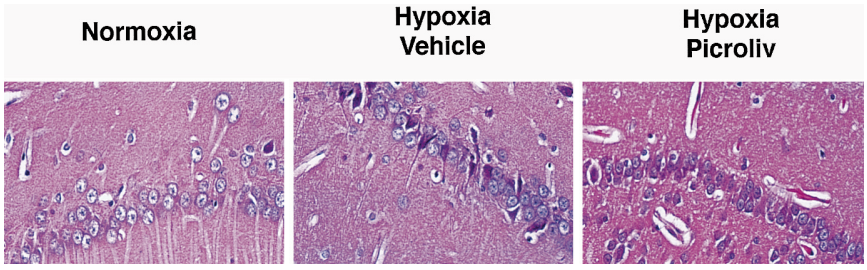

Figure 1. H&E-stained sections of hippocampus. Neurons in the CA 1 region of the hippocampus underwent apoptosis after rats were exposed to 4 days of hypoxia. Pretreatment with picroliv prevented this apoptosis.

IGF-I receptor mRNA. IGF-IR mRNA expression was observed in the hippocampus, cortex and cerebellum in normoxic rats. In response to hypoxia, a significant reduction in the intensity of signal was seen (figs 4 and 5). Picroliv treatment, however, prevented the downregulation of receptor expression, and expression was greater than controls in some regions of the brain such as the cortex (fig. 4) and the hippocampus (fig. 5), especially so in the dentate region. RT-PCR analysis of IGF-IR mRNA (fig. 6) showed similar results, and the quantitations indicate that picroliv pretreatment causes approximately two fold increase compared with controls, subjected to hypoxia. Like IGF-I and IGF-II, no significant differences were observed in the IGF-IR levels in animals not subjected to hypoxia (table 1).

Discussion

IGFs have important roles, not only in normal growth and differentiation, but also in maintenance of peak function of nervous, musculoskeletal and reproductive systems in the adult and aging individual. IGFs act as endogenous neuroprotective agents and have the ability to inhibit inappropriate induction of neuronal apoptosis [17]. Also, IGFs continue to be produced in discrete areas in the adult brain. Numerous studies, in vitro and in vivo, have led to the hypothesis that, in the adult brain, IGF-I acts not only as a trophic factor but also as a neuromodulator of higher brain functions [18]. Therefore, IGFs could serve as important targets for prevention of neuronal injury.

We have seen tissue-specific changes in the expression of IGF-I, IGF-II and IGF-IR following hypoxia. We also

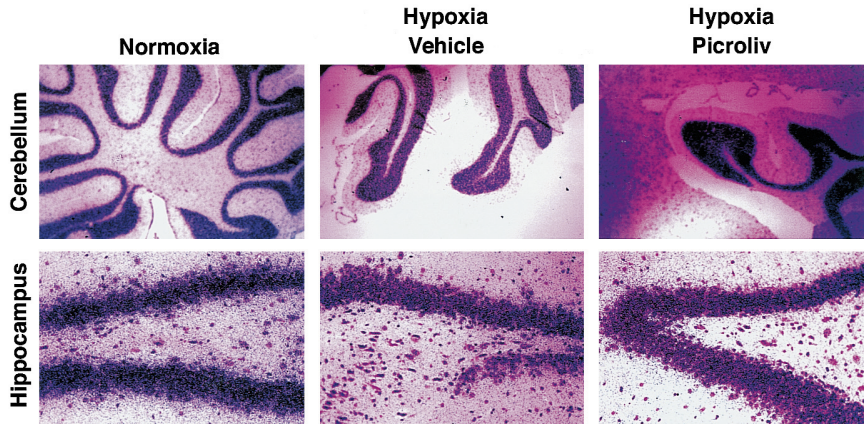

Figure 2. In situ hybridization for IGF-I mRNA in cerebellum and hippocampus of rat brains. IGF-I mRNA levels are reduced in hypoxic rat brain. Brain from picroliv-pretreated animals shows signals comparable to levels in normoxia.

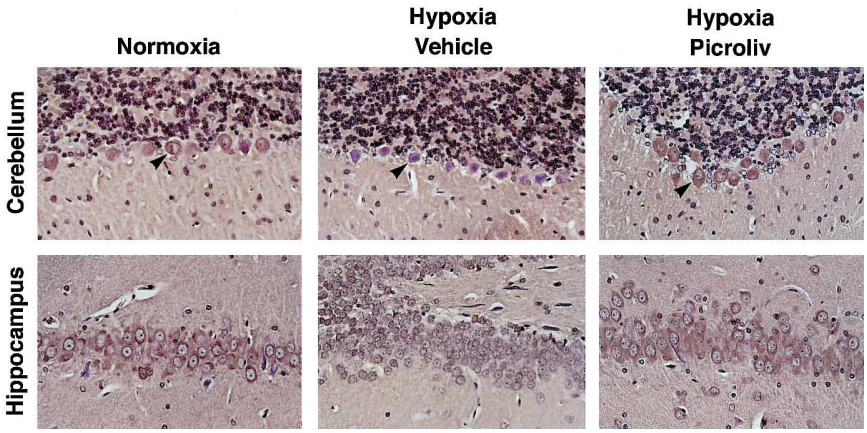

Figure 3. Immunohistochemical analysis of IGF-I in cerebellum. IGF-I protein levels are reduced in the Purkinje cells following hypoxia (arrowheads). Sections from picroliv-pretreated animals show positive staining similar to normoxic controls.

observed the beneficial effects of picroliv pretreatment in that it prevented neuronal death in the hippocampal neurons and reversed the changes in IGF-I and IGF-IR gene expression caused by hypoxia in brains of adult rats. IGF-II mRNA levels, however, remained insensitive toward hypoxia. Further, the expression of both IGF-I and IGF-IR was limited to the brain and was not found in the livers or lungs of the same animals. This is in contrast to the findings of Moromisato et al. [19], who found a significant increase in IGF-I mRNA in the heart and lung of newborn rats exposed to hypoxia (12% O_2) for 7 days, and increased IGF-IR mRNA in the lungs of these litters. However, it could well be that the baseline expression of IGFs itself was elevated in these tissues since the lungs are still developing during the neonatal period and are, therefore, more sensitive to changes in the oxygen concentration of breathed air.

Several observations suggest that IGF-I contributes to regulation of events during brain development and is a neurotrophic factor that supports survival of neurons [20]. IGF-I promotes neuronal precursor differentiation, and in the absence of IGF-I, cultured stem cell progeny do not differentiate into neurons [21]. The neuroprotective effect of IGF-I has been documented in vitro [22, 23]. IGF-I has also been evaluated in settings of hypoxia and ischemia. The IGF system is extremely sensitive to metabolic perturbations associated with cerebral hypoxia and ischemia. An immediate decrease

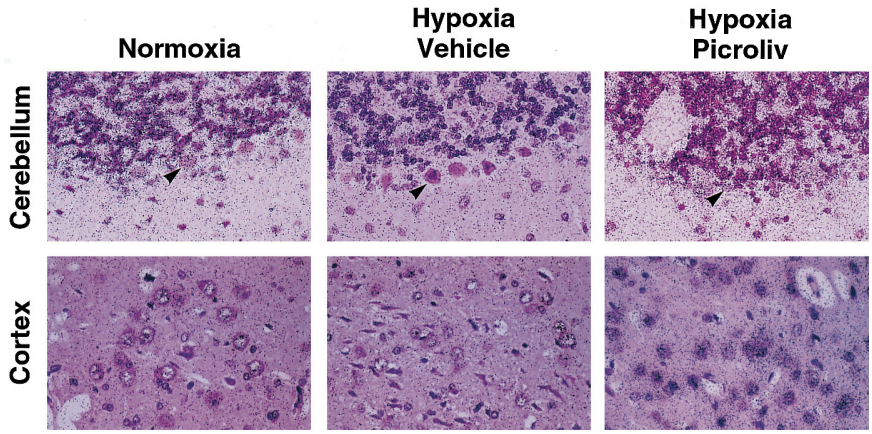

Figure 4. In situ hybridization for IGF-I receptor mRNA in cerebellum and cortex of rat brain. Hypoxia led to a reduction in IGF-I receptor mRNA levels. Following picroliv pretreatment mRNA levels appear to be even higher than in the normoxic controls, especially in the cortex. (Arrowheads point to Purkinje cells of the cerebellum.)

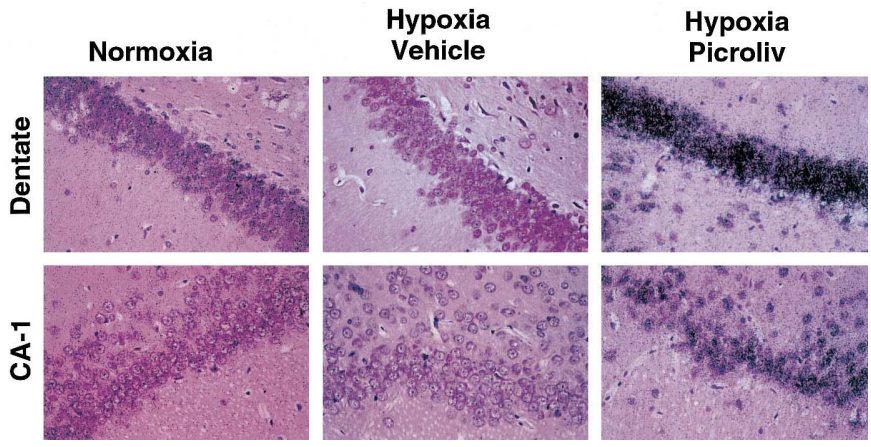

Figure 5. In situ hybridization for IGF-I receptor mRNA in dentate and CA-1 regions of hippocampus. Hypoxia caused to a reduction in IGF-I receptor mRNA levels. Levels are well maintained following picroliv pretreatment with greater signal intensity than in the normoxic controls.

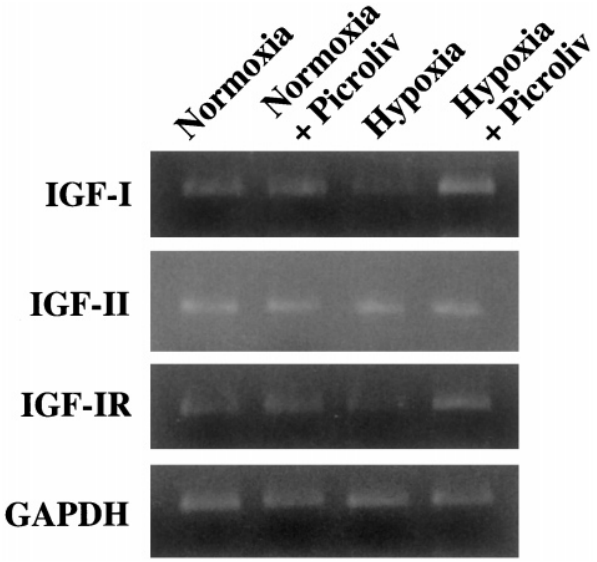

Figure 6. RT-PCR analysis of IGF-I, IGF-II and IGF-IR mRNA expressed in brain tissues. Pretreatment with picroliv during normoxia showed no changes in their expression. However, in hypoxic animals picroliv not only prevented the downregulation caused by hypoxia but resulted in increased levels of IGF-I (2–2.5-fold) and IGF-IR (2-fold) mRNAs. No significant changes were observed in IGF-II levels. Equal amounts of RNA in the RT-PCR reaction were checked by analyzing glyceryl-3-phosphate-dehydrogenase (GAPDH) expression.

in IGF-I gene expression may be partially responsible for impending neuronal death and selective vulnerability, especially in vulnerable areas such as the hippocampus [24]. Induction of hypoxia in fetal sheep decreased the plasma IGF-I concentrations, suggesting that IGF-I is important for normal fetal growth [25]. Neuronal protection was demonstrated [26] by supplementation with IGF-I after hypoxic or ischemic injury. Intraventricular injection of IGF-I after hypoxic or ischemic injury reduced neuronal loss [27]. In studies involving neonatal rats exposed to hypoxia or ischemia [24], a significant decrease in mRNAs of the IGF system (IGF-I, IGF-IR, IGFBP2 and IGFBP5) was seen. Similarly, transient hypoxic or ischemic injury to the cerebrum through the ligation of the carotid artery for 1 h followed by exposure to hypoxia for 90 min resulted in increased IGF-I expression 72–120 h later in the ligated side [28]. Increased expression of IGF-I mRNA has also been found in the hypertrophied right ventricle of hypoxic rats [29].

In our study, rats were subjected to a continuous period of hypoxia for a period of 4 days, in self-contained chambers that could hold enough food and water for that period of time. IGF-I expression was not altered in the lungs or liver at this point since the expression of IGF-I may be related to the sensitivities of each tissue type to hypoxia. Even within the brain, IGF-I mRNA and protein expression was maximal in the CA-1 region of the hippocampus and the Purkinje cells of the cerebellum, cell types known to be the most sensitive to ischemia. Biological responsiveness to IGFs occurs through IGF-IR, although IGF-II binds to this receptor with lower affinity than IGF-I [30]. Following hypoxia, there was a downregulation of mRNA expression, and this was also reversed by picroliv. This protective phenomenon correlated with reduced hippocampal neuronal death. Unlike IGF-I, the expression of IGF-I receptor was higher in the cerebral cortex after picroliv treatment and hypoxia, suggesting a differential regulation of IGF-I and its receptor in response to hypoxia, in the brain.

IGF-II induction has been observed after 5–7 days mainly in the infarcted cortex, only in cases of severe ischemic injury to developing rat brains [31]. Human hepatocellular carcinoma cells exposed to hypoxia showed increased IGF-II mRNA and induced VEGF expression [32]. In our experiments, IGF-II was not induced after hypoxia in any of the tissues examined.

The reduced levels of IGF-I and its receptor in hypoxic brains may be related to pathophysiological processes that occur during hypoxia. Exposure to hypoxia resulted in reduced IGF-I gene expression in cultured bovine aortic and pulmonary arterial endothelial cells [33]. At the same time, it has been shown that IGF-I increases VEGF mRNA, and that hypoxia-mediated and IGF-I-mediated increases in VEGF mRNA and

Table 1. Quantitation of the expression of IGF-I, IGF-II and IGF-IR in the brain tissue by densitometric analysis of RT-PCR products*.

Gene	Normoxia		Hypoxia	
	vehicle	picroliv	vehicle	picroliv
IGF-I	18.49 ± 90.13	17.37 ± 1.70	12.30 ± 0.84	29.51 ± 0.48†
IGF-II	12.41 ± 0.12	11.15 ± 0.09	13.23 ± 0.27	11.45 ± 0.11
IGF-IR	15.52 ± 0.27	15.74 ± 0.17	10.84 ± 0.25	23.85 ± 0.33†
GAPDH	25.45 ± 0.05	26.41 ± 0.30	29.99 ± 0.13	26.08 ± 0.11

*Values are mean $\pm$ SD of 6 animals in each group.
†$P < 0.01$ compared with hypoxia vehicle.

protein levels are additive [34]. In fact, we have earlier shown that picroliv enhances the upregulation of VEGF during hypoxia [9]. This activity of picroliv may well be mediated through IGF-I. The IGFBPs are other important components of the IGF system that modulate the biologic activity of IGF-I and IGF-II in an inhibitory or excitatory manner [35]. Different IGFBPs have been shown to be differentially regulated in various areas of the brain following recovery from hypoxic or ischemic injury, and modulation of IGF-1 action by IGFBPs might represent a key mechanism that restricts neuronal cell loss following hypoxic or ischemic brain injury [24, 36, 37]. We are presently investigating the role of picroliv, if any, in modulating the IGFBP system.

Administration of picroliv substantially helps in the restoration of IGF-I and IGF-IR levels in brains of rats. Picroliv, with its antioxidant and membrane-stabilizing properties, seems to act as a protective agent against hypoxic injury. Further studies are in progress to dissect out the mechanisms of picroliv-induced modulation of the IGF family.

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