



## DETECTION OF HYPOXIC CELLS WITH THE 2-NITROIMIDAZOLE, EF5, CORRELATES WITH EARLY REDOX CHANGES IN RAT BRAIN AFTER PERINATAL HYPOXIA-ISCHEMIA

M. BERGERON,\*†‡§¶ S. M. EVANS,‡ F. R. SHARP,\*† C. J. KOCH,§ E. M. LORD||  
 and D. M. FERRIERO\*¶

\*Department of Neurology, Neonatal Brain Disorders Laboratory, University of California at  
 San Francisco, San Francisco, CA 94143-0114, U.S.A.

†Department of Neurology, Veterans Affairs Medical Center, San Francisco, California, U.S.A.

‡School of Veterinary Medicine (Clinical Studies) and §School of Medicine (Radiation Oncology),  
 University of Pennsylvania, Philadelphia, Pennsylvania, U.S.A.

||Cancer Center, University of Rochester, Rochester, New York, U.S.A.

¶Department of Pediatrics, University of California at San Francisco, San Francisco, CA 94143-0114,  
 U.S.A.

**Abstract**—The hypoxia-dependent activation of nitroheterocyclic drugs by cellular nitroreductases leads to the formation of intracellular adducts between the drugs and cellular macromolecules. Because this covalent binding is maximal in the absence of oxygen, detection of bound adducts provides an assay for estimating the degree of cellular hypoxia *in vivo*. Using a pentafluorinated derivative of etanidazole called EF5, we studied the distribution of EF5 adducts in seven-day-old rats subjected to different treatments which decrease the level of oxygen in the brain. EF5 solution was administered intraperitoneally 30 min prior to each treatment. The effect of acute and chronic hypoxia on EF5 adduct formation (binding) was studied in the brain of newborn rats exposed to global hypoxia (8% O<sub>2</sub> for 30, 90 or 150 min) and in the brain of chronically hypoxic rat pups with congenital cardiac defects (Wistar Kyoto). The effect of combined hypoxia-ischemia was investigated in rat pups subjected to right carotid coagulation and concurrent exposure to 8% O<sub>2</sub> for 30, 90 or 150 min. Brains were frozen immediately at the end of each treatment. Using a Cy3-conjugated monoclonal mouse antibody (ELK3-51) raised against EF5 adducts, hypoxic cells within brain regions were visualized by fluorescence immunocytochemistry.

Brains from controls or vehicle-injected animals showed no EF5 binding. Notably, brains from animals which were chronically hypoxic as a result of congenital cardiac defects also showed no EF5 binding. A short exposure (30 min) to hypoxia or to combined hypoxia-ischemia resulted in increased background stain and few scattered cells with low-intensity immunostaining. Acute hypoxia exposure of at least 90–150 min, which in this age animal does not result in frank cellular damage, produced patchy areas of low- to moderate-intensity fluorescence scattered throughout the brain. In contrast, 90–150 min of hypoxia-ischemia was associated with intense immunofluorescence in the hemisphere ipsilateral to the carotid occlusion, with a pattern similar to that reported previously for the histological damage seen in this model.

This study provides a sensitive method for the evaluation of the level of oxygen depletion in brain tissue after neonatal hypoxia-ischemia, at times much earlier than any method demonstrates apoptotic or necrotic cell death. Since the level of *in vivo* formation of macromolecular adducts of EF5 depends on the degree of oxygen depletion in a tissue, intracellular EF5 binding may serve as a useful marker of regional cellular vulnerability and redox state after brain injury resulting from hypoxia-ischemia. © 1999 IBRO. Published by Elsevier Science Ltd.

**Key words:** brain, EF5, hypoxia, ischemia, neonatal, redox.

In the Vannucci model of neonatal hypoxia-ischemia (HI), rat pups are subjected to unilateral carotid occlusion with concurrent global hypoxia exposure.<sup>23</sup>

During the course of the insult, an early reduction of serum glucose and oxygen is observed.<sup>29</sup> Interestingly, despite similar reductions of glucose in both cerebral hemispheres,<sup>29,31</sup> only the hemisphere ipsilateral to the carotid occlusion is associated with subsequent histopathological damage.<sup>23,27</sup> These observations suggest that the initial decline in blood oxygen and brain glucose does not predict the degree of cellular damage seen after the insult. Since early reduction in regional cerebral blood flow<sup>24</sup>

\*\*To whom correspondence should be addressed.

**Abbreviations:** EF5, 2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)acetamide; HI, hypoxia-ischemia; NADH, reduced nicotinamide adenine dinucleotide; P, postnatal day; PBS, phosphate-buffered saline; WKY/NCR, Wistar Kyoto rat with congenital heart disease, normotensive and chronically hypoxic; WR, Wistar rat.

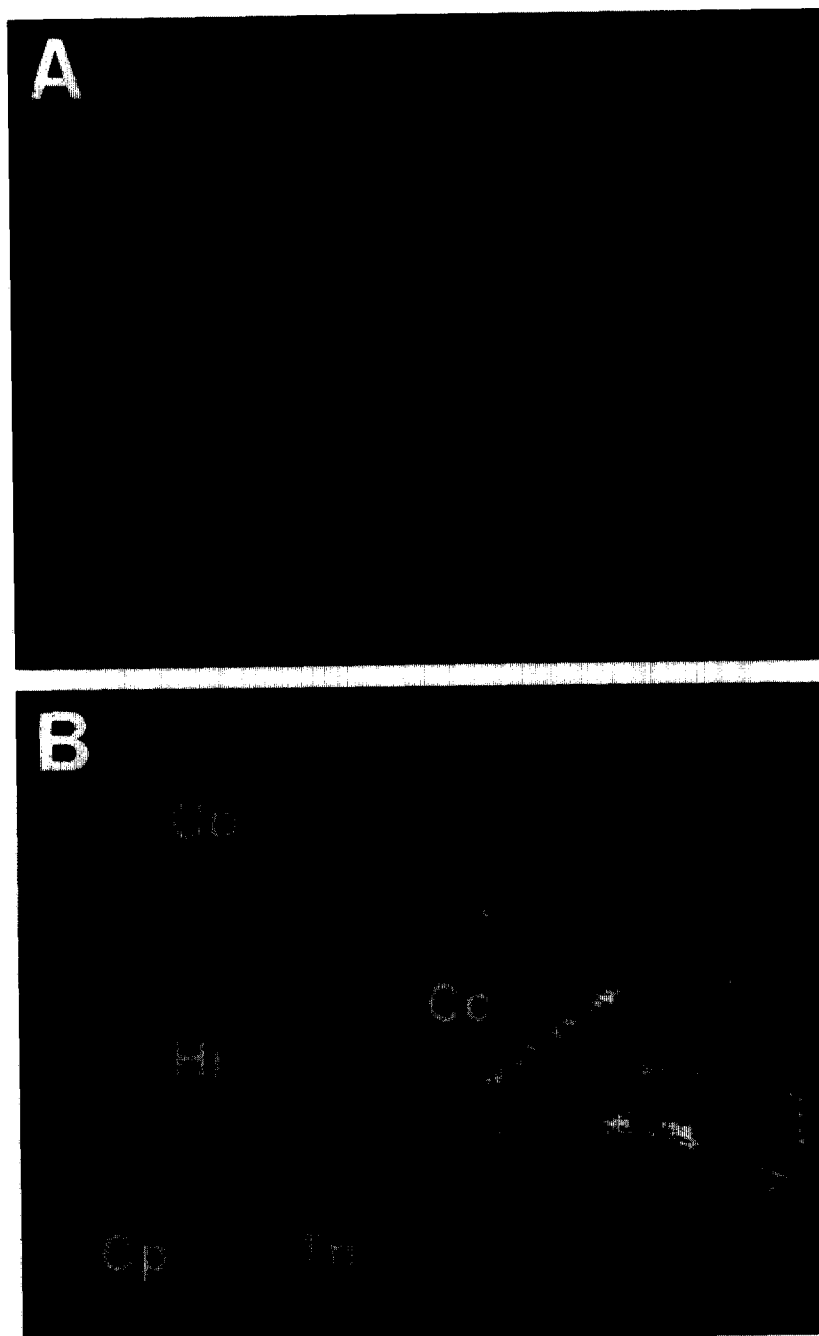


Fig. 1. Photomicrographs of 16- $\mu$ m-thick coronal sections showing the distribution profile of EF5 binding in dorsal regions of newborn P7 WR brain following 150 min of perinatal HI. (A) Brain from an EF5-injected control (normoxic) rat, showing the absence of non-specific binding and autofluorescence activity. (B) Brain from an EF5-injected rat immediately after HI. Whereas hypoxia alone (no ligation) led to a light and patchy EF5 binding pattern in the contralateral (left) hemisphere, combined hypoxia and ischemia produced a strong and diffuse EF5 binding throughout the hemisphere ipsilateral (right) to the carotid occlusion. Note the columnar pattern of EF5 binding in the ipsilateral cortex (B). Cc, corpus callosum; Co, cerebral cortex; Cp, choroid plexus; Hi, hippocampus; Th, thalamus.

and high energy phosphates have been shown in the ipsilateral but not in the contralateral hemisphere.<sup>29,31,32</sup> greater ipsilateral oxygen depletion could be responsible for consequent tissue damage and may well determine the cellular vulnerability to HI injury. To date, no study has demonstrated the

relative degree of *in vivo* oxygenation in newborn rat brain during HI. In the present study, we investigated the role of oxygen depletion as an early signal of HI brain injury and demonstrated some heterogeneity in the cell populations affected by hypoxia or HI.

To determine the relative level of brain tissue oxygenation during oxygen deprivation in newborn rats, we adapted a sensitive immunofluorescence method based on the characteristic property of nitro-heterocyclic drugs to form stable adducts with intracellular macromolecules (binding) in the absence or at low levels of oxygen.<sup>5,17</sup> These compounds have been used as markers of tissue oxygenation status in tumor cells<sup>8,17</sup> and normal tissues.<sup>25</sup> The nitroimidazole EF5, a pentafluorinated derivative of etanidazole [2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)acetamide], was used in the current study. In viable hypoxic cells but not in necrotic cells, EF5 is biochemically reduced to a product that can bind covalently to protein thiols.<sup>21</sup> This redox-dependent process is progressively inhibited at increasing oxygen tensions<sup>14</sup> and involves the cell-specific action of cellular nitroreductases such as cytochrome P-450 and cytochrome P-450 reductase.<sup>13,20,30</sup> The use of a specific monoclonal antibody recognizing macromolecular adducts of EF5<sup>17</sup> allows for their detection and localization, and thus can provide information on the relative oxygenation of a tissue at a cellular resolution. Recent studies using this antibody have shown increased EF5 binding after hypoxia exposure in multicellular tumor spheroids<sup>28</sup> and monolayer cultures<sup>11,14,17</sup> in tumor cells implanted either subcutaneously in the mouse<sup>16</sup> or on the epigastric branch of the femoral vessels in the rat.<sup>8</sup> The present study investigates the distribution and intensity of EF5 binding in hypoxic cells in the rat brain and local changes in redox state of vulnerable cells after neonatal hypoxia or HI.

## EXPERIMENTAL PROCEDURES

### Animals

All procedures were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. All efforts were made to reduce the number of animals used and to minimize animal suffering. All protocols were approved by the University of California at San Francisco Committee on Animal Research. Both male and female rats were used at postnatal day 7 (P7), since their brain maturity corresponds roughly to that of a late-term gestation human fetus or newborn infant.<sup>6</sup> Five litters each containing 10 Wistar rat (WR; Simonsen, Gilroy, CA, U.S.A.) pups at P7, and three litters each containing seven to 10 normotensive chronically hypoxemic Wistar Kyoto (WKY/NCr; Charles River, Wilmington, MA, U.S.A.) pups at P7, were used as described previously<sup>3,23</sup> with minor modifications. The rats of the inbred WKY/NCr strain spontaneously develop various combinations of heart defects, including Tetralogy of Fallot, pulmonary valve stenosis, ventricular septal defects, hypertrophic cardiomyopathy in association with hypoplasia of the ductus arteriosus and occasional anomalies in the aortic arch system.<sup>15</sup>

Rat pups received an intraperitoneal injection (0.1 ml/10 g) of either 10 mM EF5 solution (made up in 0.9% sodium chloride, pH 7.4) or an equivalent volume of vehicle solution. This low dose of EF5 (whole-body concentration of 100  $\mu$ M) was chosen since it results in a uniform body distribution within 30 min<sup>8,16</sup> and optimal detection sensitivity over a wide range of cellular oxygen levels.<sup>14,17</sup> EF5

Table 1. Effect of different duration of hypoxia and hypoxia-ischemia treatments on EF5 binding in newborn rat brain at postnatal day 7

Brain region	Duration of treatment (min)			
	0	30	90	150
<b>Cortex</b>				
Contralateral	-	-/+	+	+
Ipsilateral	-	-/+	+/+++	+++
<b>Hippocampus</b>				
Contralateral	-	-/+	++	++
Ipsilateral	-	-/+	+/+++	+++
<b>Striatum</b>				
Contralateral	-	-/+	+	+
Ipsilateral	-	-/+	+/+++	+++
<b>Thalamus-hypothalamus</b>				
Contralateral	-	-/+	+	+
Ipsilateral	-	-/+	+/+++	+++

Contralateral: hypoxia only. Ipsilateral: combined hypoxia and ischemia. Scores are based on the relative density and intensity of cells positively immunostained for EF5 adducts within each respective brain region. Scores represent a compilation of observations ( $n=4$  animals for each time-point) made by two different observers. -: no detectable cellular staining; +: occasional immunopositive cells with low- to moderate-intensity staining; ++: moderate number of cells with moderate- to high-intensity staining; +++: high cell density of moderate- to high-intensity staining.

was synthesized by Dr M. Tracy and colleagues at SRI International (Menlo Park, CA). Thirty minutes after EF5 injection, rat pups were anesthetized with a gas mixture containing 1% halothane in 30% O<sub>2</sub>/70% N<sub>2</sub>O, and underwent right common carotid artery coagulation through a ventral midline neck incision. The wound was sutured and pups returned to their dam for 1.5 h. To document any adverse effect of EF5 administration, all animals were tested behaviorally (at 30 min and 1.5 h after injection) for their ability to ambulate, nurse, right themselves and respond to physiologic stimuli (tail pinch). Pups were then placed in an 8% O<sub>2</sub>/92% N<sub>2</sub> humidified atmosphere at a constant temperature of 37°C for 30, 90 or 150 min (HI groups; coagulation, global hypoxia). Sham-operated animals underwent the same operative procedure except that the carotid artery was not ligated (hypoxia groups; no coagulation, global hypoxia). Immediately after the end of each treatment, rat pups were decapitated and their brains were quickly frozen at -70°C. Control untreated animals (no coagulation, no global hypoxia) and a group of coagulation-only animals (with no hypoxia;  $n=4$ ) were also studied. Since the latter two groups showed no difference in cell integrity and EF5 binding profile, the control animals shown in the present study refer to untreated control animals (no coagulation, no global hypoxia). In general, rat pups from each litter were divided into untreated controls (one to two per litter), hypoxia-treated (two to three per litter) and HI groups (five to six per litter).

### Detection of EF5 binding

The detection of EF5 binding was performed as described previously<sup>16</sup> with modifications. Sixteen-micrometer-thick coronal sections were cut on a cryostat, collected on to polylysine-coated slides and fixed in 4% paraformaldehyde for 1 h at 4°C. Sections were washed three times for 10 min in cold 0.05 M phosphate-buffered saline (PBS; pH 7.4) and

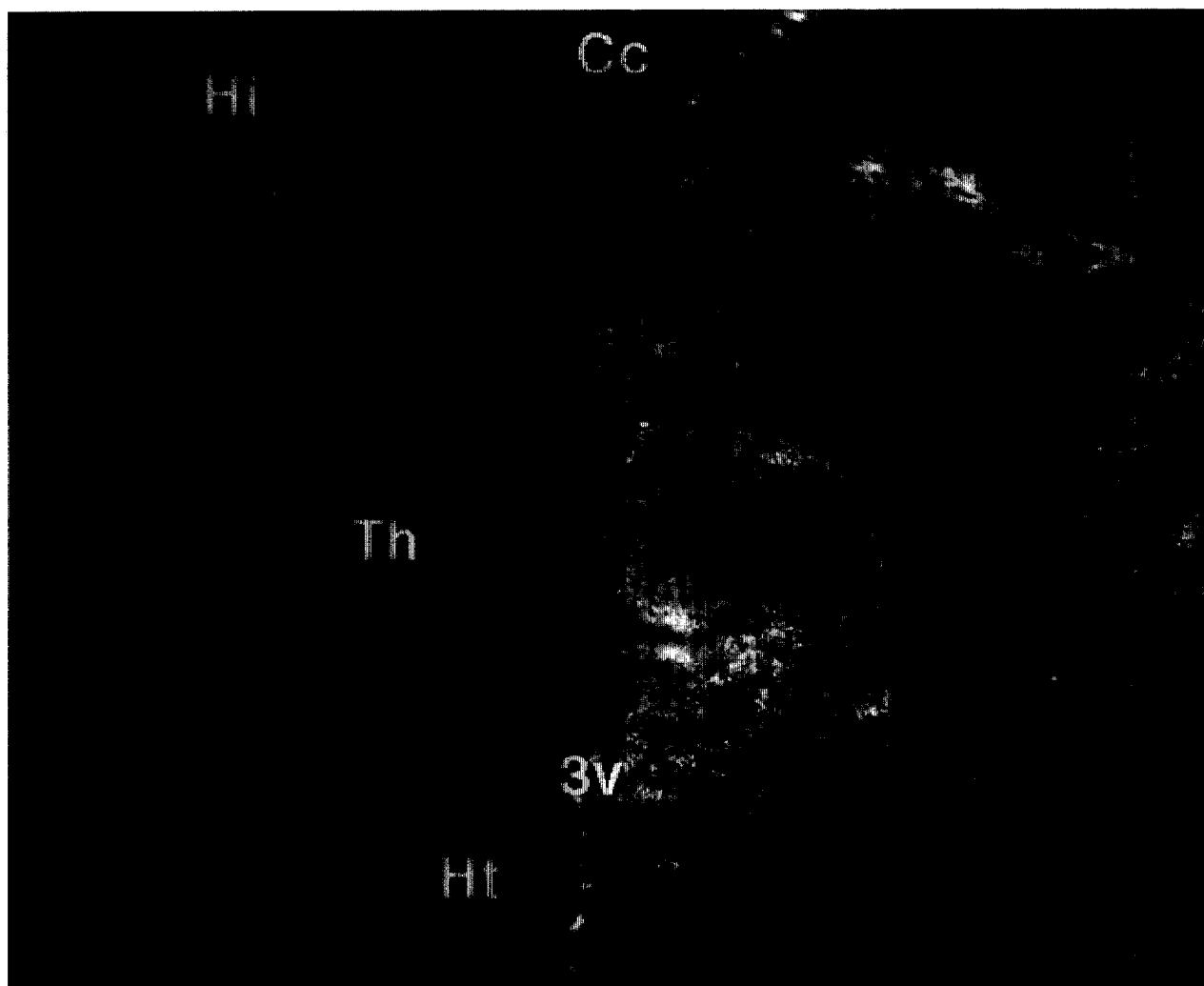


Fig. 2. Photomicrographs of 16- $\mu$ m-thick coronal sections showing the distribution profile of EF5 binding in the ventral portion of newborn P7 WR brain following 150 min of perinatal HI. Whereas acute hypoxia exposure (no ligation) resulted in scattered EF5 binding in the contralateral hemisphere (left side), combined hypoxia and ischemia produced a marked increase in EF5 binding throughout the hemisphere ipsilateral to the carotid occlusion (right side). Cc, corpus callosum; Hi, hippocampus; Ht, hypothalamus; Th, thalamus; 3v, third ventricle.

then incubated overnight at 4°C in a blocking solution (PBS containing 5% non-fat dry milk, 1.5% whole rat serum and 2.5 mM sodium azide). EF5 binding to macromolecules was determined by fluorescence immunocytochemistry. A mouse anti-rat monoclonal antibody (ELK3-51) was made against bioreductive adducts of EF5 as described previously<sup>14,17</sup> and was conjugated with the fluorescent dye Cy3. The dye:protein ratio was approximately 4:1. Sections were incubated with a 75  $\mu$ g/ml dilution of ELK3-51 monoclonal antibody in PBS containing 1.5% bovine serum albumin, 0.3% Tween-20 and 2.5 mM sodium azide in the dark for 6 h at 4°C. Sections were then washed twice for 1 h at 4°C in PBS containing 0.3% Tween-20 and 2.5 mM sodium azide, followed by a 1-h wash in PBS at 4°C in the dark. Sections were coverslipped with Fluoromount-G (Southern Biotech, Assoc., Birmingham, AL). High-power color photomicrographs were obtained with a Leitz vario-orthomat microscope using a Ploemopak 2.1 fluorescence illuminator. For low-power photomicrographs, a Nikon fluorescence microscope equipped with a  $\times 10$  Fluor objective and a tetramethyl-rhodamine filter set was used. Histopathologi-

cal examination of each newborn rat brain was performed blindly on alternate coronal sections stained with Cresyl Violet for Nissl bodies.

## RESULTS

### *Effect of EF5 on behavior and brain histology in normal rats*

There were no adverse physiological or behavioral changes in newborn rats administered EF5 compared with vehicle-injected animals. In particular, animals injected with EF5 showed intact grasping and righting reflexes, normal suckling and respiration rhythm, and normal ambulation and tail-pinch response. Brains from control rat pups (no hypoxia, no carotid occlusion) administered either EF5 or vehicle solution showed no abnormalities on Nissl staining. This is consistent with the lack of toxic effect of EF5 on

the plating efficiency in cultured cell lines,<sup>7</sup> and toxicity studies performed in rats, cats and dogs (Evans S. M., personal communication).

Immunofluorescence detection of bound macromolecular adducts of EF5 in the brain of normal newborn (P7) WR pups indicated the absence of non-specific EF5 binding and minimal autofluorescence activity of the ELK3-51 antibody (Figs 1A, 3A, 4A). Because there was no difference in fluorescence intensity between normal controls pretreated with either EF5 or vehicle solution, only brains from controls receiving EF5 are shown.

#### *EF5 binding after chronic hypoxia*

Since EF5 binding to macromolecules increases with decreasing oxygen tension,<sup>14,17</sup> we compared the effect of different *in vivo* treatments which decrease the level of oxygen in newborn rat brain. To study the effect of chronic hypoxia on EF5 macromolecular adduct formation in immature brain, P7 rat pups bearing a combination of congenital cardiac defects were used (WKY/NCr). The percentage of systemic oxygen saturation in these animals at P7 is approximately 80%.<sup>3</sup> Interestingly, chronic hypoxemia associated with congenital heart defects (WKY/NCr) had no effect on EF5 binding in all brain regions examined, including the cerebral cortex (Fig. 3B), hippocampus (Fig. 4B) and hypothalamus (Fig. 4E), compared to normoxic WR pups (Figs 3A, 4A).

#### *EF5 binding after acute hypoxia*

To study the effect of acute global hypoxia exposure on brain EF5 binding, P7 rats were subjected to 8% O<sub>2</sub>/92% N<sub>2</sub> for 30, 90 or 150 min (Table 1). Thirty-minute exposure to global hypoxia slightly increased the background stain compared with controls, but produced very little defined cellular staining (Table 1). In contrast, 90 min of acute global hypoxia produced low-intensity regions of EF5 binding scattered throughout the brain parenchyma (Table 1). A similar staining pattern was observed after 150 min of global hypoxia. More specifically, longer global hypoxia exposure ( $\geq 90$  min) resulted in scattered patches of low- to medium-intensity staining throughout the cerebral cortex (Figs 1B, 3C, E), the pyramidal cell layer of the hippocampus (Figs 1B, 2, 4C), and the thalamus and hypothalamus (Figs 1B, 2, 4F). In addition, low-intensity EF5 binding was often detected in the choroid plexus (Figs 1B, 2) and in the ependymal cells lining the ventral portion of the third ventricle (Fig. 4F; left of the midline). Similarly, in the Vannucci model, where the hemisphere contralateral to the carotid occlusion is subjected to acute hypoxia exposure only (without ischemia), the same fluorescence pattern was observed (Figs 1B, 2, 3C, E, 4C). Histopathological evaluation of alternate brain sections stained with Cresyl Violet demonstrated no detectable cell loss for all durations of hypoxia exposure. Since acute hypoxia and HI treat-

ments produced similar EF5 binding profiles in WR and WKY/NCr animals, only results from the treated WR pups are shown in Figs 1-4.

#### *EF5 binding after hypoxia-ischemia*

The effect of combined global hypoxia and ischemia was next investigated in rat pups subjected to right carotid artery coagulation with concurrent exposure to 8% O<sub>2</sub>/92% N<sub>2</sub> for 30, 90 or 150 min. As observed above after 30 min of global hypoxia treatment, 30 min of HI treatment slightly increased the background stain without producing much defined cellular staining (Table 1). In contrast, 90 min of HI treatment resulted in significantly increased EF5 binding throughout the hemisphere ipsilateral to the carotid occlusion (Table 1). A similar EF5 binding pattern was observed after 150 min of HI treatment, except that the intensity of cellular staining was slightly higher (Table 1). In the cerebral cortex of both WR (Figs 1B, 2, 3D, F, 4D, F) and WKY/NCr (not shown) rats, longer duration of HI treatment ( $\geq 90$  min) produced an intense EF5 binding, which often displayed a columnar pattern (Figs 1B, 3D). The presence of EF5 macromolecular adducts in these hypoxic-ischemic cells was found in both the cytoplasmic and nuclear compartments (Fig. 3F). Morphologically, these cells were large and round, some of which had long processes, such as those found in neurons. In the hypoxic-ischemic hippocampus, EF5 binding was most prominent in the pyramidal cell layer of the CA1-CA3 subfields of Ammon's horn and in both blades of the hilus of the dentate gyrus (Fig. 4D). Markedly increased cellular EF5 binding was also found throughout the ipsilateral hypoxic-ischemic striatum, thalamus, choroid plexus (Figs 1B, 2) and in ependymal cells lining the ventral portion of the third ventricle (Fig. 4F, right side of the midline). Vehicle-treated rat pups subjected to the different durations of HI treatment demonstrated no detectable fluorescence in either hemisphere (not shown). Although intense immunofluorescence was observed with EF5 binding immediately after 90-150 min of HI treatment, histopathological evaluation of Nissl-stained coronal sections indicated no detectable cell loss.

#### DISCUSSION

The present study is the first demonstration of regional oxygenation and redox state in brain tissue based upon immunohistochemical staining methods in newborn rats subjected to hypoxia or HI. Different patterns of EF5 binding were observed at the cellular level, depending upon the different degrees of hypoxia exposure. There was no EF5 binding observed in either WR (normoxemic) or WKY/NCr (hypoxemic) brains under normoxic conditions. This suggests that chronic hypoxemia associated with congenital heart defects (WKY/NCr) does not lead to severe oxygen depletion or difference in redox state in

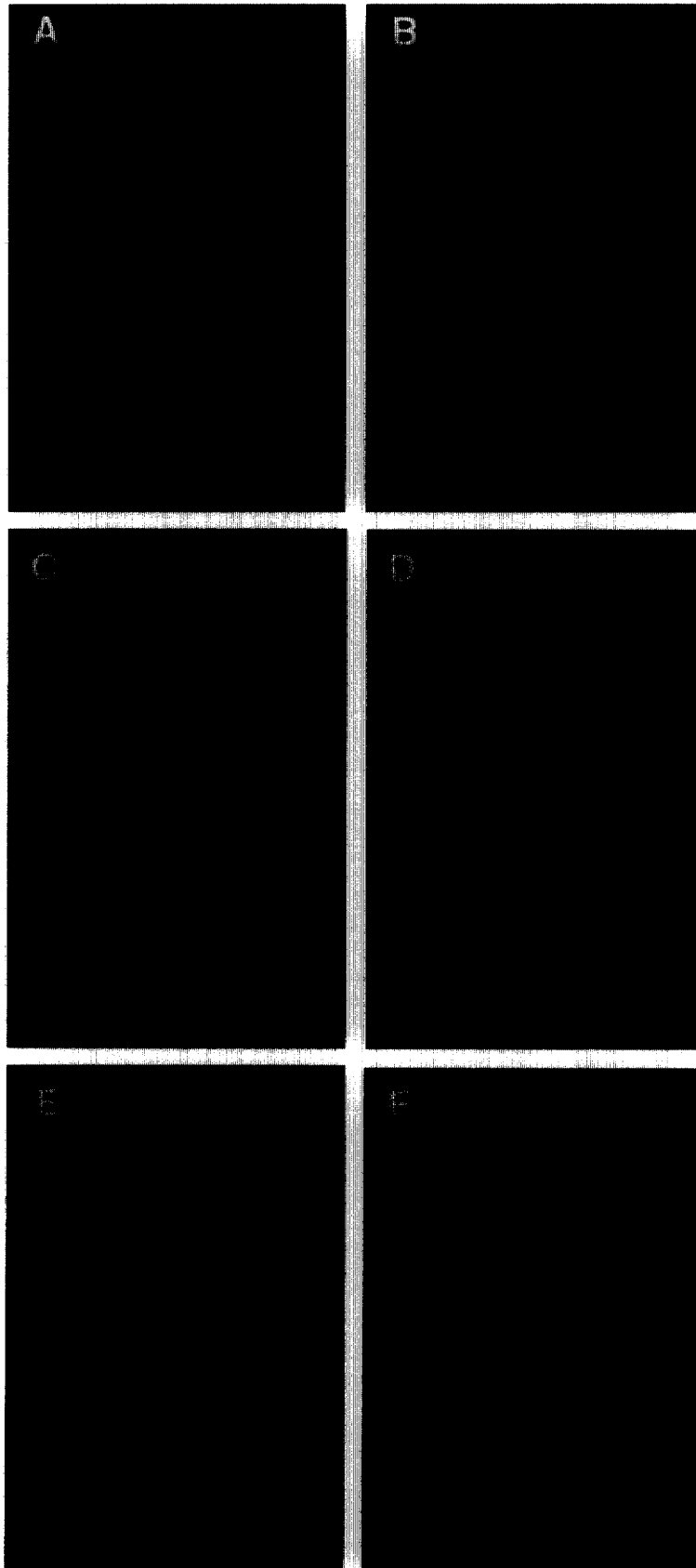


Fig. 3.

newborn rat brain, consistent with preserved tissue integrity.<sup>3</sup> It is also possible that newborn WKY/NCr rats develop compensatory mechanisms that allow adequate oxygen supply to the brain under normoxic conditions. Interestingly, whereas a short duration of acute global hypoxia (8% O<sub>2</sub>, 30 min) did not produce any significant cellular EF5 binding, longer global hypoxia exposure (8% O<sub>2</sub>, 90–150 min) produced multifocal areas of low-intensity EF5 binding in both WR and WKY/NCr strains. Although 30 min of HI treatment did not result in significant cellular EF5 binding, 90 min of HI resulted in intense and diffuse cellular EF5 binding throughout the ipsilateral hemisphere, including the cerebral cortex, hippocampus, striatum, thalamus and hypothalamus, regions known to be most susceptible to neonatal hypoxic-ischemic injury.<sup>23,27</sup> Increasing the duration of HI treatment to 150 min produced a small additional increase in the intensity of cellular staining, but the regional pattern of EF5 binding was the same as that observed after 90 min of HI treatment. These data are consistent with previous studies in cultured cell lines, showing increased EF5 binding with decreasing oxygen tensions.<sup>14,17</sup> Our observations suggest that the level of *in vivo* formation of EF5 adducts in neonatal rat brain is closely related to the oxygen partial pressure in brain tissue.

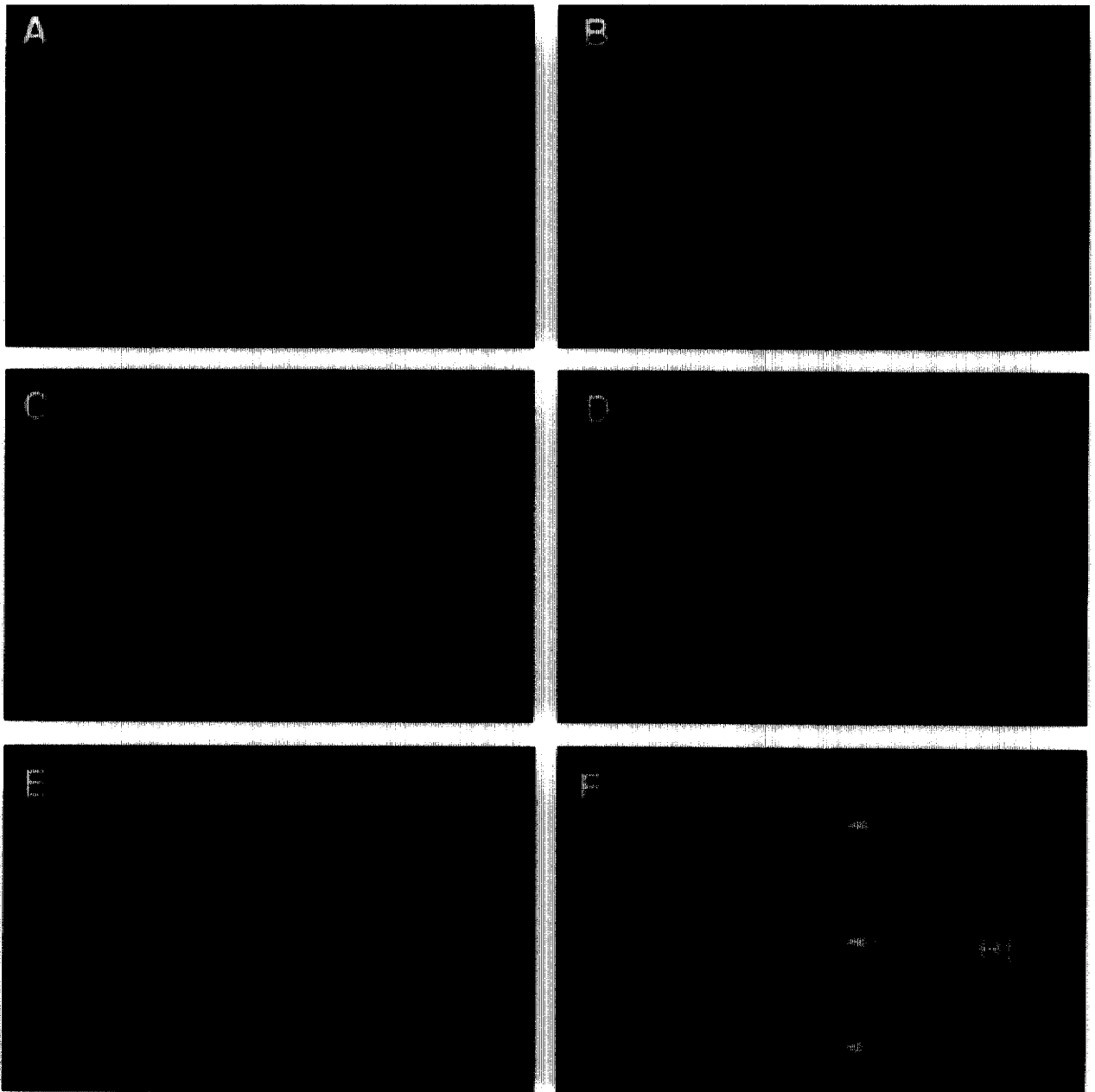
In the newborn rat brain, acute global hypoxia alone (8% O<sub>2</sub> for up to 3 h) does not lead to any evidence of cellular damage. However, ischemia produced by the combination of unilateral carotid occlusion and exposure to global hypoxia for more than 75–90 min results in subsequent brain injury.<sup>23,27</sup> Depending on the severity of the initial HI insult, evidence of delayed neuronal damage and tissue necrosis in the ischemic hemisphere usually occurs several hours after the end of hypoxic-ischemic exposure.<sup>2,12,27</sup> In the present study, interhemispheric differences in the level of EF5-bound adducts were readily detectable immediately at the end of 90 and 150 min of HI treatment, a time-point ( $T=0$  h after treatment) at which there was no observed cellular injury in both hemispheres. The previously reported observations of severe glucose depletion and unchanged high-energy phosphates in the contralateral hypoxic hemisphere,<sup>29,31</sup> and our current observations of low levels of EF5 binding in the same hemisphere, suggest that a more severe oxygen depletion in the brain is necessary to cause energy failure and cellular damage. Accordingly, the ipsi-

lateral ischemic hemisphere, which shows both severe glucose depletion after 60–120 min of HI treatment<sup>29,31</sup> and a marked increase in EF5 binding after ~90 min of HI, is associated with decreased levels of high-energy phosphates<sup>29</sup> and subsequent tissue damage.<sup>12,23,27</sup> These observations suggest that EF5 binding may serve as an early marker of cellular vulnerability to hypoxic-ischemic brain injury.

The present findings extend the previous demonstration of changes in the redox state of newborn rat brain undergoing either hypoxia or HI.<sup>29,31,32</sup> EF5 activation and subsequent binding in hypoxic cells depend on the initial action of nitroreductases, which require reducing equivalents such as NAD(P)H to function.<sup>13,30</sup> It has been shown previously that the cytoplasmic NAD<sup>+</sup>/NADH ratio decreased significantly from controls during acute hypoxia alone,<sup>31,32</sup> and we found that under similar circumstances only scattered areas of EF5 binding were detected. In contrast, the cytoplasmic NAD<sup>+</sup>/NADH ratio in the ipsilateral hemisphere, which was shown to be significantly lower than that of the contralateral hemisphere for a hypoxia exposure of at least 90 min,<sup>32</sup> is associated with significant increases in the extent and intensity of EF5 binding. These observations suggest that EF5 binding may reflect an increased level of nitroreductase activity which depends, at least in part, on the presence of a minimum threshold level of reducing equivalents in the cell. Increased activation of nitroreductases in the brain, such as the cytochrome P-450 system,<sup>19,22</sup> could be of significance during HI, since it produces highly reactive and cytotoxic metabolites, some of which may be involved in neuronal degeneration.

The final fluorescence intensity of tissue regions is an integration of the hypoxia-dependent binding which has occurred over the entire duration of an experiment. In the present study, the temporal threshold (~90 min) for eliciting a significant increase in EF5 adduct formation after HI treatment coincides with the duration of HI required to cause subsequent tissue damage and cerebral infarction in the ipsilateral hemisphere.<sup>10,23,27,32</sup> Interestingly, tissue columns without EF5 binding were observed next to columns of intense EF5 binding in the ipsilateral cerebral cortex after 90–150 min of HI treatment. This pattern is similar to that described previously for NADH fluorescence<sup>29</sup> and suggests that, during the course of HI exposure, the tissue columns where EF5 binding was not detectable never became

Fig. 3. EF5 binding in the cerebral cortex from newborn P7 WR pups and chronically hypoxic rats (WKY/NCr) after EF5 administration and 150 min of HI. (A) WR untreated control, i.e. no hypoxia, no ischemia. (B) WKY/NCr untreated control. (C) WR contralateral side (hypoxia only). (D) WR ipsilateral side (hypoxia and ischemia). (E, F) High-power photomicrographs of contralateral (E) and ipsilateral (F) cortex shown in C and D, respectively. Chronic hypoxia associated with congenital heart defects (WKY/NCr; in B) had no effect on EF5 binding in newborn rat brain compared with the normoxic WR pup (A). Since HI produced a similar EF5 binding profile in WR and WKY/NCr, only results from hypoxic-ischemic WR pups are shown (C, D). Note the columnar pattern of hypoxic cells in the ipsilateral hemisphere (D).



**Fig. 4.** EF5 binding in the hilus of the dentate gyrus (A–D), and in the hypothalamus and ependymal cells lining the third ventricle (E, F) from newborn P7 WR pups and chronically hypoxic rats (WKY/NCr) after EF5 administration and 150 min of HI. (A) WR untreated control, i.e. no hypoxia, no ischemia. (B) WKY/NCr untreated control. (C) WR contralateral side (hypoxia only). (D) WR ipsilateral side (hypoxia and ischemia). While constitutive EF5 binding was absent in the normoxic WR control (A), acute hypoxia (C) but not chronic hypoxia (WKY/NCr; in B) produced some patches of low- to medium-intensity immunoreactivity in the pyramidal cell layer (C). Combined HI produced an intense and diffuse immunostaining throughout the dentate gyrus (D) and hippocampus. Areas of the ventromedial hypothalamus and the ependymal cells lining the third ventricle are shown in E (WKY/NCr untreated control) and F (WR brain after HI), in which contralateral is on the left side of the third ventricle and ipsilateral is on the right side. In F, note the strong EF5 binding in the ipsilateral ependyma (arrows) and periventricular and ventromedial hypothalamic nuclei (Ht), compared with the faint patchy staining in the contralateral side (left from the midline).

hypoxic and/or did not reduce enough to produce a detectable signal of EF5 binding. A transition from mitochondrial reduction to oxidation concomitant with decreased cytoplasmic  $\text{NAD}^+/\text{NADH}$  ratio dur-

ing HI exposure has been shown to coincide temporally with the duration of HI (~90 min) required to convert selective neuronal necrosis into cerebral infarction.<sup>32</sup> The tissue columns without EF5 binding



could be indicative of mitochondrial oxidation counterbalancing the increased cytoplasmic reduction. However, this appears unlikely, since the cytoplasmic reduction which occurs before mitochondrial oxidation<sup>32</sup> would have produced EF5 binding. Therefore, the cortical columns of no or low-level EF5 binding may indicate areas of relatively intact oxygen supply and undisrupted cellular redox state.

Early mitochondrial failure represents a critical event in the development of irreversible ischemic neuronal damage.<sup>1</sup> Indeed, suppression of mitochondrial activity occurs well before any evidence of cellular injury in selectively vulnerable brain regions following transient ischemia in adult rats and gerbils.<sup>1,26</sup> In newborn rat brain, regional disruption of mitochondrial cytochrome c oxidase (cytochrome aa<sub>3</sub>) activity either slightly precedes or coincides with the threshold of HI necessary to elicit irreversible injury.<sup>18</sup> Nitric oxide has been shown to compete with molecular oxygen for the cytochrome c oxidase complex, resulting in its inhibition.<sup>4</sup> Because nitric oxide production increases as a consequence of energy failure and depolarization associated with ischemia,<sup>9</sup> the changes in redox state resulting from inhibition of mitochondrial cytochrome c oxidase could be responsible, at least in part, for increased EF5 binding in the ischemic hemisphere. Therefore, since normal cellular redox state depends directly on

intact mitochondrial functions, the EF5 binding assay may be useful for the early evaluation of mitochondrial integrity through changes in cellular redox state.

## CONCLUSIONS

The level of *in vivo* formation of macromolecular adducts of EF5 depends on the degree of oxygen depletion and the redox state of a tissue. Since the temporal threshold (~90 min) for eliciting a significant increase in EF5 adduct formation coincides with the duration of HI required to cause subsequent cerebral infarction, the presence of EF5 adducts in cells, at times much earlier than methods demonstrate apoptotic and necrotic cell death, may serve as an early marker of regional cellular vulnerability and disrupted redox state (abnormal mitochondrial function) after neonatal HI.

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