

Localization of specific erythropoietin binding sites in defined areas of the mouse brain

(erythropoietin receptor/oxygen-regulated gene expression/hypoxia/radioligand binding)

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ABSTRACT The main physiological regulator of erythropoiesis is the hematopoietic growth factor erythropoietin (EPO), which is primarily produced in the kidney of adult mammals and is induced in response to hypoxia. Binding of EPO to the EPO receptor (EPO-R), a member of the cytokine receptor superfamily, controls the terminal maturation of red blood cells. So far, EPO has been reported to act mainly on erythroid precursor cells. However, we have detected mRNA encoding both EPO and EPO-R in mouse brain by reverse transcription-PCR. Exposure to 0.1% carbon monoxide, a procedure that causes functional anemia, resulted in a 20-fold increase of EPO mRNA in mouse brain as quantified by competitive reverse transcription-PCR, whereas the EPO-R mRNA level was not influenced by hypoxia. Binding studies on mouse brain sections revealed defined binding sites for radiiodinated EPO in distinct brain areas. The specificity of EPO binding was assessed by homologous competition with an excess of unlabeled EPO and by using two monoclonal antibodies against human EPO, one inhibitory and the other noninhibitory for binding of EPO to EPO-R. Major EPO binding sites were observed in the hippocampus, capsula interna, cortex, and midbrain areas. Functional expression of the EPO-R and hypoxic upregulation of EPO suggest a role of EPO in the brain.

Erythropoietin (EPO) is a 30.4-kDa glycoprotein which represents the major regulator of erythropoiesis (reviewed in refs. 1–3). The main site of EPO production switches during development from the fetal liver to the adult kidney. Fibroblast-like type I interstitial cells have been identified as the EPO-producing cell population in the kidney (4, 5). In the liver, both a subset of hepatocytes and the nonparenchymal Ito cells, also known as fat-storing or perisinusoidal cells, have been reported to be the source of EPO production (6, 7). EPO gene expression is regulated in an oxygen-dependent manner: hypoxic exposure results in elevated EPO production in mammals and in the EPO-producing human hepatoma cell lines HepG2 and Hep3B (1–3). Binding of EPO to the EPO receptor (EPO-R), a member of the cytokine receptor superfamily (8), leads to enhanced red blood cell production by suppressing programmed cell death of erythroid progenitor cells (reviewed in ref. 9). So far, EPO has been shown to act mainly on erythroid cells. However, apart from its key function in erythropoiesis, EPO may also influence the functional behavior of nonerythroid cells: EPO might have mitogenic and chemotactic effects on endothelial cells (10) and fetal liver stromal cells (11), both cell types harboring the EPO-R (12, 11). Stimulation of differentiation and increased DNA synthesis in response to EPO has been observed in megakaryocytes *in vitro* (13, 14), which is consistent with functional expression of EPO-R

on those cells (15). In addition, EPO showed neurotrophic effects on cultured embryonal neurons and supported survival of injured rat neurons *in vivo* (16). Furthermore, *in vitro* expression of the EPO-R has been detected in rat PC12 cells, an adrenal medullary pheochromocytoma-derived cell line which displays some neuronal properties in culture; the EPO-R cDNA sequence from PC12 cells was found to be identical to the one from erythroid cells (17).

Assuming that EPO-dependent modulation of neuronal cells in culture reflects the *in vivo* situation in the brain, one would expect local EPO expression in this organ since kidney-derived EPO is unlikely to cross the blood-brain barrier. Indeed, accumulation of EPO mRNA in the brain of hypoxic rats has been observed by a ribonuclease protection assay (18). Furthermore, human tumors of the central nervous system such as meningioma (19) and cerebellar hemangioblastoma (20), both associated with secondary erythrocytosis, have been found to synthesize EPO. In the present work, we quantified the hypoxic upregulation of EPO mRNA in the murine brain and localized specific EPO binding sites in defined areas of the brain by *in situ* binding studies using radiiodinated EPO.

MATERIALS AND METHODS

Animals. Mice were bred in our conventional animal facilities. EPO production was induced by exposing the mice to 0.1% carbon monoxide for 4 hr (21). Blood was collected from stimulated and control animals and, after perfusion and decapitation, brain and kidney were removed and frozen in liquid nitrogen. Serum EPO concentrations were measured by RIA (22). Total RNA from brain and kidney was isolated with the RNazol kit (Cinna/Biotecx, Houston, TX). Resuspended total RNA was quantified by spectrophotometric absorbance at 260 nm and tested for integrity by agarose gel electrophoresis.

Reverse Transcription (RT)-PCR. Total RNA (1 μ g) from brain, kidney, and murine erythroleukemia (MEL) cells was reverse-transcribed with Promega's RT system. One-seventh of the reaction mixture was used as template for PCR amplification of either EPO or EPO-R cDNA with the primers 5'-ATCTGCGACAGTCGAGTTCT-3' (sense) and 5'-GTATCCACTGTGAGTGTTCC-3' (antisense), located in exon 2 and exon 5, respectively, of the murine EPO gene, and 5'-GGACACCTACTGGTATTGG-3' (sense) and 5'-GACGTTGTAGGCTGGAGTCC-3' (antisense), located in exon 8 and the 3' untranslated region, respectively, of the murine EPO-R gene (23). RT-PCR products were 395 bp for EPO and 452 bp for EPO-R. EPO mRNA was quantified by competitive RT-PCR (24). The competitor EPO template was obtained by a 1.4-kb *Sca I-Sma I* deletion of a mouse genomic EPO clone which, after amplification with the same primer set, resulted in a 492-bp fragment (H.H.M., R.H.W., L.A.R., and

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Abbreviations: EPO, erythropoietin; EPO-R, EPO receptor; mAb, monoclonal antibody; RT, reverse transcription.

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M.G., unpublished work). Varying amounts of synthetic RNA competitor template were added to the total RNA samples prior to RT-PCR. The relative amount of amplified target cDNA versus competitor cDNA was quantitated by visual inspection of ethidium bromide-stained agarose gels of the amplified products.

¹²⁵I-EPO Binding on Slide-Mounted Sections. Anesthetized adult male mice were perfused with 7 ml of ice-cold 0.9% NaCl through the left ventricle. The brain and the quadriceps muscle were removed and washed in ice-cold phosphate-buffered saline for 15 min. Subsequently, the tissues were frozen in liquid nitrogen-cooled isopentane for 20 min and transferred quickly into a precooled cryocut chamber (-17°C). After drying, the brain and the skeletal muscle were mounted on precooled chucks and embedded. The tissues were then cut in 17- μm sections at -17°C , thaw-mounted on precooled gelatin-coated slides, washed twice with phosphate-buffered saline for 10 min, and dried with an air stream (25). Nonspecific binding sites were blocked by preincubation with a protein mixture consisting of 0.5 mM ribonuclease A (Sigma), 1 mM trypsin inhibitor (Sigma), 0.05 mM wheat germ agglutinin (Boehringer Mannheim), and 1 mM ovalbumin (Sigma). After the preincubation solution was removed, ¹²⁵I-labeled recombinant human EPO (specific activity: 300–900 Ci/mmol; Amersham; 1 Ci = 37 GBq) was dissolved in phosphate-buffered saline containing 0.1% bovine serum albumin and added to the slices, which subsequently were incubated for 90 min at room temperature. The concentration of the ¹²⁵I-EPO in the incubation solution was 400–500 pM, close to the K_d value found in erythroid colony-forming-unit cells (26). Following incubation, the slices were washed twice in cold phosphate-buffered saline for 10 min, dipped in water, and dried in an air stream in order to prevent diffusion of the reversibly bound ligand. Dried slices were exposed to Hyperfilm ³H (Amersham) for 10 to 14 days at 4°C . In competition experiments, unlabeled recombinant human EPO (Boehringer Mannheim) was mixed at 1000-fold molar excess with ¹²⁵I-EPO prior to incubation. Preincubation of ¹²⁵I-EPO with monoclonal antibodies (mAbs) was performed in a molar ratio of 1:100 at 37°C for 60 min in a rotary shaker (150 rpm).

RESULTS AND DISCUSSION

Transcripts encoding EPO and EPO-R were found in total brain RNA. Stimulation of EPO production in 8-week-old mice ($n = 9$) was achieved by exposing the animals to an atmosphere containing 0.1% carbon monoxide, a procedure that causes functional anemia. The brains of stimulated animals were removed after *in vivo* perfusion, and total RNA isolated from individual mice was analyzed for the presence of EPO and EPO-R mRNA. RT-PCR analysis revealed that both genes are transcribed in the mouse brain (Fig. 1). The identity of the amplified products was confirmed by restriction fragment length analysis and Southern blotting.

These findings encouraged us to test whether EPO gene expression in brain is regulated in an oxygen-dependent manner as it is in kidney and liver. To this end, we quantified specific EPO mRNA in the brain of carbon monoxide-exposed mice and unstimulated control animals by means of competitive RT-PCR. Hypoxic stimulation of mice was confirmed by a 40-fold increase of immunoreactive serum EPO concentrations as shown by RIA (Fig. 2A). The amount of specific EPO mRNA isolated from brain of unstimulated control mice was 1–2 fg/ μg of total RNA; exposure to carbon monoxide resulted in an \approx 20-fold accumulation of EPO mRNA (Fig. 2B), indicating that the gene encoding EPO is capable of responding to hypoxia in some cerebral cells. In comparison, the renal EPO mRNA concentration of normoxic mice was about 10 fg/ μg of total RNA and showed an up to 200-fold increase in stimulated kidneys (H.H.M., R.H.W., L.A.R., and M.G., unpublished

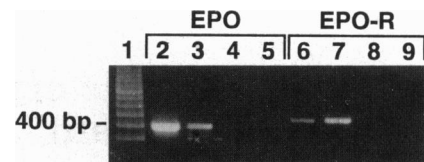


FIG. 1. Expression of EPO and EPO-R mRNA in brain from 8-week-old mice. RT-PCR analysis of EPO mRNA (lanes 2–5) and of EPO-R mRNA (lanes 6–9) was performed with 1 μg of total RNA from brain (lanes 3 and 7) and kidney (lane 2) of a carbon monoxide-stimulated, perfused mouse (positive control for EPO mRNA) and from MEL cells (lane 6) (positive control for EPO-R mRNA; ref. 27). Two negative controls were carried out for each RT-PCR set by omitting either reverse transcriptase (lanes 4 and 8) or total RNA (lanes 5 and 9). DNA migration of a 100-bp DNA ladder (Pharmacia) is indicated (lane 1).

work). This observation implies that the adaptive molecular mechanisms in response to hypoxia in the brain correspond to, but might not be identical with, the oxygen-dependent regulation of the EPO gene in kidney and liver. Furthermore, assuming that the EPO mRNA level correlates with the amount of EPO protein production, one would expect a modest increase in brain EPO compared with renal EPO after hypoxic induction. Since EPO is unlikely to cross the blood-brain barrier, this difference might represent an adaptation to the different supply mechanisms of EPO to its target organs: while kidney-derived EPO is delivered into the blood for transport to the bone marrow, brain-derived EPO is restricted to act locally in this organ, presumably in a paracrine fashion.

Specific binding sites for EPO in brain tissue were visualized by binding studies in which ¹²⁵I-labeled recombinant human EPO was incubated with slide-mounted unfixed brain sections of perfused 8-week-old mice. Binding of ¹²⁵I-EPO was observed in distinct areas of the brain as shown in frontal sections (Fig. 3A). Competition of ¹²⁵I-EPO with a 1000-fold molar excess of unlabeled EPO resulted in a strong decrease of the signal (Fig. 3B) compared with the adjacent brain section exposed only to ¹²⁵I-EPO (Fig. 3A), indicating the specificity of binding sites for EPO in the brain. These results were confirmed by using two mAbs raised against recombinant human EPO which either inhibit (mAb E14) or do not inhibit (mAb D7) binding of EPO to the EPO-R (28). The two mAbs have comparable affinities for human EPO (K_d of 0.3 nM). Preincubation of ¹²⁵I-EPO with mAb E14 strongly inhibited binding to EPO-R, thereby abolishing the autoradiographic signal compared with an adjacent control brain slice (Fig. 3C and D). In contrast, binding of ¹²⁵I-EPO to EPO-R was not influenced after preincubation with the noninhibitory mAb D7

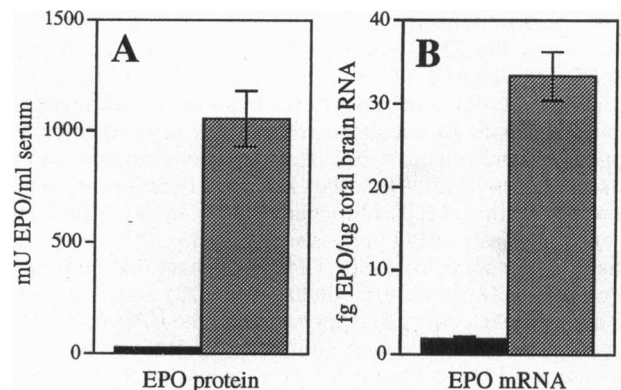


FIG. 2. Quantification of serum EPO protein and brain EPO mRNA. (A) Serum EPO levels from three carbon monoxide-stimulated (gray bars) and three control mice (black bars) were measured by RIA. (B) EPO mRNA in brain of these animals was quantified by competitive EPO RT-PCR.

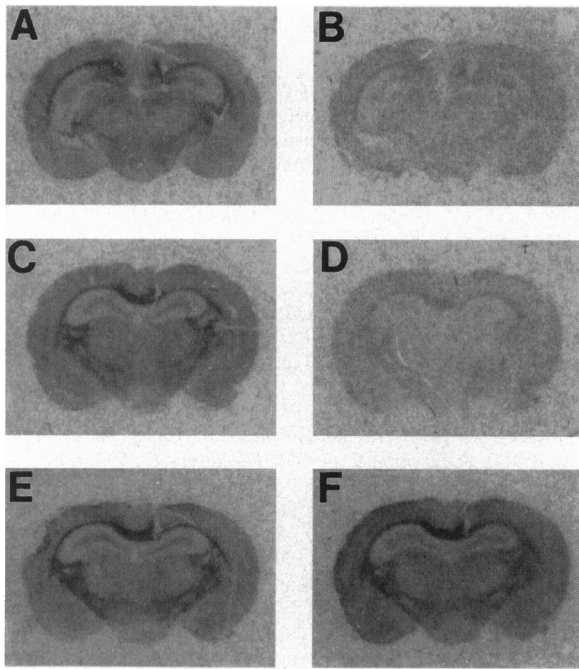


FIG. 3. Specific binding of ^{125}I -EPO to mouse brain sections. Frontal brain section of an 8-week-old mouse incubated with ^{125}I -EPO (A) and adjacent section incubated with ^{125}I -EPO and a 1000-fold molar excess of unlabeled EPO (B). Control frontal sections (C and E) and adjacent sections incubated with ^{125}I -EPO and either the inhibitory mAb E14 (D) or the noninhibitory mAb D7 (F). Since we could not detect muscular EPO-R transcripts by RT-PCR, skeletal muscle sections were used as negative controls (not shown).

(Fig. 3 E and F). In an additional control experiment, we observed that preincubation with a nonrelated mAb raised against rat renin did not alter the binding of EPO to EPO-R (data not shown). In contrast to EPO, hypoxic induction of EPO-R in brain was not detected either by RT-PCR analysis or by radioligand binding studies on brain sections from hypoxic mice (data not shown).

^{125}I -EPO bound to defined areas in the brain (Fig. 4). The most intense staining was visible in capsula interna, corpus callosum, fimbria hippocampus, zona incerta, alveus (not shown), and mamillothalamic tract (not shown). Intermediate

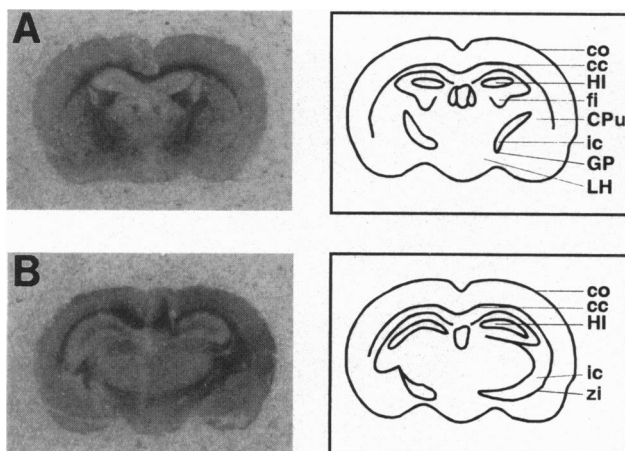


FIG. 4. Specific ^{125}I -EPO binding sites in two frontal mouse brain sections. The distance between the frontal section (A) and the dorsal section (B) is 200 μm . The sections were prepared and processed as described in Fig. 3. Nomenclature (29): CPu, caudatoputamen; co, cerebral cortex; cc, corpus callosum; fi, fimbria hippocampus; GP, globus pallidus; HI, hippocampus; ic, internal capsule; LH, lateral hypothalamic area; zi, zona incerta.

autoradiographic staining was also observed in neocortical regions such as the outer cortical layers and in the hippocampus, which is an archaocortical area. The hippocampus showed very distinct staining: while staining of the cellular layers containing pyramidal cells was intense, the labeling of adjacent layers containing fiber systems was decreased. Further staining was visible in brainstem, mesencephalon, and lateral posterior thalamic nuclei (all three areas not shown), caudatoputamen, globus pallidus, and lateral hypothalamus. Autoradiographic staining of the cerebellum was dependent on the sectioning level: binding of ^{125}I -EPO was observed in sections containing the granular layer but was not detectable in other cerebellar areas. Figs. 3 and 4 reveal that the main ^{125}I -EPO binding site is the white matter, which consists of myelinated nerve fibers, oligodendrocytes, and fibrous astrocytes. A report showing functional EPO-R expression in cultured cells with neuronal characteristics (17) suggests that axons might be one possible candidate carrying the EPO-R. Furthermore, expression of EPO-R reported in mouse embryonic stem cells (30) and in the mouse postimplantation embryo (31) has interesting implications for the role of EPO in early development, including hematopoiesis and neurogenesis. This notion is further supported by a report describing the tissue-specific mRNA expression of the endogenous EPO-R gene and the human EPO-R transgene in the brain of transgenic mouse embryos (32).

Multiple EPO- and non-EPO-producing cell lines of various species were found to have the capability of sensing oxygen tensions and responding to hypoxia, implying that oxygen sensing is a widespread mechanism in mammalian cells (33, 34). While this manuscript was in preparation, Masuda *et al.* (35) reported the partial purification of biologically active EPO from primary cerebral cells derived from rat fetuses, demonstrating that after immortalization with the simian virus 40 large tumor antigen, these cultured cells accumulated EPO mRNA in an oxygen-dependent manner. They also provided immunochemical evidence that astrocytes might represent the EPO-producing cell population. These *in vitro* results further support the notion that EPO plays an as yet undefined role in the mammalian brain. At present, the physiological function of EPO in brain is an open question. It is known that hypoxia induces changes in membrane conductivity of neuronal cells for K^+ , Na^+ , and Ca^{2+} ions (reviewed in refs. 36 and 37). This effect reduces the cellular requirement for ATP and might prevent cell damage during hypoxia. We speculate that EPO might be involved in the regulation of membrane conductivity during oxygen deprivation to preserve intracellular ATP. In preliminary results, a rapid increase in intracellular Ca^{2+} concentration (17) and in Ca^{2+} flux (38) was observed after application of EPO to PC12 cells. Further electrophysiological experiments are needed to elucidate the role of EPO/EPO-R interaction in brain cells. In addition, localization of EPO binding sites in the hippocampus, an area that is especially vulnerable to reduced oxygen supply (39), further supports the notion that EPO and its receptor might be involved in the protection of cerebral cells from hypoxic damage.

In summary, expression of EPO-R mRNA in brain tissue, localization of specific EPO binding sites *in situ*, and detection of hypoxic upregulation of EPO mRNA in the brain suggest a functional role of EPO and EPO-R in mammalian brain and imply that EPO is a polyfunctional rather than a monofunctional growth factor, like many other hematopoietic factors that bind to receptors of the cytokine superfamily.

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