Magnesium and Posthypoxic Changes of Nitrergic Population in Rat Hippocampus

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Abstract: We used NADPH-diaphorase staining to study effects of magnesium pre-treatment during long-lasting hypoxia on the brain structure of rats. NADPH-diaphorase is an enzyme co-localized in neurons with NO-synthase that is responsible for NO synthesis. NO participates in hypoxic-ischaemic injury of the brain. Hypoxia was induced in consecutive days from the 2nd till the 11th day of postnatal life in a hypobaric chamber (for 8 hours per day). Magnesium was administered before each hypoxia exposition. At the age of 12 days, the animals were transcardially perfused with 4% buffered neutral paraformaldehyde under the deep thiopental anaesthesia. Cryostat sections were stained to identify NADPH-diaphorase positive neurons that were then quantified in five hippocampal regions. In comparison to the control animals, intermittent hypoxia brought about higher density of NADPH-diaphorase positive neurons in all studied areas of the hippocampal structure: in CA1 and CA3 areas of the hippocampus and in hilus, in the dorsal and ventral blades of the dentate gyrus. Magnesium pre-treatment during hypoxia reduced number of NADPH-diaphorase positive neurons in all studied areas.

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Introduction

According to clinical observations and theoretical experiments, hypoxic brain damage represents one of hypoxia is also highly significant for the development of epilepsy. It explains why hypoxia attracted research interest in the past and is highly relevant nowadays. The clinical experience suggests that acute and chronic epileptogenic effects of hypoxia are strongly age dependent and that the immature brain is more susceptible than the adult one, and seizures induced by hypoxia in the early life are often refractory to medical therapy (Greenwood et al., 2000; Lin et al., 2002; Vannucci and Hagberg, 2004).

Many studies have attempted to find factors or substances, with therapeutic effects or a protective potential when used as a premedication. In the recent years many research groups focused at the nitric oxide (NO) which was proposed to be a non conventional neuromodulator and the association between NO and hypoxia has been widely documented (Garnier et al., 2002; Haga et al., 2003; Perlman, 2006).

NO is synthesized from L-arginine (with citruline as a co product) by the enzyme nitric oxide synthase (NOS), which is present in four different isoforms at least. The endothelial isoform (eNOS), is membrane associated and can be found in endothelial cells; the inducible isoform (iNOS) comes predominantly in macrophages; the mitochondrial isoform (mtNOS) is present in the inner mitochondrial membrane. The fourth isoform (nNOS) is constitutively expressed by subsets of neurons in the central and peripheral nervous systems, and its activity is calcium-calmodulin dependent (Black et al., 1995; Guix et al., 2005). Only 2% of brain neurons contain NOS, but these neurons ramify sufficiently to contact almost all brain cells (Bredt and Snyder, 1990). NO is an unstable molecule with a half-life of a few seconds and acts in the absence of a cell surface receptor (Bertini and Bentivoglio, 1997).

To study the neurons producing NO (so called nitrergic neuronal system), the nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) staining was used. The NADPH-d histochemical technique is based on the presence of an enzyme that catalyzes the NADPH-dependent conversion of a soluble tetrazolium salt to an insoluble, visible formazan. It has been shown, that almost all the NADPH-d positive cells exhibit also NO synthase activity and co-localization of these enzymes is dramatic, especially in the brain (Dawson et al., 1991).

Many reports have shown that in several models of hypoxia/ischemia, magnesium sulphate plays a neuroprotective role (Saris et al., 2000). There are several possible steps that magnesium can influence, including enhanced cerebral blood flow to ischemic areas, inhibition of calcium influx into cells, noncompetitive blockade of N-methyl-D-aspartate (NMDA) receptors, and favourable recovery of cellular energy metabolism during reoxygenation/reperfusion period (Nowak et al., 1984; Paoletti et al., 1995; Kristian and Siesjo, 1998). Magnesium can reduce the size of brain infarct, provide cerebral vasodilatation or act as a calcium channel antagonist

in different experimental models (Altura and Altura, 1984, Iseri and Fench, 1984; Chi et al., 1990; Kemp et al., 1999).

To study the influence of magnesium on hypoxia-associated changes of nitrergic system in rat hippocampus, the standard ontogenetic line (35, 25 and 12-day-old rats – commonly used in our laboratory) has been studied. The results of 35 and 25-day-old groups have been reported recently (Maresova et al., 2005; Jandova et al., 2006a) and this paper would like to present the conclusion of the youngest group. The 12-day-old animals correspond to early human postnatal phase and show some important differences when compared with older animals. The immature hippocampus (as well as the central nervous system en masse) is considerably plastic, the maturation of mediator system is incomplete and CNS answers to stress (allostasis) are quite different, compared to adult animals (Jensen and Wang, 1996; Contestabile, 2000; Mantelas et al., 2003; Tagliaferro et al., 2003).

Material and Methods

Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies.

In our study 16 animals (male rats, Wistar strain) in 4 experimental groups were used: (1) hypoxic group (H) involving rats exposed together with their mother for 8 hours per day (except day 6 and 7) to repeated hypobaric hypoxia in a hypobaric chamber at a simulated altitude of 7 000 m since their day of birth till the 11^{th} day; (2) hypoxic group as above with magnesium sulphuricum (300 mg/kg i.p.) injection before the hypoxia exposition (H+Mg); (3) control rats not exposed to hypoxia repeatedly injected (9 times) with the same dose of magnesium sulphuricum (C+Mg); (4) control rats received corresponding volumes of normal saline solution (C).

For histochemical staining for NADPH-diaphorase, control and treated rats were under deep thiopental anaesthesia transcardially perfused at P12 (one day after the end exposition to hypobaric hypoxia) with 4% neutral paraformaldehyde in phosphate-buffered saline (PBS, 0.1 M, pH 7.4). Brains were removed, immersed in the same fixative for one hour, transferred overnight into 20% sucrose for cryoprotection. A set of serial frontal cryostat section (40 μm thick) was obtained. The brain sections were then subjected to NADPH-diaphorase staining. The free-floating sections were placed in 0.1 M phosphate buffer and incubated in 0.1 M phosphate buffer containing 0.5 mg/ml β-NADPH (Sigma), 0.2 mg/ml Nitro blue tetrazolium (NBT, Sigma) and 0.3% Triton X-100 (Sigma) for 4 h at 37 °C in thermostat. Following the reaction, the sections were rinsed in 0.1 M phosphate buffer and kept at 8 °C for 16 h. The histochemically reacted sections were mounted on pre-cleaned 0.5% gelatine-coated Microscope Slides (Menzel-Gläser), air dried, cover-slipped with Microscope Cover Glasses (Menzel-Gläser) using D.P.X. Neutral Mounting Medium (Aldrich) (Wang et al., 2001).

Hippocampal formation that included CA1 and CA3 area of hippocampus, dorsal blade (DB DG), ventral blade (VB DG) and hilus of the dentate gyrus between the AP plane 2.5 mm and 4.0 mm posterior to the bregma, was subjected to quantification of nitrergic neurons per section area under the light microscope Olympus Provis AX 70.

In each animal, 25–30 sections were quantified. For the statistical evaluation, the *t*-unpaired test and ANOVA were used (level of significance was set at $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***)).

Results

Repeated hypobaric perinatal hypoxia enhanced the number of NADPH-d positive neurons in all investigated areas, in CA1 hippocampal area by 147% ($p<0.001$), in CA3 area by 194% ($p<0.001$), in the hilus by 164% ($p<0.001$), in the ventral blade by 260% ($p<0.001$) and in the dorsal blade of the dentate gyrus by 151% ($p<0.001$), compared to the control group which we consider as baseline (Figure 1).

Magnesium pre-treatment in control rats brought about higher number of nitrergic neurons in all areas of the hippocampus: in CA1 hippocampal area by 190% ($p<0.001$), in CA3 area by 262% ($p<0.001$), in the hilus by 211% ($p<0.001$), in the ventral blade by 164% ($p<0.001$) and in the dorsal blade of the dentate gyrus by 157% ($p<0.001$) (Figure 2).

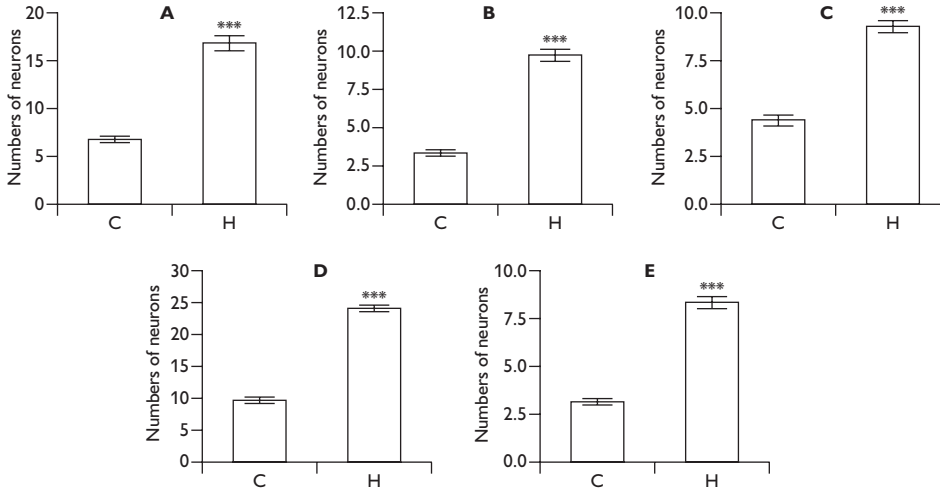

Figure 1 – (A) Number OF NADPH-d positive neurons in CA1 area of hippocampus per section area, (B) number of NADPH-d positive neurons in CA3 area of hippocampus per section area, (C) number of NADPH-d positive neurons in dorsal blade of the dentate gyrus per section area (D), number of NADPH-d positive neurons in ventral blade of the dentate gyrus per section area, (E) number of NADPH-d positive neurons in hilus of the dentate gyrus per section area, C = control group, H = hypoxic group, mean ± SEM, level of significance $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).

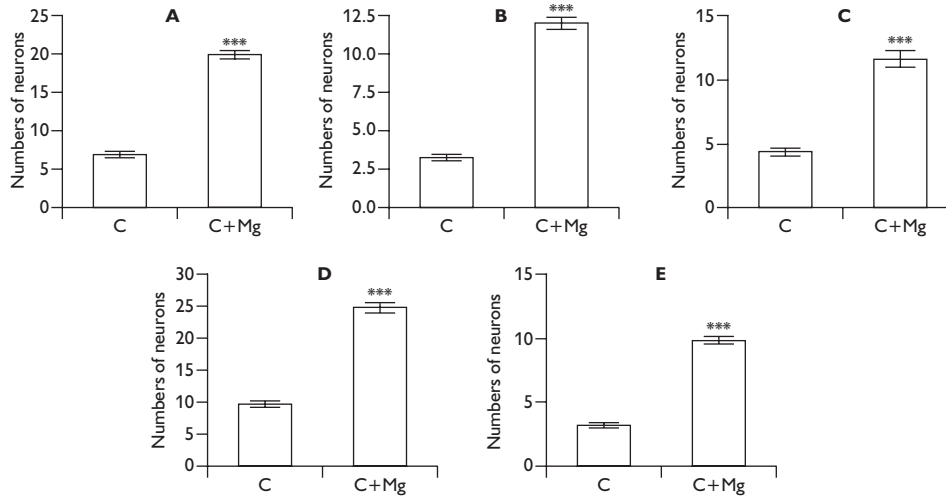

Figure 2 – (A) Number of NADPH-d positive neurons in CA1 area of hippocampus per section area, (B) number of NADPH-d positive neurons in CA3 area of hippocampus per section area, (C) number of NADPH-d positive neurons in dorsal blade of the dentate gyrus per section area (D), number of NADPH-d positive neurons in ventral blade of the dentate gyrus per section area, (E) number of NADPH-d positive neurons in hilus of the dentate gyrus per section area, C = control group, C+Mg = control group with magnesium administration, mean ± SEM, level of significance p<0.05 (*), p<0.01 (**), p<0.001 (***).

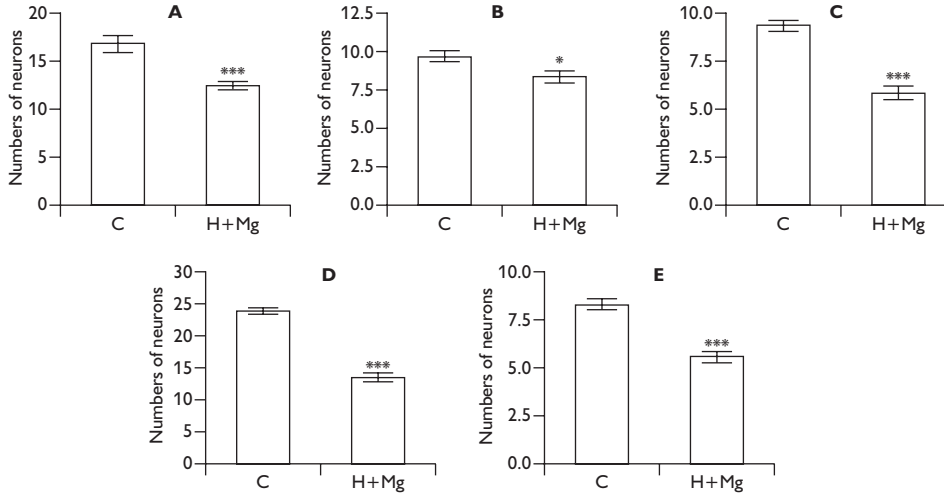

Figure 3 – (A) Number of NADPH-d positive neurons in CA1 area of hippocampus per section area, (B) number of NADPH-d positive neurons in CA3 area of hippocampus per section area, (C) number of NADPH-d positive neurons in dorsal blade of the dentate gyrus per section area (D), number of NADPH-d positive neurons in ventral blade of the dentate gyrus per section area, (E) number of NADPH-d positive neurons in hilus of the dentate gyrus per section area, H = hypoxic group, H+Mg = hypoxic group with magnesium pre-treatment, mean ± SEM, level of significance p<0.05 (*), p<0.01 (**), p<0.001 (***).

Modulatory influence of magnesium pre-treatment was analysed in rats exposed to intermittent hypoxia. The number of NADPH-d positive neurons in the CA1 area of the hippocampus was lower by 36% ($p<0.001$), in CA3 hippocampal areas by 14% ($p<0.05$), in the hilus by 33% ($p<0.001$), in the ventral blade by 19% ($p<0.001$) and in the dorsal blade of the dentate gyrus by 44% ($p<0.001$) in comparison with hypoxia exposed but not pre-treated animals (Figure 3).

Discussion

Hypoxia and ischemia of the brain leads to the oxidative stress, characterized by imbalance between antioxidant- and oxidant-generating systems, which causes oxidative damage (Buonocore et al., 2001). During the period of reoxygenation/reperfusion following hypoxia/ischemia, free radicals are generated that are responsible for much of the organ damage. This results in generation of toxic reactive oxygen intermediates such as peroxynitrite, hydroxyl radical and reactive NO species that further contribute to lipid peroxidation and membrane breakdown (Beckman, 1991), mitochondrial impairment, excitotoxicity, and apoptosis (Blomgren and Hagberg, 2006). On the other hand, NO itself may inhibit lipid peroxidation (Nicolescu et al., 2002), induces vasodilatation, angiogenesis and inhibits platelet aggregation and leukocyte activation (Moncada and Erusalimsky, 2002).

Since NO is likely to play an important role as a mediator of excitotoxic neuronal injury after hypoxia, it is important to determine the NOS activity during development and establish whether this expression predicts the areas of the brain that are vulnerable to injury from hypoxia. NOS is the only enzyme with nicotine adenosine dinucleotide phosphate (NADPH)-diaphorase activity preserved in formaldehyde fixed tissue, and so this histochemical procedure can be used to identify NOS-containing cells (Hope et al., 1991). NADPH-d reactivity was detected in various regions of the nervous system of mammals including the rat. The coexistence of NADPH-d reactivity and neurotransmitter or neuropeptide reactivity was demonstrated in several neuronal populations (Wang et al., 2001).

In the present work, our findings showed that long-lasting intermittent perinatal hypoxia increased the number of NADPH-d positive neurons in 12-day-old rats in CA1, CA3 areas of the hippocampus and in ventral and dorsal blades of the dentate gyrus. These results support theory of the overproduction of NO via elevated NOS activity during and after hypoxic exposure (Mishra et al., 2000; van den Tweel et al., 2005). Postnatal period of 12 days is a critical period for structural and functional maturation of the rat brain. Moreover, it represents the synaptogenic period, equivalent to the synaptogenic stage during early human postnatal phase (Contestabile, 2000; Tagliaferro et al., 2003). The effect of hypoxia as we observed in CA1 and CA3 areas of the hippocampus rat and in the hilus of the dentate gyrus was similar to that described in 35-day-old and in 25-day-old rats (Maresova et al., 2005). On the contrary, recent studies describe reducing effect of hypoxia on the density of nitrergic neurons in adult rats only (Benesova et al., 2004, 2005).

Although nNOS was reported to be an enzyme expressed constitutively, several lines of evidence led to the conclusion that this enzyme can be regulated during processes related to plasticity and cellular damage (Deng and Cadet, 1999). This enzyme was reported to be up-regulated in different models of brain injuries, such as lipopolysaccharide injection (Harada et al., 1999) and methylmercury administration (Ikeda et al., 1999).

Histochemical activity of NOS isoform may be modulated by various factors. These factors have different origin and importance in mammalian organism. Effects of nicotine or kainic acid can serve as an example. The latter substance was reported to have an excitotoxic effect and its intraperitoneal administration brings about lower number of NADPH-d positive cell in immature rat's hippocampus. On the contrary, nicotine, given by acute administration brings about higher the number of NADPH-d positive cells in immature hippocampus. The most affected areas were CA1 and CA3 areas of the hippocampus, both blades of the dentate gyrus remained unaffected (Jandova et al., 2006b; Riljak et al., 2006, 2007). These papers documented well, that NOS/NADPH-d activity is highly sensitive to many substances and that the reaction of nitrergic system is unpredictable, region specific and highly age-dependent.

Nitric oxide is a critical mediator of neuronal injury as evidenced by the administration of pharmacological inhibitors of NOS that reduce neuronal injury from focal ischemia, NMDA-dependent excitotoxicity and cerebral hypoxia (Huang et al., 1994; Bolanos et al., 1997; Numagami et al., 1997).

In our experiment we would try to block NOS expression by the administration of magnesium, which was successfully used to reduce the ischemic brain damage and the BBB disruption in severe hypoglycemia and traumatic brain injury and to treat preeclampsia-ecclampsia (Cotton et al., 1992; Schmid-Elsaesser et al., 1999; Kaya et al., 2001; Esen et al., 2003).

Magnesium pre-treatment of rats exposed to intermittent hypobaric hypoxia evoked decline of nitrergic neurons density in all studied areas of the hippocampus. Possible protective effect of the magnesium administration can be hypothetically explained by an inhibition of gene expression of nitric oxide synthase. NMDA receptor and voltage-gated calcium channels blockade by magnesium bring about reduction in intracellular calcium concentration, which can suppress synthesis of calcium dependent nNOS and subsequently lower the synthesis of NO (Maulik et al., 2001).

On the contrary, magnesium administration to 12-day-old rats not exposed to hypoxia condition brought about higher number of NADPH-d positive cells in all studied areas of the hippocampal formation. These findings were surprising because the older experimental non-hypoxia treated animals (the results published previously) respond to magnesium administration reversely. The precise mechanisms of magnesium influence on immature intact animals are still not completely clear. The application of magnesium may probably induce

osmotic changes/stress and in this way can magnesium interference in whole body homeostasis. This hypothesis is in line with our finding that rat pups, which were treated with magnesium and their body weight decreased about 25% in comparison with control animals (administration of normal saline solution in equal volume). The changes of weight were not detected in groups of older animals, which were studied in previous experiments.

Conclusion

Hypoxia and magnesium can strongly influence the hippocampal nitrergic system of immature rats. This effect is age and region specific. The precise relation between hypoxia/magnesium and nitric oxide is still not completely clear. Only further studies can help to clarify the role of nitric oxide in the brain under physiological and pathological conditions.

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