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Diazepam neuroprotection in excitotoxic and oxidative stress involves a mitochondrial mechanism additional to the $GABA_AR$ and hypothermic effects

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ABSTRACT

The aim of the present investigation was to analyze the molecular mechanism(s) of diazepam neuroprotection in two models of selective neuronal death in CA1 sector of hippocampus: *in vivo* following transient gerbil brain ischemia and *in vitro* in rat hippocampal brain slices subjected to glutamatergic (100 μM NMDA) or oxidative (30 μM tertbutyl-hydroksyperoxide (TBH)) stress. In the *in vivo* model the diazepam treatment (two doses of 10 mg/kg i.p. 30 and 90 min after the insult) resulted in more than 60% of CA1 hippocampal neurons surviving the insult comparing with 15% in untreated animals. To test whether the protective effect of diazepam was due to the postulated drug-induced hypothermia we followed the fluxes of body temperature during postischemic reperfusion: diazepam reduced temperature from 36.6 ± 1 °C to 33.4 ± 2 °C. Equivalent hypothermia induced and maintained in animals after ischemia did not prevent neuronal cell loss to the same extent as diazepam did (42.8 ± 9.2% and 72.4 ± 14.5% of live neurons, respectively). *In vitro*, under constant temperature conditions, diazepam exerted neuroprotective effects following a "U-shaped" dose–response curve, with concentration efficacy window of 0.5–10 μM. Five micro-molar diazepam showed significant protection by reducing over 50% the number of (dead) propidium iodide labeled cells even in the presence of $GABA_A$ receptor antagonist bicuculline. Next, we have shown that diazepam reduced the efflux of cytochrome c out of mitochondria both in compromised CA1 neurons *in vitro* and in isolated mitochondria treated with 30 μM THB. Our results suggest that the neuroprotective action of diazepam relies on additional mechanism(s) and not solely on its hypothermic effect. We suggest that diazepam evokes neuroprotection through its central receptors located on the $GABA_A$ receptor complex and, possibly, through its peripheral receptor, the translocator protein TSPO (previously called the peripheral benzodiazepine receptor) located in the outer mitochondrial membrane.

1. Introduction

Diazepam is a simple-to-administer and inexpensive drug that belongs to the broad group of pharmacological agents called benzodiazepines, which are widely prescribed for the treatment of anxiety, insomnia or seizure disorders. Furthermore, the experiments performed *in vivo* and *in vitro* revealed that diazepam has a strong neuroprotective potential with respect to hippocampal neurons following ischemic injury. Diazepam decreases infarct volume and may attenuate anoxic central nervous system (CNS) white matter dysfunction (Aerden et al., 2004; Fern et al., 1993). A recently completed clinical trial (EGASIS) confirmed that diazepam has a favorable safety profile in acute stroke patients (Lodder et al., 2006). Moreover, its neuroprotective effect is stronger in cardioembolic stroke, probably because of spontaneous emboli fragmentation and subsequent reperfusion (Heuts-van et al., 1996; Lodder et al., 2006). This observation seems critical from the clinical point of view, since among many compounds that provided protection in both *in vitro* and *in vivo* models of ischemia only tPA have shown efficacy in clinical trials.

Despite its promising neuroprotective properties, the exact mechanism of diazepam protection is not fully understood. Diazepam protective influence on the central nervous system has been commonly associated with the GABA neurotransmission enhancement through central benzodiazepine (BZD) receptors (CBRs) (Tallman, 1980) or through hypothermia induction. CBRs are part of a macromolecular complex that contains a γ-amino butyric acid receptor and a chloride ion channel (DeLorey and Olsen, 1992). BZD binds to the α-subunit of γ-amino butyric acid type A receptors ($GABA_A$) and as a positive allosteric modulator

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Abbreviations: BZD, benzodiazepines; CA, cornu ammonis; CBR, central benzodiazepine receptors; CNS, central nervous system; CsA, cyclosporin A; $GABA_AR$, γ-amino butyric acid receptor; OHC, organotypic hippocampal culture; PI, propidium iodide; MTP, mitochondrial permeability transition pore; NMDA, N-methyl-D-aspartate; TBH, tertbutyl-hydroksyperoxide; TSPO, translocator protein.

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facilitates the inhibitory effect of GABA (DeLorey and Olsen, 1992). A number of studies have indicated that $GABA_A$ receptors are down-regulated following cerebral ischemia and that increases in GABA activity are attenuating neuronal death. Moreover, under ischemic conditions the affinity of brain GABA receptors for GABA agonists is diminished and diazepam raises the affinity of $GABA_A$ receptor for GABA. Yet, the data from various *in vitro* models of ischemic brain injury have shown that the activation of GABA-ergic system exacerbated neuronal death induced by excitotoxity (Erdo et al., 1991), depolarizing conditions (Lukasiuk and Pitkanen, 2000) and oxygen/glucose deprivation (OGD) (Muir et al., 1996). Moreover, studies *in vivo* with topiramate and vigabatrin, drugs enhancing the action of GABA, did not result in significant differences in outcomes (using functional or histological measures) following focal cerebral ischemia (Madden, 1994).

Apart from $GABA_A$ receptor modulation, diazepam is known to induce hypothermia. Since prolonged mild hypothermia persistently reduces neuronal death in CA1 hippocampal region (Colbourne et al., 1999), this mechanism was also considered as partly responsible for the neuroprotection exerted by diazepam (Kuhmonen et al., 2002; Schwartz et al., 1994). However, diazepam reduced neuronal death even if microinjected directly into hippocampus, where it did not cause systemic hypothermia (Schwartz et al., 1995).

Since both mechanisms mentioned before do not fully explain the mechanism of protection evoked by diazepam *in vivo* and *in vitro*, the present study aimed to determine whether this effect is, at least to an extent, associated with benzodiazepine mitochondrial receptor. There are data showing that diazepam also acts on a pharmacologically distinct type of receptor, the translocator protein (TSPO, previously called the peripheral benzodiazepine receptor or the mitochondrial benzodiazepine receptor) (Veenman et al., 2004). TSPO is located primarily in the outer mitochondrial membrane and is not linked functionally or structurally to the $GABA_A$ receptor. In the central nervous system TSPO is mainly found in glial cells (Gallager et al., 1981; Schoemaker et al., 1982; Wilms et al., 2003) and neurons (Anholt et al., 1984; Doble et al., 1987). TSPO functions in regulation of neurosteroids transport and synthesis (Papadopoulos et al., 1997), apoptosis, cell proliferation (Li et al., 2007; Schlumpf et al., 1995), immune response (Lazzarini et al., 1996, 2001, 2006), and microglia activation (de Silva et al., 1973; Massoco and Palermo-Neto, 1999). TSPO can be found in conjunction with the voltage-dependent anion channel and adenine nucleotide transporter, both components of the mitochondrial permeability transition pore (MTP). MTP is a multiprotein complex located at the contact site between the inner and outer mitochondrial membranes which is intimately involved in the initiation and regulation of apoptosis (Chelli et al., 2004; Marselli et al., 2004; Veenman et al., 2004). The induction of TSPO expression under neuropathological conditions suggests that this molecule may be involved in the stress response to ischemia. However, the precise role of TSPO in the injured neural tissue is unknown. Importantly, diazepam shows similar affinity to both, the central and the peripheral benzodiazepine receptor.

To assess the mechanisms responsible for the strong neuroprotective effect of diazepam two models of selective neuronal death in the CA1 region of hippocampus were used. In the model of 5 min global brain ischemia in gerbils we show that transient reduction of body temperature and activation of $GABA_A$ receptors are only one element of diazepam mode of action. In the organotypic hippocampal culture (OHC) and in isolated mitochondria we were able to show that diazepam inhibits cytochrome c efflux from mitochondria and that these effects were not blocked by inhibition of $GABA_A$ receptor antagonist—bicuculline. Our data indicate that good neuroprotection offered by diazepam following the ischemic stroke is based on its involvement in three complementary pathways leading to the increased neuronal inhibition and reduction of cytochrome c leakage from mitochondria.

2. Materials and methods

2.1. Animal model

All the experimental procedures were approved by the Local Committee for Ethics in Animal Experiments.

Male Mongolian gerbils weighing 50–60 g were used. The ischemic insult was induced as described previously (Domanska-Janik et al., 2004). Briefly, 5 min ligation of the common carotid arteries (CCA) under anesthesia with halothane in $N_2O:O_2$ (70:30) was applied in strictly controlled normothermic conditions. The cerebral blood flow was monitored by laser Doppler (Muro, Inc.) and only animals which had <6% of normal cortical blood flow were used for the experiments. Groups of gerbils received injections of selected drugs: 2.5 mg/kg cyclosporine A (CsA) during the insult directly to the left carotid artery (Domanska-Janik et al., 2004) or two doses of 10 mg/kg diazepam i.p. 30 and 90 min after the insult. Sham operated animals were injected with the same volume of the vehicle. The rectal temperature was measured during surgery and CCA occlusion, for the duration of the first 3 h of reperfusion and after 24 h. Animals were allowed a recovery period of one week, then were perfused with ice-cold 4% paraformaldehyde in PBS under pentobarbital anesthesia and brains used for histological examinations (Fig. 1A). The histological evaluation was performed on paraffin-embedded, 10-μm thick sections stained with hematoxylin/eosin. The extent of cell damage of CA1 hippocampal regions was quantified as the mean number of remaining intact neurons in the coronal sections using Axioscop 2 bright field microscope (Zeiss, Munich, Germany). To account for the variability in neuronal numbers with respect to different stereotaxic reference points, we used an MC 10095 camera (Carl Zeiss Jena GmbH) to capture at least three well-defined 300 μm CA1 fields along the septo-temporal length of the hippocampus and counted those using a computer-assisted image analysis system (KS 300, Carl Zeiss Jena GmbH). The counts from these three regions were averaged.

2.1.1. Hypothermia induction

Two groups of animals were cooled down to 32–33 °C for 6 h starting immediately after the induction of ischemia (hypothermia 0′) and 20 min after the insult (hypothermia 20′), respectively. The rectal temperature was measured for 3 h after ischemia and additionally 24 h after injury.

2.2. Organotypic hippocampal culture

Hippocampal slices were prepared from 7–10 days old Wistar rats according to the method of Stoppini et al. (1991) with slight modifications. After brief anesthesia with Vetbutal (pentobarbital; Sigma) ice-cooled pups were plunged into 70% alcohol solution, decapitated with scissors, and then brains were quickly removed to ice-cold HBSS (Gibco). Hippopcampi were separated and cut into 400 μm slices using McIlwain tissue chopper. Slices were transposed to a Millicell-CM (Millipore) membranes for further growth. Millicell-CM membranes in 6-well plates were pre-equilibrated with 1 ml of culture medium (HEPES pH 7.2, 50% DMEM, 25% HBSS, 25% Horse Serum (Gibco), 2 mmol/l L-glutamine, 5 mg/ml glucose, 1% amphotericine B and 0.4% penicillin–streptomycin) prepared according to Aitken et al. (1995) and Gähwiler et al. (1997). Cultures were started in a 25% horse serum-containing medium which was gradually replaced (from DIV 4 until 7) by a serum-free,

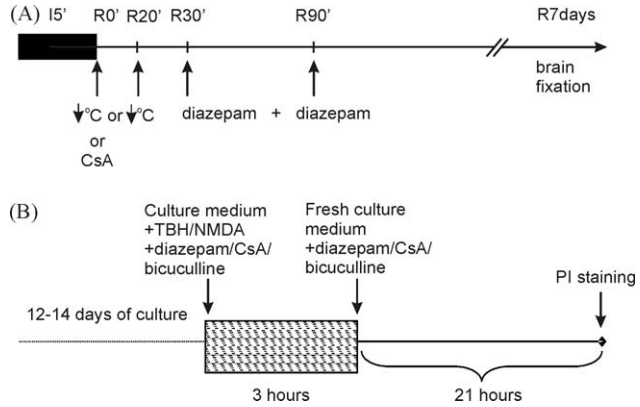

Fig. 1. Experimental protocols used to study: (A) diazepam, CsA or hypothermia protection *in vivo* after 5 min, transient gerbil brain ischemia (I5′) and reperfusion (R). (B) diazepam, bicuculline or CsA treatment *in vitro* in the model of organotypic hippocampal culture (OHC) after 30 μM TBH or 100 μM NMDA injury. PI—propidium iodide, R0′, R20′, R30′, R90′ and R7d—time of postischemic reperfusion 0, 20, 30, 90 min and 7 days, respectively.