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NEUROPROTECTIVE EFFECT OF DIMEBON AGAINST ISCHEMIC NEURONAL DAMAGE

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Abstract—Dimebon (dimebolin or latrepirdine), originally developed as an anti-histaminic drug, has been investigated and proposed as a cognitive enhancer for treating neurodegenerative disorders such as Alzheimer's and Huntington's diseases, and more recently schizophrenia. This study was conducted to evaluate the potential neuroprotective effect of dimebon during brain ischemia using rat hippocampal slices subjected to oxygen and glucose deprivation followed by a reoxygenation period (OGD/Reox) or glutamate excitotoxicity. Dimebon, incubated during the OGD/Reox period, caused a concentration -dependent protective effect of hippocampal slices; maximum protection (85%) was achieved at 30 μM. Mitochondrial membrane depolarization, reactive oxygen species of oxygen (ROS) production, nitric oxide synthase (iNOS) induction and translocation of p65 to the nucleus induced by OGD/Reox were significantly reduced in dimebon-treated hippocampal slices. In the glutamate-induced excitotoxicity model, dimebon also afforded a concentration-dependent protective effect that was significantly higher than that obtained with memantine, a non-competitive *N*-methyl-D-aspartate (NMDA) antagonist. When changes in the intracellular calcium concentration were evaluated in Fluo-4-loaded rat hippocampal neurons, glutamate-induced calcium transients were reduced by 20% with dimebon. These results suggest that dimebon could counteract different pathophysiological processes during ischemic brain damage and, could therefore, be considered as a novel therapeutic strategy for cerebral ischemia-reoxygenation injury.

Key words: dimebon, oxygen–glucose deprivation, neuroprotection, ischemia, hippocampal slices, hippocampal neurons.

INTRODUCTION

Behind heart disease and cancer, stroke is one of the most frequent causes of death and disability worldwide, with a significant clinical and socioeconomic impact (Rosamond et al., 2008; Urban et al., 2010). Its pathophysiology involves multiple biochemical mechanisms that trigger a cascade of detrimental cellular pathways such as large amounts of free radicals, calcium overload, inflammation, glutamate-mediated excitotoxicity and neurotoxicity in the penumbral cortex during reperfusion (Doyle et al., 2008). In spite of the high impact of this disease, the treatment to minimize tissue injury after stroke is still unsatisfactory and, thus, there is a need to develop effective treatments.

Dimebon (2,8-dimethyl-5-(2-(6-methylpyridin-3-yl)ethyl)-2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole dihydrochloride) is a non-selective antihistaminic drug, previously used in Russia that showed potential to improve cognitive functions (Bachurin et al., 2001). In a Phase II Alzheimer's disease (AD) trial, dimebon treatment was associated with benefits on cognition, global function and behavior (Doody et al., 2008). In non-human primates dimebon was shown to improve components of working memory (Webster et al., 2011). In recent studies, it was demonstrated that dimebon antagonizes brain 5-HT6 receptors and enhances mitochondrial activities; these actions could contribute to explain dimebon's cognitive-enhancing properties (Schaffhauser et al., 2009; Moreira et al., 2010; Zhang et al., 2010). However, in spite of all these positive results, a subsequent Phase III trial reported no significant improvement in AD patients (Miller, 2010).

Dimebon has been described as a multifunctional drug, endowed with different mechanisms of action with potential relevance to AD pathology such as inhibition of

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Abbreviations: ΔΨm, mitochondrial membrane potential; ANOVA, analysis of variance; AD, Alzheimer disease; DCFH-DA, 2′,7′-dichlorfluorescein-diacetate; DMEM, Dulbecco's-Modified Eagle's Medium; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; HEPES, hydroxyethyl piperazineethanesulfonic acid; iNOS, inducible nitric oxide synthase; KRB, Krebs bicarbonate dissection buffer; MK801, dizocilpine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor kappa B; NMDA, *N*-methyl-D-aspartate; OGD, oxygen and glucose deprivation; Reox, reoxygenation; ROS, reactive oxygen species; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; s.e.m., standard error of the mean; TMRE, tetramethylrhodamine ethyl ester.

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acetylcholinesterase and butyrylcholinesterase, blockade of *N*-methyl-D-aspartate (NMDA) receptors and inhibition of the mitochondrial permeability transition pore opening (Bachurin et al., 2001, 2003; Lermontova et al., 2001; Grigorev et al., 2003). Preclinical studies have revealed several activities of dimebon in *in vitro* and *in vivo* models such as neurite promotion outgrowth in cultured hippocampal and cortical neurons (Protter et al., 2009) and hippocampal neurogenesis enhancement (Pieper et al., 2010). However, to date, the exact mechanisms whereby dimebon exerts its pro-cognitive and neuroprotective effects remain unknown.

Considering that vascular factors predisposing to cerebrovascular disease or stroke are being increasingly related to AD pathology (Gordon-Krajcer et al., 2007; Garcia-Alloza et al., 2011), it seemed appropriate to investigate whether dimebon could also exert neuroprotection during brain ischemia. Our results indicate that dimebon provides neuroprotection in an *in vitro* model of ischemia based on rat hippocampal slices subjected to oxygen and glucose deprivation followed by reoxygenation (OGD/Reox); its protective effect was related to the reduction of mitochondrial depolarization, glutamate-induced toxicity, reactive oxygen species (ROS) and inducible nitric oxide synthase (iNOS), and p65 translocation to the nucleus inhibition. Furthermore, in isolated rat hippocampal neurons, dimebon reduced intracellular Ca^{2+} transients induced by glutamate. To our knowledge, this is the first report that evidences dimebon's neuroprotective effect on the cascade of events leading to neuronal death in a hypoxia–ischemia *in vitro* model.

EXPERIMENTAL PROCEDURES

Reagents

Dimebon (2,8-dimethyl-5-(2-(6-methylpyridin-3-yl)ethyl)-2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole dihydrochloride) was purchased from Biotrend (Cologne, Germany). Memantine, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and dimethyl sulfoxide (DMSO) were from Sigma (Madrid, Spain). Dulbecco's-Modified Eagle's Medium (DMEM), fetal bovine serum and penicillin/streptomycin were purchased from Invitrogen (Barcelona, Spain). Fluo-4 AM was from Molecular Probes (Invitrogen, Barcelona, Spain).

Use of animals

All experimental procedures were performed following the *Guide for the Care and Use of Laboratory Animals* and were previously approved by the institutional Ethics Committee of the Autonomous University of Madrid, Spain, according to the European Guidelines for the use and care of animals for research in accordance with the European Union Directive of 22 September 2010 (2010/63/UE) and with the Spanish Royal Decree of 1 February 2013 (53/2013). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Preparation of rat hippocampal slices

Adult male Sprague–Dawley rats (275–325 g) from a colony of our animal quarters were used. *In vitro* damage caused by OGD/Reox and the protection elicited by dimebon was studied in acutely isolated rat hippocampal slices. We used the protocol described by our research group (Egea et al., 2007) with slight modifications. Rats were quickly decapitated under sodium pentobarbital anesthesia (60 mg/kg, i.p.), forebrains were rapidly removed from the skull and placed into ice-cold Krebs bicarbonate dissection buffer (KRB) (pH 7.4), containing (in mM): NaCl 120, KCl 2, $CaCl_2$ 0.5, $NaHCO_3$ 26, $MgSO_4$ 10, KH_2PO_4 1.18, glucose 11 and sucrose 200. The chamber solutions were pre-bubbled with either 95% O_2/5% CO_2 or 95% N_2/5% CO_2 gas mixtures, for at least 45 min before slice immersion, to ensure O_2 saturation or removal. The hippocampi were quickly dissected, and slices (350 µm thick) were rapidly prepared using a McIlwain Tissue Chopper. Then, the slices were transferred to vials of sucrose-free dissection buffer, bubbled with 95% O_2/5% CO_2 in a water bath at room temperature for 60 min, to allow tissue recovery from slicing trauma before starting the experiments (equilibration period). The slices corresponding to the control group were incubated 15 min in a KRB solution with the following composition (in mM): NaCl 120, KCl 2, $CaCl_2$ 2, $NaHCO_3$ 26, $MgSO_4$ 1.19, KH_2PO_4 1.18 and glucose 11; this solution was equilibrated with 95% O_2/5% CO_2. OGD was induced by incubating the slices for a 15-min period in a glucose-free Krebs solution, equilibrated with a 95% N_2/5% CO_2 gas mixture; glucose was replaced by 2-deoxyglucose. After this OGD period, the slices were returned back to an oxygenated normal Krebs solution containing glucose for 120 min (re-oxygenation period). Experiments were performed at 37 °C. A control and an OGD group were included in all experiments and four dimebon or memantine concentrations were tested in each experiment. When treated, OGD slices received dimebon and remained there during the OGD and re-oxygenation period (see Fig. 1A for experimental protocol).

In order to investigate the mechanisms involved in glutamate-induced toxicity, we used a protocol previously described (Molz et al., 2008) with slight modifications. After the preincubation time with dimebon or memantine (see protocol in Fig. 5A), hippocampal slices were incubated with glutamate (1 mmol/L) for 1 h in KRB. After this period, the medium was withdrawn and replaced by a nutritive culture medium composed of 50% of KRB, 50% of DMEM, 20 mM of HEPES, 100 units/mL penicillin and 100 µg/mL streptomycin, at 37 °C in a CO_2 atmosphere and slices were maintained for additional 4 h to evaluate cell viability.

Viability assay

Hippocampal cell viability was determined through the ability of the cells to reduce MTT according to the previously described protocol by Lorrio et al. (2013). Briefly, hippocampal slices were collected immediately