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Research report

Antioxidant effects of JM-20 on rat brain mitochondria and synaptosomes: Mitoprotection against Ca^{2+}-induced mitochondrial impairment

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ABSTRACT

Because mitochondrial oxidative stress and impairment are important mediators of neuronal damage in neurodegenerative diseases and in brain ischemia/reperfusion, in the present study, we evaluated the antioxidant and mitoprotective effect of a new promising neuroprotective molecule, JM-20, in mitochondria and synaptosomes isolated from rat brains. JM-20 inhibited succinate-mediated H_2O_2 generation in both mitochondria and synaptosomes incubated in depolarized (high K^+) medium at extremely low micromolar concentration and with identical IC_{50} values of 0.91 μM. JM-20 also repressed glucose-induced H_2O_2 generation stimulated by rotenone or by antimycin A in synaptosomes incubated in high sodium-polarized medium at extremely low IC_{50} values of 0.395 μM and 2.452 μM, respectively. JM-20 was unable to react directly with H_2O_2 or with superoxide anion radicals but displayed a cathodic reduction peak at −0.71 V, which is close to that of oxygen (−0.8 V), indicating high electron affinity. JM-20 also inhibited uncoupled respiration in mitochondria or synaptosomes and was a more effective inhibitor in the presence of the respiratory substrates glutamate/malate than in the presence of succinate. JM-20 also prevented Ca^{2+}-induced mitochondrial permeability transition pore opening, membrane potential dissipation and cytochrome *c* release, which are key pathogenic events during stroke. This molecule also prevented Ca^{2+} influx into synaptosomes and mitochondria; the former effect was a consequence of the latter because JM-20 inhibition followed the patterns of carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP), which is a classic mitochondrial uncoupler. Because the mitochondrion is considered an important source and target of neuronal cell death signaling after an ischemic insult, the antioxidant and protective effects of JM-20 against the deleterious effects of Ca^{2+} observed at the mitochondrial level in this study may endow this molecule with the ability to succeed in mitochondrion-targeted strategies to combat ischemic brain damage.

1. Introduction

Oxidative stress is implicated in the pathogenesis of progressive neuron deterioration associated with excitotoxicity observed in chronic and acute brain diseases or in aging (Chen et al., 2011;

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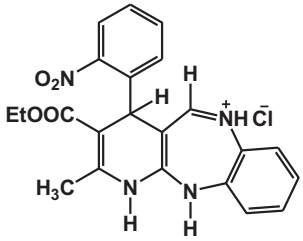

Fig. 1. Chemical structure of JM-20 (3-ethoxycarbonyl-2-methyl-4-(2-nitrophenyl)-4,11-dihydro-1*H*-pyrido[2,3-b][1,5]benzodiazepine).

Manzanero et al., 2013; Perluigi et al., 2013; Wang and Qin, 2010). Because mitochondria are the major sites for reactive oxygen species (ROS) generation in neurons (Lin and Beal, 2006), these organelles are a focus for determining new neuroprotective agents; evidence has accumulated that suggest a crucial role for mitochondrial defects in the pathogenesis of these conditions by initiating and promoting oxidative stress.

Recently, we obtained a new family of 1,5-benzodiazepines that structurally differ from the currently available 1,5-benzodiazepines due to the presence of a 1,4-dihydropyridine moiety fused to the benzodiazepine ring. JM-20 (3-ethoxycarbonyl-2-methyl-4-(2-nitrophenyl)-4,11-dihydro-1*H*-pyrido[2,3-*b*][1,5] benzodiazepine), which is a member of this compound family (Fig. 1), has an anxiolytic profile similar to that of diazepam (Figueredo et al., 2013). At a low micromolar concentration, we observed JM-20 prevents the Ca^{2+}-induced mitochondrial permeability transition, membrane potential dissipation, and pro-apoptotic protein cytochrome *c* release, and inhibits the hydrolytic activity of F_1F_0-ATP synthase in rat liver mitochondria (Nuñez-Figueredo et al., 2014a). We hypothesized that these mitoprotective effects may be involved in the neuroprotection elicited by JM-20 in several *in vitro* models relevant to cerebral ischemia (Nuñez-Figueredo et al., 2014a). We also reported an important protective effect of JM-20 against hydrogen peroxide (H_2O_2)- or glutamate-induced PC-12 cell death or KCN-mediated chemical hypoxia (Nuñez-Figueredo et al., 2014a). Because oxidative stress and mitochondrial impairment are key mediators of the ischemic process, particularly after reperfusion (Chan, 2005; Niizuma et al., 2009), we examined the antioxidant effects of JM-20 in rat brain mitochondria and in a synaptosomal preparation. To support the mitoprotective hypothesis of JM-20 neuroprotection, we also assessed its mitoprotective effect on Ca^{2+}-induced mitochondrial dysfunction using isolated mitochondria from rat brains. We observed that JM-20 acts equally as a potent antioxidant in mitochondria or in synaptosomes most likely due to its high electron-affinity at the organelle level. Our results also indicate that the mitoprotection against Ca^{2+}-mediated mitochondrial impairment may involve Ca^{2+} uptake inhibition at the mitochondrial level, which could play a role in the mechanism of action of JM-20 as a neuroprotectant.

2. Materials and methods

2.1. Compounds and reagents

All chemicals and enzymes used were of the highest grade available and were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified. Stock solutions of JM-20 were prepared daily in dimethyl sulfoxide (DMSO) and added to the reaction media at 1/1000 (v/v) dilution. Control experiments contained DMSO at 1/1000 dilution. JM-20 was synthesized, purified and characterized as previously reported (Figueredo et al., 2013).

2.2. Isolation of synaptosomal preparation and mitochondria

The forebrains from male Wistar rats (3–4 months old) were rapidly removed and homogenized in isolation buffer containing 0.32 M sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA) (K^+ salt), and 10 mM Tris–HCl (pH 7.4). The homogenate was centrifuged at 1330 × *g* for 3 min. The supernatant was carefully retained and centrifuged at 16,000 rpm (21,200 × *g*) for 10 min. The pellet was resuspended, carefully layered on the top of a discontinuous Percoll gradient and centrifuged for 5 min at 30,700 × *g* (Sims, 1990; Sims and Anderson, 2008). The synaptosomal and mitochondrial fractions were incubated in depolarized buffer (high K^+: 100 mM KCl, 75 mM mannitol, 25 mM sucrose, 5 mM phosphate, 0.05 mM EDTA, and 10 mM Tris–HCl, pH 7.4) and synaptosomal was also incubated in polarized buffer (high Na^+: 140 mM NaCl, 20 mM HEPES, 10 mM D-glucose, 1.2 mM Na_2HPO_4, 1 mM $MgCl_2$, 5 mM $NaHCO_3$, and 5 mM KCl, pH 7.4). The mitochondrial respiratory control ratio (state 3/state 4 respiratory rate ratio) was over 5, measured using 5 mM glutamate and 5 mM malate as NADH-linked substrates. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil.

2.3. ROS production

The mitochondrial release of H_2O_2 was assessed by the Amplex Red oxidation method. The synaptosomal or mitochondrial fractions (0.1 mg protein/ml) were incubated in the polarized or depolarized buffer supplemented with 10 μM Amplex Red and 2 units/ml of horseradish peroxidase (HRP). The fluorescence was monitored at excitation (563 nm) and emission wavelengths (587 nm) using a Spectra Max M5 microplate reader (Molecular Devices, Orleans Drive Sunnyvale, CA, USA). Catalase (EC 1.11.1.6) at 10 U/ml was used as a positive control. Each experiment was repeated 5 times with different synaptosomal or mitochondrial preparations.

2.4. Superoxide anion radical scavenging assay

Superoxide anion radical ($O_2^{\bullet-}$) generated by the xanthine/xanthine oxidase (EC 1.17.3.2) system was determined spectrophotometrically by monitoring the reduction products of nitroblue tetrazolium (NBT) (Nagai et al., 2001). The reaction mixture consisted of 3 mM xanthine, 3 mM EDTA, 15 mM NBT, and JM-20 (0.5, 1, 3, 5, 25 μM final concentrations) in 50 mM phosphate buffer (pH 7.4). After incubation at 25 °C for 2 min, the reaction was initiated by adding 0.05 U/ml xanthine oxidase and carried out at 25 °C for 10 min. The absorbance was measured at 560 nm. The reaction mixture without xanthine oxidase was used as a blank. Superoxide dismutase enzyme (SOD, EC 1.15.1.1) at 2 U/ml was used as positive control. The superoxide anion radical level was expressed as the percentage of NBT reduction *versus* control (set at 100%), which did not contain JM-20 or SOD.

The effect of JM-20 on enzyme activity was studied by estimating the uric acid formation from xanthine after 10 min incubation at 25 °C, while absorbance was measured at 295 nm. The absorbance was monitored using a Spectra Max M5 microplate reader (Molecular Devices, Orleans Drive Sunnyvale, CA, USA).

2.5. Oxygen (O_2) measurement

The O_2 consumption rates were measured polarographically using high-resolution respirometry (Oroboros Oxygraph-O2 K, Oroboros Instruments, Innsbruck, Austria). Synaptosomal or mitochondrial fractions (0.1 mg/ml) were incubated with polarized