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Peripheral benzodiazepine receptor ligand, PK11195 induces mitochondria cytochrome *c* release and dissipation of mitochondria potential via induction of mitochondria permeability transition

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Abstract

Mitochondrial permeability transition pore plays an important role in the processes of cell apoptosis and necrosis. The peripheral benzodiazepine receptor, a mitochondria outer-membrane protein, is involved in the regulation of mitochondrial permeability transition. In the present study, we test if PK11195, a peripheral benzodiazepine receptor ligand, can lead to the opening of mitochondrial permeability transition pores, and subsequently causes mitochondria cytochrome *c* loss and mitochondria uncoupling. In isolated cardiac mitochondria, PK11195 (50, 100, 200 μM) caused a dose-dependent mitochondrial swelling, cytochrome *c* loss, and the dissipation of mitochondrial potential. Cyclosporin A (0.2 μM), a specific inhibitor of mitochondrial permeability transition, completely prevented the mitochondrial swelling induced by PK11195, and maintained the cytochrome *c* content and membrane potential. These data suggest that peripheral benzodiazepine receptor ligand, PK11195 caused mitochondrial uncoupling and cytochrome *c* loss via induction of mitochondrial permeability transition.

Keywords: Peripheral benzodiazepine receptor; PK11195; Mitochondria permeability transition

1. Introduction

Mitochondria play a key role in cell apoptosis and necrosis. Cytochrome *c* release is a critical early event to trigger apoptosis cascade. Opening of mitochondrial permeability transition pores (mPTP) is an important step in this process (Crompton, 1999; Halestrap et al., 1998, 2004; Lemasters et al., 1998). The mPTP is considered to include the voltage-dependent anion channel (VDAC, located in mitochondrial outer-membrane), the adenine nucleotide translocator (ANT, across the mitochondrial outer and inner membrane at mitochondrial contact site), and the cyclophilin D (CyP-D) in the matrix. Recent studies showed that peripheral benzodiazepine receptor, hexokinase, and creatine kinase, may also be involved in the mPTP (Halestrap, 2002). The mitochondrial permeability transition (MPT) caused by the opening of mPTP may act as a "central executioner" of cells subjected to a range of insults (such as oxidative stress, growth factor removal, or exposures to cytokines), determining not only whether cells live or die, but also whether death occurs by apoptosis or necrosis (Hirsch et al., 1997; Susin et al., 1997).

The peripheral benzodiazepine receptor is present in peripheral tissues such as adrenals, kidney and heart, as well as in the brain (Anholt et al., 1985; De Souza et al., 1985). The peripheral benzodiazepine receptor is different from the central benzodiazepine receptor, which is coupled to GABA receptors and responsible to the classical sedative, anxiolytic and anticonvulsant effect (Gavish et al., 1992; McEnery et al., 1992). Peripheral benzodiazepine receptor is a 169-amino acid protein with five trans-membrane domains associated with the mitochondrial outer membrane (Liauzun et al., 1998) which has been suggested to be involved in the control of several mitochondrial functions including the respiratory chain and ion channel activities, in the regulation of apoptosis, which occurs during cardiac injury (Bono et al., 1999; Leducq et al.,

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2003) and in the modulation of immune functions, steroidogenesis and neurodegenerative process (Ferzaz et al., 2002; Galiegue et al., 2003). Recently, peripheral benzodiazepine receptor is reported to be implicated in mPTP formation (Beutner et al., 1996; Marzo et al., 1998; Zamzami et al., 1998). Cytochrome *c* loss from mitochondria is an important early event to trigger apoptosis. Opening of mPTP is well known to cause cytochrome *c* loss from mitochondria. Based on the entropy-driven and enthalpy-driven nature of ligand receptor interactions, Isoquinoline carboxamide PK11195 has been classified as an antagonist (Le Fur et al., 1983). In the current study, we use PK11195 to test if blockade of peripheral benzodiazepine receptor is able to induce MPT and cause cytochrome *c* loss and mitochondria uncoupling.

2. Materials and methods

2.1. Reagents and animal

1-(2-Chlorophenyl-*N*-methyl-1-methylpropyl)-3-isoquinolinecarboxamide (PK11195) and Subtilisin Carlsberg were purchased from the Sigma company (St. Louis, MO). Cyclosporin A was obtained from Fluka Biochemical company (Milwaukee, WI). Mouse monoclonal anti-cytochrome *c* antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse IgG and horseradish peroxidase (HRP)-linked antibody were purchased from Cell signaling Technology (Beverly, MA). 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) were purchased from Molecular Probes, INC. (Eugene, OR). All other compounds were purchased from chemical sources. Male Sprague–Dawley (SD) rats weighing 200–250 g were supplied by the Animal Center of Xuzhou Medical College, China. All experiments were approved by the Animal Care and Use Committee at the College.

2.2. Preparation of rat heart mitochondria

Cardiac mitochondria were isolated from male SD rats using Vin's protocol (Vin et al., 2003) with minor modifications. Briefly, rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (50 mg/kg). Hearts were removed, homogenized in ice-cold buffer A (100 mM sucrose, 46 mM KCl, 10 mM TES, 2 mM EGTA, 0.5% (w/v) BSA, 5 mM $MgCl_2$, 1 mM ATP, pH 7.2) containing 2% w/v protease (Subtilisin Carlsberg), and incubated on ice for 5 min. In order to remove protease from the medium, the supernatant was centrifuged at 8500 *g* for 15 min. The pellet was resuspended in the ice-cold buffer B without protease. The homogenate was centrifuged at 500 *g* for 10 min at 4 °C. The supernatant was filtered through muslin and centrifuged at 8000 *g* for 15 min. Mitochondrial pellets were resuspended in buffer B and kept in ice for the rest of the experiment. All mitochondrial isolation procedures were carried out at 4 °C. Protein concentration was determined by modified Lowry's method (Peterson, 1977), using BSA as the standard.

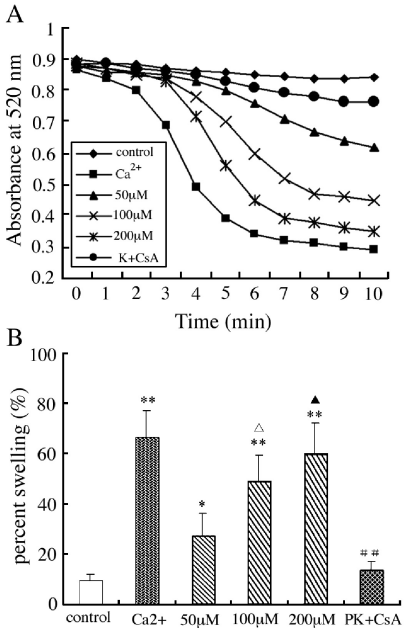

Fig. 1. Effects of PK11195 on mitochondrial permeability transition. A: Mitochondria were incubated in the presence of 50, 100, or 200 μM PK11195 or 100 μM PK11195 plus 0.2 μM Cyclosporin A (PK+CsA group), PK11195 induced mitochondrial swelling in a dose-dependent manner. Mitochondrial swelling induced by PK11195 (100 μM) was inhibited by 0.2 μM Cyclosporin A, swelling was measured by absorbance at 520 nm. B: Quantitative assessment of effects of PK11195 on MPT. Results are expressed as a ratio of the decrease of absorbance to the initial absorbance. Results are expressed as the mean±S.E.M (n=6). *p<0.05 and **P<0.01 versus control group; $^{\triangle}P$<0.05 versus 50 μM group; $^{\blacktriangle}P$<0.05 and $^{\#\#}P$<0.01 versus 100 μM group.

2.3. Determination of mitochondrial permeability transition

The MPT was detected by the change of absorbance after chemical addition with spectrophotometer (Clarke et al., 2002; Di Lisa et al., 2001; Korge et al., 2002). MPT causes mitochondrial swelling, and a decrease in absorbance at 520 nm (Abs520 nm). Fresh heart mitochondria were added to a buffer containing 300 mM sucrose, 5 mM succinate and 10 mM MOPS, pH 7.4 with Tris, and the final volume is 1.0 ml and protein concentration is 1 mg/ml. The reference cuvette has the same buffer without mitochondria. The change of absorbance was measured for 10 min with spectrophotometer at 520 nm and the temperature was maintained at 30 °C during assay. Different concentration of PK11195 (50, 100, 200 μM) was added into incubation buffer, respectively. In additional experiments, 0.2 μM cyclosporin A, an inhibitor of MPT was added 5 min before the addition of 100 M PK11195. Calcium (150 μM) induced MPT opening as positive control group. At the end of assay, the mitochondria and supernatant was collected by centrifugation for the following analysis.

2.4. Electron microscopy microscope analysis

Mitochondria were fixed by mixing equal amounts (v/v) of the mitochondrial suspensions with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. After a primary fixation of