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European Journal of Pharmacology 560 (2007) 117–122
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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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Received 27 September 2006; received in revised form 12 December 2006; accepted 18 December 2006
Available online 19 January 2007

**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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doi:10.1016/j.ejphar.2006.12.027

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.

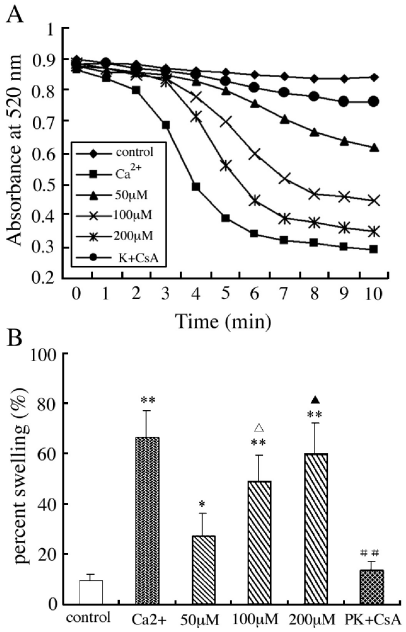


Fig. 1. Effects of PK11195 on mitochondrial permeability transition. A: Mitochondria were incubated in the presence of 50, 100, or 200 μM PK11195 or 100 μM PK11195 plus 0.2 μM Cyclosporin A (PK+CsA group), PK11195 induced mitochondrial swelling in a dose-dependent manner. Mitochondrial swelling induced 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

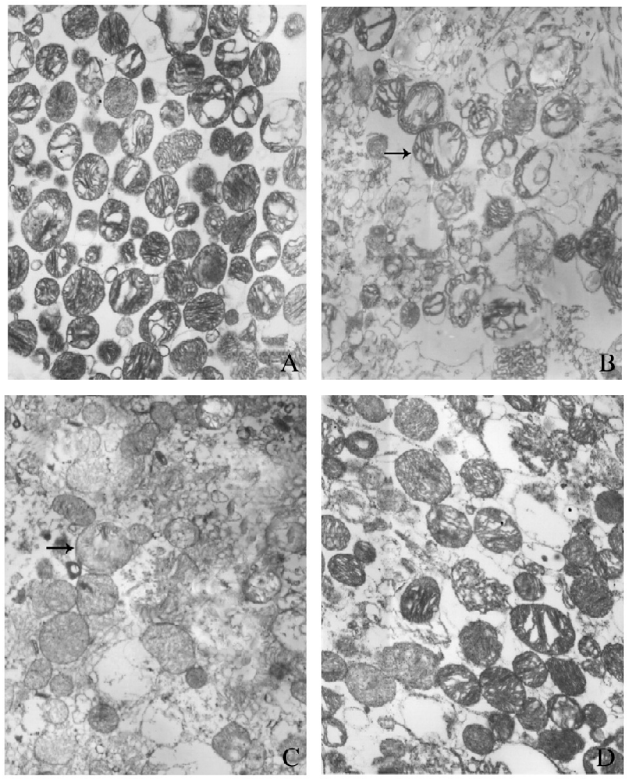


Fig. 2. Effects of PK11195 on ultrastructural observation of mitochondria. (A) Untreated mitochondria; (B) mitochondria exposed to 150 μM $Ca^{2+}$; (C) mitochondria exposed to 100 μM PK11195; (D) mitochondria exposed to 100 μM PK11195 plus Cyclosporin A; (arrow) typical swelling mitochondria. Electron micrographs are at a magnification of ×8000.

approximately 5 min, the mitochondria were pelleted by centrifugation and subsequently fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h at 4 °C. After rinsing in cacodylate buffer, mitochondrial pellets were postfixed in 1% cacodylate-buffered osmium tetroxide for 2 h at room temperature, then dehydrated in a graded series of ethanol, briefly transferred to propylene oxide, and embedded in Epon–Araldite. Ultrathin sections (60–80 nm thick) were cut with a diamond knife, placed on Formvar carbon-coated copper grids (200 mesh), stained with uranyl acetate and lead citrate, and observed with H-600 transmission electron microscope.

### 2.5. Mitochondrial cytochrome c release

The mitochondrial pellet and supernatant was separated by centrifugation. Cytochrome *c* content in the pellet and supernatant was determined using Western Blotting. Mitochondrial proteins (50 to 100 μg) were dissolved in sample buffer and boiled for 5 min, were subjected to 15% sod. dodecyl sulfonate (SDS)–polyacrylamide gels. After electrophoresis, the samples were transferred to a nitrocellulose sheet. The blots were then blocked with 5% nonfat milk and incubated with the primary and HRP-conjugated secondary antibodies. The first antibody was mouse monoclonal antibody to denatured cytochrome *c* (1:1000). The secondary antibody was horseradish peroxidase-conjugated goat anti-mouse antibody (1:2000). The bands were detected by NBT/BCIP, laser scanning densitometry was used for the semi-quantitative determination of the proteins.

### 2.6. Mitochondrial membrane potential

The changes in mitochondrial membrane potential were monitored with the dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) (Reers et al., 1991). Mitochondria were stained with JC-1 (5 μM) at 37 °C for 15 min and rinsed with Tyrode solution by centrifugation. The intensity of fluorescence was determined using a laser scanning confocal microscope (Germany, Lecia Tcs SP2). JC-1 monomer (green) fluorescence was excited at 488 nm and detected the emissions from 505 to 530 nm. JC-1 aggregate (red) fluorescence was excited at 543 nm and recorded the emissions fluorescence at 560 nm. Ten or more areas were selected from each image and the average intensity for each region was quantified (Lecia, LCS Universal Imaging). The ratio of red to green fluorescence for

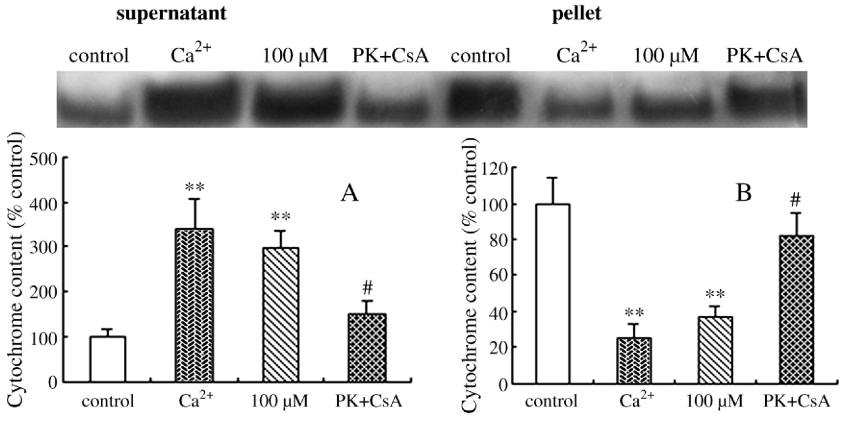


Fig. 3. Effects of PK11195 on the release of cytochrome *c* from mitochondria. Top: Western blotting for cytochrome *c* was performed on supernatant (left) and pellet (right) of isolated mitochondrial. A and B show the semi-quantitative laser densitometric analyses (bottom) of Western blots of CytoC in cytosolic and mitochondrial fractions respectively. $^{**}P<0.01$ versus control group; $^{\#}P<0.05$ versus 100 μM group.

Table 1
Effects of PK11195 on mitochondrial membrane potential

| Groups | JC-1 ratio (aggregate/monomer) |
|---|---|
| Control | 0.94±0.23 |
| $Ca^{2+}$ | 0.25±0.05 |
| 100 μM | 0.34±0.09[a] |
| PK+CsA | 0.62±0.13[b] |

Quantification of mitochondrial membrane potential expressed as a ratio of JC-1 aggregate to monomer fluorescence in different treatments ($n$=10 images). [a]$P$<0.01 versus control group; [b]$P$<0.05 versus 100 μM group.

each region was calculated. The decreased ratio was interpreted as a decrease in mitochondrial membrane potential (Troyan et al., 1997).

### 2.7. Statistical analysis

Statistical analysis was performed by one-way ANOVA, Newman–Keuls was applied to test for the differences between individual groups. $P$<0.05 was considered statistically significant.

## 3. Results

### 3.1. Effects of PK11195 on mitochondrial permeability transition

Treatment mitochondria with 50, 100, or 200 μM PK11195 led to a significant decrease in absorbance at 520 nm compared to non-chemical-treated mitochondria (control group). 100 μM PK11195 caused more mitochondria swelling than 50 μM PK11195, and 200 μM PK11195 caused more mitochondria swilling than 100 μM PK11195. Cyclosporin A (0.2 μM) prevented MPT induced by PK11195 (100 μM) (Fig. 1A, B).

### 3.2. Effects of PK11195 on ultrastructural observation of mitochondria

The mitochondrial samples were observed with a transmission electron microscope. Control group showed well-preserved mitochondria with an electron-dense matrix and well-arranged cristae (Fig. 2A). In the specimens treated with $Ca^{2+}$ (Fig. 2B) or 100 μM PK11195 (Fig. 2C), most of mitochondria showed morphological change. In particular, they showed swelling, hypertrophy, cristolysis, and matrix dilution. In the presence of 100 μM PK11195 plus cyclosporin A (Fig. 2D), the most of mitochondria displayed the characteristic ultrastructure of the intact organelle.

### 3.3. Effects of PK11195 on cytochrome c release from mitochondrial

PK11195 resulted in the translocation of cytochrome $c$ from the mitochondria to the cytosol. Compared to untreated mitochondria, PK11195 (100 μM) treatment markedly decreased cytochrome $c$ content in mitochondrial pellets, whereas the content of cytochrome $c$ in cytosol was significantly increased. Cyclosporin A treatment prevented cytochrome $c$ loss from mitochondria induced by PK11195 (Fig. 3).

### 3.4. Effects of PK11195 on mitochondrial membrane potential

The exposure of mitochondria to PK11195 caused dissipation of mitochondrial membrane potential significantly (Table 1). Mitochondrial membrane potential in 100 μM PK11195-treated mitochondria is 0.34±0.09, much lower than that in untreated group (0.94±0.23). The mitochondrial membrane potential was maintained in PK11195 with cyclosporin A (0.62±0.13). It is not surprising to see that calcium overloading led to mitochondrial uncoupling in our system (0.25±0.05).

## 4. Discussion

In the physiological condition, $Ca^{2+}$ overloading and ROS generation are the most important triggers for MPT opening. [$Ca^{2+}$] over 100 μM was necessary to stimulate MPT (Schild et al., 2001). Thus, we used 150 μM $Ca^{2+}$ to trigger MPT in our system. MPT can be opened in two modes: low conductance and high conductance. Under the low-conductance mode, MPT opening is reversible, allowing permeation of small solutes that depolarize potential transiently. In the high-conductance mode, MPT openings fall into two classes: transient and long-lasting, the latter often being irreversible. Under this condition, ions and water flux into mitochondrial matrix and lead to matrix swell, eventually cause mitochondrial outer-membrane rupture. Outer-membrane leakage makes it possible for the proapoptotic molecules such as cytochrome $c$, Smac/DIABLO, AIF, Endo G, and Htra2/Omi to be released from mitochondria and induce cell apoptosis. The peripheral benzodiazepine receptor is located in the outer mitochondrial membrane and has been proposed to be a component of mPTP. Our data showed that PK11195 induced MPT without any calcium addition. This is consistent with the putative functions have suggested for the peripheral benzodiazepine receptor as modulation of voltage-dependent calcium channels (Bolger et al., 1990). PK11195 has been shown to possess affinity for voltage-operated calcium channels in the plasma membrane (Campiani et al., 1996). PK11195 may also have direct effects on voltage-operated calcium channels, apart from their effects via peripheral benzodiazepine receptor, probably either by facilitating endogenous calcium binding to its own sites and/or by acting directly on the mPTP. This hypothesis is in agreement with the suggested molecular pore structure, consisting of two copies of the 18-kDa peptide (PK11195-binding site) associated with dimer porin molecules and with two molecules of the adenine nucleotide carrier (Zoratti and Szabo, 1995). Cyclosporin A blocks PK11195 induced MPT, suggesting that PK11195 induced mitochondrial swell with MPT opening.

According to Martinou et al. (2000), the most common mode of cytochrome $c$ release is following the outer mitochondrial membrane rupture as a result of mitochondrial matrix swelling

due to the opening of the MPT. In our study, the extent of cytochrome *c* loss is consistent with the mitochondrial matrix swelling, as shown by mitochondrial ultrastructural alterations analysis. PK11195-treated mitochondria showed complete or partially complete round structures, consistent with their becoming permeable, and swollen mitochondria, in contrast with well-preserved control organelles, showing an electron-dense matrix and well-arranged cristae. Some studies also suggested that PK11195 induce mitochondrial permeability transition in isolated cardiac mitochondria as well as in cardiac myocytes (Chelli et al., 2001).

MPT opening leads to mitochondrial inner-membrane leakage. Thus, proton, pumped from mitochondrial matrix into inter-membrane space, can flow back to mitochondrial matrix without generating ATP. This leads to mitochondrial uncoupling and dissipation of the mitochondrial membrane potential (Green and Reed, 1998; Kroemer et al., 1998). Using fluorescence dye JC-1 to reflect the change of mitochondrial membrane potential (Salvioli et al., 1997), we found that treatment of isolated rat mitochondria with PK11195 resulted in the dissipation of mitochondrial potential dissipation, supporting that PK11195 induced MPT. Cyclosporin A blocks PK11195 induced mitochondrial swell and cytochrome *c* loss, further supporting that PK11195 is a MPT inducer in our system.

Cytochrome *c* plays a shuttle role in electron transportation between complex III and complex IV at mitochondrial electron transport chain. The loss of cytochrome *c* will block electron transport at the distal electron transport chain, and lead to the electron accumulated at respiratory complex at upstream electron transport chain. This will facilitate electron leakage and reactive oxygen species generation from mitochondrial respiratory chain (Borutaite and Brown, 2003).

In conclusion, blockade of peripheral benzodiazepine receptor with PK11195 triggers MPT, caused subsequent mitochondrial uncoupling and cytochrome *c* loss. Modulation of peripheral benzodiazepine receptor may represent an alternative way to regulate MPT during pathophysiological condition.

## Acknowledgement

This work was supported by the National Natural Science Foundation of China (No. 30571782) and the Foundation of Educational Committee of Jiangsu Province (No. 05KJD32023).

We are extremely grateful to Qun Chen, M.D., Ph.D. (Case Western Reserve University, Louis Stokes VA Medical Center) for the assistance in the revision of the manuscript.

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