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Melatonin modulates permeability transition pore and 5-hydroxydecanoate induced K_{ATP} channel inhibition in isolated brain mitochondria

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ABSTRACT

There is increasing recognition of the magnitude of mitochondria in neurodegenerative disorders. Mitochondria play a key role in apoptotic and necrotic cell death. Melatonin (Mel), an indoleamine produced in several organs including the pineal gland has been known for its neuroprotective actions. In our study, we have investigated whether the mitochondrial ATP sensitive potassium (mtK_{ATP}) channel blocker 5-hydroxydecanoate (5-HD) and calcium (Ca^{2+}) affects permeability transition pore (PTP) alterations in isolated brain mitochondria treated with melatonin (Mel) and cyclosporin A (CsA). Mitochondrial swelling, mitochondrial membrane potential ($\Delta\psi_m$), ROS measurement and mitochondrial respiration were evaluated in isolated brain mitochondria. In our results, mitochondrial swelling stimulated by exposing Ca^{2+} ions and 5-HD associated by mPTP opening as depicted by modulation of CsA and Mel. In addition, Ca^{2+} and 5-HD decreased $\Delta\psi_m$, depleted intracellular ROS, and inhibition of mitochondrial respiration (state 3 and state 4) in isolated brain mitochondria. Addition of Mel and CsA has shown significant restoration in mitochondrial swelling, $\Delta\psi_m$, intracellular ROS measurement and mitochondrial respiration in isolated brain mitochondria. Therefore, we speculate the modulatory effect of Mel and CsA in mitochondria treated with 5-HD and Ca^{2+} hinders the mPTP-mediated mitochondrial dysfunction and cellular oxidative stress. We conclude that inhibition of mPT is one likely mechanism of CsA's and its neuroprotective actions. Development of neuroprotective agents including Mel targeting the mPTP therefore bears hope for future treatment of severe neurodegenerative diseases.

1. Introduction

There is growing identity of the magnitude of mitochondria in neurodegenerative ailments. Indeed, over the past two decades or subsequently, mitochondrial dysfunction has increased from end juncture epiphenomenon owing to intermediate modulator to an insight that it may be a crucial effector of disease. Mitochondria play a key role in both apoptotic and necrotic cell death (Waseem and Parvez, 2015). The release of pro-apoptotic proteins including cytochrome *c* (cyt-*c*) is known as a mitochondrial substrate that finally activates the downstream effectors caspases leading to cellular obliteration or damage (Molpeceres et al., 2007). This process is linked with opening of the mitochondrial PTP, a "megachannel" across both the inner and the outer mitochondrial membrane.

It has been implicated that PTP can exist in several sub-conductance states between 40 and 1500 pS, and can be pharmacologically induced by a large variety of substances. In an open state, it allows the molecules or ions up to a molecular weight of about 1.5 kDa to permeate (Chinopoulos and Adam-Vizi, 2012) across the pore. Therefore, PTP opening leads to a collapse of the mitochondrial membrane potential, causing alteration of oxidative phosphorylation and adenosine triphosphate (ATP) depletion (Vianello et al., 2012). It has been reported that Ca^{2+} is known to be the foremost physiological activator of the PTP. Other factors that are established to cause PTP activation are ROS and RNS, mitochondrial depolarization and high levels of inorganic phosphate (Pi). On the contrary, hyperpolarization of the mitochondrial membrane in addition to high levels of ATP and adenosine-5-diphosphate (ADP) are recognized to block the pore (Kupsch et al., 2007).

In view of the fact that PTP-mediated cell death is involved in the advancement of several diseases, much endeavour is spent to explore modulators of this phenomenon. In recent years, many investigators have implicated that an instant mitochondrial ion channel, the ATP sensitive potassium channel (mtK_{ATP} channel) of the inner mitochondrial membrane interacts with the PTP (Cheng et al., 2010). It was shown that mtK_{ATP} are important determinants of resistance to ischemic damage (Dahlem et al., 2006) and apoptosis (Racay et al., 2009). They may also be able to diminish ischemic injury. The ameliorative consequences

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of ischemic injury can be eliminated by administration of inhibitors of mtK_{ATP} channels (Kupsch et al., 2007), such as glibenclamide and 5-hydroxydecanoate (5-HD). The mechanisms that are responsible for such effects of mtK_{ATP} channel modulators are still debatable. Recent studies imply a connection between the state of the mtK_{ATP} channel and the open probability of the PTP (Prendes et al., 2014). Among these actions, activation by Ca^{2+} and blockade by CsA are studied best (Akopova et al., 2011). As the PTP seems to be a key player in the apoptotic cascade, a direct or an indirect blocker of the PTP is attractive. Some neuroprotective substances including dopamine agonist (Ropinirole and Pramipexole), and melatonin (Mel) were identified to fulfill this decisive factor (Andrabi et al., 2004; Parvez et al., 2010; Siemen and Ziemer, 2013).

Mel is the major secretory product of the pineal gland, and is well known for its neuroprotective effects (Jumnongprakhon et al., 2014). As an antioxidant molecule, it has been identified in species throughout the animal kingdom and, in the last decade, it has also been found in plants. It is substantially effectual for preventing loss of neurons in in vitro and in vivo models of brain impairment where oxidative stress is involved. It has been clearly demonstrated that Mel has the propensity to ameliorate brain damage by enhancing antioxidant capacity thereby attenuating free radical induced damage and can therefore be employed in management of neuronal or brain damage wherein mitochondrial functions are compromised (Manchester et al., 2015). It has been investigated that the ameliorative effect of Mel against mitochondrial dysfunction may be due to the direct interaction with mPTP and/or by preserving mitochondrial redox status (Hibaoui et al., 2009). Mel instantly crosses to the BBB (Naseem and Parvez, 2014) and was found to minimize the infarct area and neuronal mPTP (Andrabi et al., 2004). It contributes to the elevation were diminished by Mel in cultured neurons, similar to the pattern seen with CsA. Mel is well reported as an endogenous modulator and a provocation of brain impairment subsequent to ischemic seizures have also been identified in rats that are deficient in Mel production (Virmani et al., 2003; Andrabi et al., 2015; Rasheed et al., 2016). Furthermore, the direct antioxidant capacity and numerous other mechanisms such as interactions with Ca^{2+} dependent proteins are also regarded to be involved in the Mel-mediated neuroprotection. A mechanism of a direct mPTP inhibition by Mel may be included to the elucidation for its potential neuroprotective action as mitochondria are promptly regarded as the cellular communicating stations that amalgamate numerous signals including free radical production and Ca^{2+} release and thereby alteration in mtK_{ATP} channel. There is very less report available for the effects of Mel against neuronal injury mediated by mtK_{ATP} channels and PTP. In our study, we investigated whether the mtK_{ATP} channel inhibitor 5-HD and Ca^{2+} affects PTP alterations in isolated brain mitochondria treated with Mel and CsA. It raises the question if mPTP inhibition and activation is one of the mechanisms that mediate the neuroprotective effect of Mel.

2. Methods

2.1. Reagents

Adenosine-5-diphosphate (ADP), bovine serum albumin (BSA), butylated hydroxy toluene (BHT), calcium chloride, dihydroethidium (DHE), disodium hydrogen phosphate, dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), ethylene glycol-O,-O′-bis, (2-aminoethyl) tetraacetic acid (EGTA), ethanol, glutamate, malate, potassium chloride, sodium dihydrogen phosphate, sodium succinate, tetramethylrhodamine ethyl ester (TMRE), trichloro acetic acid (TCA), were purchased from Sigma Chemicals, St. Louis, MO, USA (Invitrogen, USA) and Merck, India respectively. Routine chemicals were purchased from Sigma Chemicals, St. Louis, MO, USA, SRL Pvt. Ltd. (Mumbai, India) and Merck Limited (Mumbai, India). Oxaliplatin was obtained as gift sample from Dabur Pharmaceuticals Pvt. Ltd. (Delhi, India). Melatonin, cyclosporin A (CsA), cyclosporin H (CsH) and 5-hydroxydecanoic acid (5-HD) were purchased from Sigma Chemicals, St. Louis, MO, USA and Santa Cruz Biotechnology Inc., USA respectively.

2.2. Incubation procedures

Rat brain mitochondria were isolated and incubated with Ca^{2+} and 5-HD followed by pre-incubation with CsA or CsH and Mel at 25 °C temperature in swelling medium (125 mM KCl, 20 mM Tris, 1 mM $MgCl_2$, 1 μM EGTA, 5 mM glutamate, 5 mM malate and 10 mM Pi, pH 7.2), respiratory buffer (225 mM sucrose, 1 mM EDTA, 10 mM KCl, 5 mM $MgCl_2$, 10 mM Tris and 5 mM Pi, pH 7.4), standard medium (3 mM HEPES buffer, pH 7.4, containing 70 mM sucrose, 230 mM mannitol, 1 μM EDTA) and succinate at 5 mM and 10 mM. Other experimental conditions are described in the figures and legends. The dose of experimental drugs was based on previously published reports (Kupsch et al., 2007; Virmani et al., 2003; Waldmeier et al., 2002). Briefly, CsA, CsH and Mel were dissolved in ethanol to obtain 1 mM and 10 mM stock solutions respectively, which was diluted appropriately at the time of incubation. Isolated brain mitochondria were pre-incubated with CsA (10 μM), CsH (10 μM) and Mel (1 mM) for 30 min prior to the addition of 500 μM of Ca^{2+} and 5-HD.

2.3. Animals

Male Wistar rats weighing between 150 and 200 g were obtained from the Central Animal House Facility (CAHF) of Jamia Hamdard (Hamdard University), New Delhi, India. The rats were acclimatized for a week prior to the start of the experiments. They were kept at ambient temperature (22 ± 2 °C) with relative humidity at 65 ± 10% and at a photoperiod of 12 h light/dark cycle. Standard pellet rodent diet and water were provided to the animals ad libitum. The animal handling and treatment strategies including experiments were carried out according to the standard guidelines of Institutional Animal Ethics Committee (CPCSEA, registration number: 173/CPCSEA and date of registration: Jan 28th, 2000; Project Proposal number 764).

2.4. Isolation and preparation of brain mitochondria

Rat brain mitochondria were isolated by the differential centrifugation method (Kupsch et al., 2007; Ling et al., 2012). Briefly, the brain from the rat were separated and homogenized in 10 volumes (1:10 *w*/*v*) by using high performance digital based homogenizer (IKA, T-25 digital ULTRA TURRAX, Bangalore, Karnataka, India) in an ice-cold isolation buffer containing 250 mM sucrose, 10 mM HEPES, 1 mM EGTA and 0.1% fat free BSA adjusted by Tris to pH 7.4, and centrifuged at 1000*g* for 8 min at 4 °C. The supernatant was collected and centrifuged at 10,000*g* for 10 min at 4 °C. Thereafter, the obtained pellet was resuspended and washed twice with washing medium containing 250 mM sucrose, 10 mM HEPES and 0.1 mM EGTA adjusted by Tris to pH 7.4, and centrifuged at 12,300 ×*g* for 10 min. Finally, the pellet was resuspended again in an isolation medium containing 250 mM sucrose, 10 mM HEPES and 0.1% fat free BSA adjusted by Tris to pH 7.4, centrifuged at 12,300*g* for 10 min. The protein concentration of the stock suspension was 2–3 mg/ml as determined by Waseem and Parvez (2015).

2.5. Measurements of mitochondrial swelling

Mitochondrial protein content was determined by the Bradford method (1976). BSA (0.01%) was used as a standard. Aliquots of brain mitochondria (300 μg) were suspended in 1 ml of swelling medium (pH 7.2) containing 125 mM KCl, 20 mM Tris, 1 mM $MgCl_2$, 1 μM EGTA, 5 mM glutamate, 5 mM malate and 10 mM Pi (Kupsch et al., 2007). Mitochondrial swelling was monitored by measurement of light scattering at 540 nm by a Hitachi (High Technology Corporation, Tokyo, Japan) spectrophotometer. Permeability transition was induced