ISSN 1009－3095 *Journal of Zhejiang University SCIENCE* V.3，No.4，P.475－479，Sep.－Oct.，2002
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Influence of green tea polyphenols on mitochondrial permeability transition pore and Ca^{2+} transport

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Received May 6，2001；revision accepted Sep.26，2001

Abstract： The authors investigated the influence of green tea polyphenols (GTPs) on the liver mitochondria permeability transition pore (PTP) opening through mitochondria swelling and change of mitochondria membrane potential. The data showed that GTPs had obvious protective effect on the Ca^{2+}-induced PTP opening in a dose-dependent manner detected by mitochondria swelling. The results were obtained by measuring the change of mitochondria membrane potential through Rh 123. Further experiments were conducted to examine the detailed influence of GTPs on Ca^{2+} import and export of mitochondria. The results showed that GTPs had remarkably inhibitory effect on the Ca^{2+}-induced Ca^{2+} import in mitochondria; and that they could accelerate Ca^{2+}-release from mitochondria. Our data provide an alternate interpretation of the potent protective function of GTPs on cell against apoptosis.

Key words： Mitochondria，PTP green tea polyphenols，mCICR，Ca^{2+} transport，Mitochondria $\Delta\Psi_m$
Document code： A **CLC number：** Q244

INTRODUCTION

Green tea is one of the most popular beverages in the world. A number of epidemiological studies showed that it had inhibitory effects against many diseases such as a variety of cancers and neurodegenerative diseases (Bushman et al.，1998；Ahmad et al.，1999). Green tea polyphenols (GTPs) is a mixture of several catechins including (-)-epigallocatechin gallate (EGCG)，ECG，EGC and EC (Bushman et al.，1998). In recent years，the cancer-therapeutic potential of GTPs is increasingly being appreciated. The U.S. National Cancer Institute plans to develop tea compounds as chemical preventive drugs for human cancer (Steele et al.，1999). Such drugs has also been used in China and other countries for ordinary health protection. However，the mechanism of how GTPs act is still poorly understood.

Mitochondria，which were once simply thought to be generators of energy for a cell，have been considered as important sensors and amplifiers in various physiological and pathological processes. A most important development is the recognition that mitochondria play a central role in the regulation of cell death. Many mitochondria defects had been shown to contribute to the pathogenesis of human degenerative disease，aging and cancer，etc. (Green et al.，1998；Lenaz，1998).

Permeability transition pore (PTP) is a portentous pore located at the contact site between the inner and outer mitochondria membranes，and is central to the mitochondria function in cellular activity (Luchas et al.，1997；Zoratti et al.，1995). PTP opening has two states：low- and high- conductance state. The low-conductance state PTP (Ca^{2+}-induced release of Ca^{2+} from mitochondria (mCICR)) is a mitochondria Ca^{2+} release channel in the regulation of the physiological cellular Ca^{2+} signal. High-conductance state (PTP) acts as a good candidate pathway for the release of Cyt. C or AIF (apoptosis inducing factor) (Luchas et al.，1997)，play universal role of central executioner in apoptosis. Mitochondria PTP opening and mitochondria Ca^{2+} transport are drawing more and more attention (Lemasters et al.，1998). Here，we present the firstly report that GTPs have significant

influence on the mitochondria PTP and mCICR.

MATERIALS AND METHODS

Materials

Hepes, Mannitol and bovine serum albumin (BSA) were obtained from Serva. GTPs was extracted from Chinese green tea. Other reagents were local products of analytical grade.

Isolation of mitochondria

Liver mitochondria were obtained (from an about 200 g fasted female Wistar rat) overnight by a standard procedure (Luo et al., 1998) using 225 mmol/L mannitol, 75 mmol/L sucrose, 20 mmol/L Hepes, 0.5 mmol/L EGTA and 0.1% BSA supplemented with protease inhibitors as isolation medium (pH 7.2). The mitochondria were washed and then resuspended gently in MSB buffer (220 mmol/L mannitol, 70 mmol/L sucrose, 5 mmol/LHepes, pH 7.2, 0.1 mg/ml BSA) and stored in ice for up to 6 hours.

Assay for mitochondria permeability transition

Isolated mitochondria (1 mg/ml) were incubated at 20℃ in resuspension buffer. $\Delta\Psi_m$ was assessed by measuring the $\Delta\Psi_m$ - dependent uptake of rhodamine 123 (Rh 123) by using a fluorescence spectrophotometer (Hitachi F-4010) with excitation at 503 nm and emission at 533 nm after addition of 1 μmol/L Rh 123 to the mitochondria suspension (Fontaime et al., 1998). Mitochondria swelling as monitored from the decrease in OD_{540} at 20℃ by using spectrophotometer (Shimadzu UV-2101PC).

Determination of Ca^{2+} uptake and release by mitochondria

Arsenazo Ⅲ (AⅢ) is a kind of Ca^{2+} probe characterized by Ca^{2+} sensitivity, high selectivity, and membrane impermeability Ca^{2+} movement across the inner mitochondria membrane was followed at 20℃ by dual wavelength spectrophotometer (Shimadzu 557), using AⅢ as Ca^{2+} indicator as described by Frei et al. (Frei et al., 1985). The variation of Ca^{2+} in reactive system showing fluctuations of mitochondria Ca^{2+} were calibrated by using the resulting changes of absorbance of AⅢ at 685~675nm.

RESULTS

GTPs have obvious inhibitory effect on PTP opening

PTP opening can lead to increased permeability of the inner membrane, resulting in influx of medium and mitochondria swelling, and decreasing optical density (OD) of the mitochondria. Treatment with 100 μmol/L Ca^{2+} decreased the OD of the mitochondria (Fig.1). After different concentration of GTPs was introduced to pre-incubate with the teaction system, a certain amount of Ca^{2+} was added to induce mitochondria swelling. Surprisingly, GTPs had clear dose-dependent inhibitory effect on the mitochondria swelling as shown in Fig.1. When the concentration of GTPs was up to 500 μmol/L, the PTP opening was almost inhibited completely; then mitochondria swelling no longer occurred even when more Ca^{2+} was added. The protevtive action of GTPs occurred within 1 minute of preincubation.

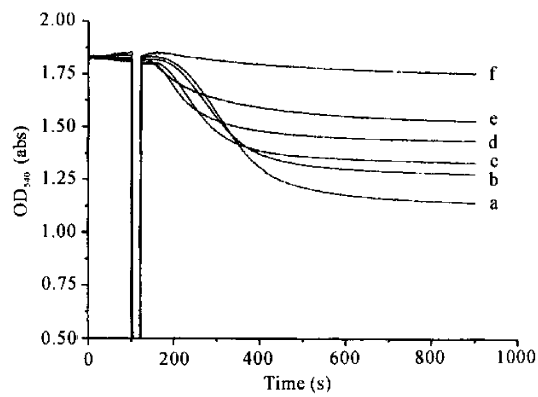

Fig.1 Effects of GTPs on Ca^{2+}-induced PTP opening. Mitochondria treated with 100 μmol/L of Ca^{2+} was used as control (curve a). After pre-incubation with GTPs at concentration of 50 μmol/L (curve b), 100 μmol/L (curve c), 250 μmol/L (curve d), 500 μmol/L (curve e) and 1mmol/L (curve f) for 2 min, mitochondria swelling was detected from OD_{540} changes.

Another interesting phenomenon was that at a certain concentration of GTPs (0.1 - 0.5 nmol/L), they accelerate mitochondria swelling but prevent the remainder PTP opening

(Fig. 1). Maby, GTPs have dual action on the PTP opening.

PTP opening also causes disappearance of mitochondria membrane potential ($\Delta\Psi_m$), resulting in release of $\Delta\Psi_m$ special probe Rh123 from mitochondria, and increase in the RH123 fluorescence in the reactive system. As shown in Fig. 2a, 100 μmol/L Ca^{2+} augments Rh123 fluorescence. Similar to mitochondria swelling, GTPs have obvious dose-dependent inhibitory effect on opening (Fig. 2b).

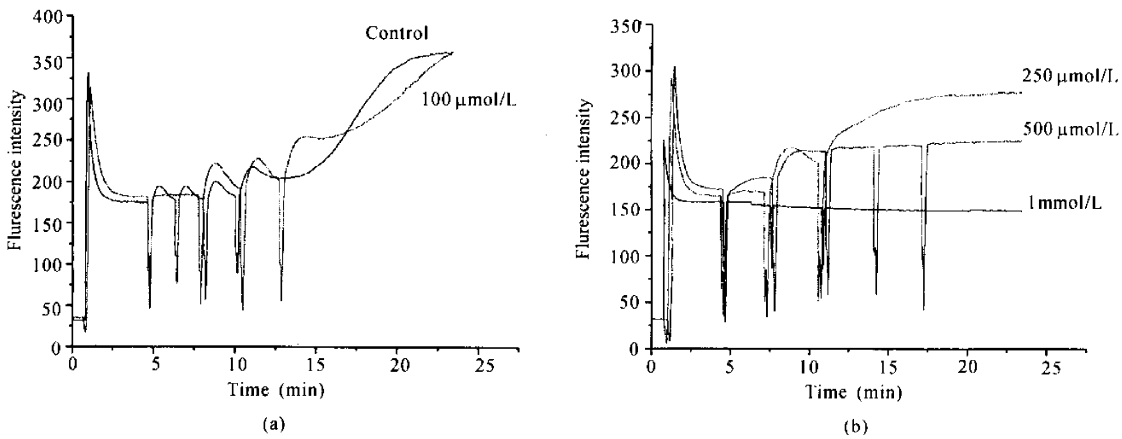

Fig. 2 Effects of GTPs on Ca^{2+}-induced disappearance of mitochondria membrane potential ($\Delta\Psi_m$). After addition of 1μmol/L Rh123 to mitochondria suspension and pre-incubation mitochondria with GTPs at concentration of (a) 100 μmol/L Ca^{2+}; (b) 250 μmol/L, 500 μmol/L and 1 mmol/L for 2 min, to the system, then detect the decrease of OD540 by using spectrophotometer

The influence of GTPs on influx and efflux of mitochondria Ca^{2+}

AⅢ is a Ca^{2+} sensitive membrane impermeable probe which can reflect the continuous change of Ca^{2+} (Frei et al., 1985). After stimulation with 100 μmol/L Ca^{2+}, AⅢ absorbance declined rapidly, then gradually rose above the baseline, indicating that mitochondria Ca^{2+} efflux had been evoked by rapid uptake of Ca^{2+}. This is Ca^{2+} mediated mCICR, i.e., Ca^{2+} influx induced Ca^{2+} release from mitochondria.

Mitochondria Ca^{2+} uniporter are closely related to Ca^{2+}-induced mCICR and the PTP opening by influx Ca^{2+} (Ichas et al., 1997). Preincubation of mitochondria with increased GTPs concentration resulted in an obvious dose-dependent inhibitory effect on Ca^{2+} influx (Fig. 3). These results indicated that GTPs may be an inhibitor of the mitochondria Ca^{2+} uniporter, similar to the previously reported RR.

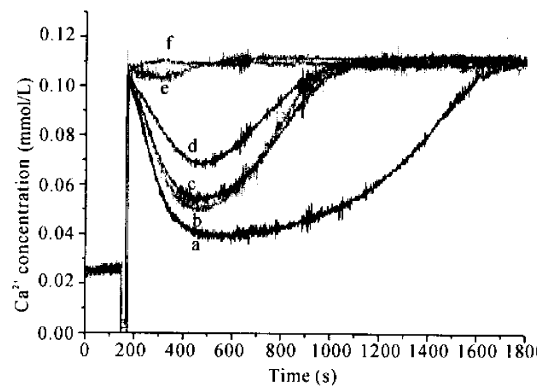

Fig. 3 Effects of GTPs on Ca^{2+}-induced Ca^{2+} import. After prencubation of mitochondria with GTPs at concentrations of 0 μmol/L (curve a), 50 μmol/L (curve b), 100 μmol/L (curve c), 250 μmol/L (curve d), 500 μmol/L (curve e) and 1 mmol/L (curve f) for 2 min, both 100 μmol/L Ca^{2+} and 10 AⅢ were added to the system to detect the changes of free Ca^{2+} in the medium.

PT pore and Ca^{2+}/Na^{+} exchanger are mitochondria Ca^{2+} export pathways (Lemasters et al., 1998). Adding GTPs to the teactive system at the bottom of AⅢ absorbance induced with 100 μmol/L Ca^{2+} led to a concentration dependent accelerative effect on the Ca^{2+} release (Fig. 4). These results may be due to the interaction of GTPs with PT pore or Ca^{2+}/Na^{+} exchange pore or its indirect influence on the Ca^{2+} export mechanism.

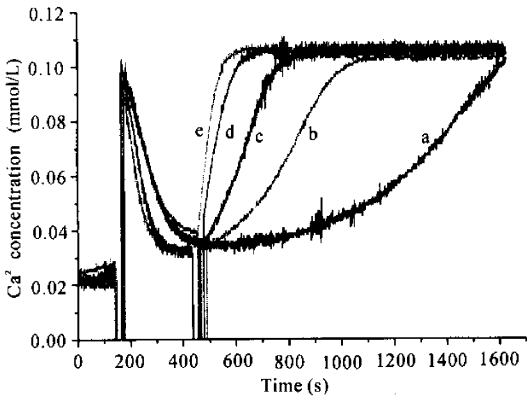

Fig. 4 Effects of GTPs on Ca^{2+}-induced Ca^{2+} release from mitochondria. After preincubation of mitochondria with the MIB buffer for about 2 min, 100 μmol/L Ca^{2+} and 10 μmol/L AⅢ were added to the system, GTPs at concentration of 0 μmol/L (curve a), 50 μmol/L (curve b), 100 μmol/L (curve c), 250 μmol/L (curve d), 500 μmol/L (curve e) and 1 mmol/L (curve f) were added to the medium till free Ca^{2+} concentration reached the bottom, respectively.

DISSCUSSION

Intense research had been focused on mitochondria because of their close relation with cell death (Desagher et al., 2000; Gottlieb et al., 2000). Ca^{2+} transport is one of the main events for mitochondria to regulate cellular physiological reaction. PTP plays an important role in the mitochondria regulation. Mitochondria Ca^{2+} transport carries out its physiological or pathological responses by inducing low or high conductance PTP (Pozzan et al., 2000; Huang et al., 2000).

Many animal bioassays, cell culture systems and epidemiological studies revealed that GTPs had remarkable protective effects against various diseases. But little is know about the detailed mechanisms of GTPs acting in cell signal system (Islam et al., 2000). Mitochondria had been implicated in a variety of disease states. Among the possible mechanisms of damage, production of ROS (reactive oxygen species) had been proposed to play roles in many diseases (Lenaz et al., 1998). So, to explore if GTPs are involved in mediating mitochondria state is intriguing, since GTPs had also been proven to act as efficient scavenger of ROS.

To our knowledge, this is the first time report of investigation into the relationship between GTPs and mitochondria PTP. Our results clearly showed that GTPs had profound influence on mitochondria PTP and Ca^{2+} transport. GTPs' overall inhibitory effect on the PTP opening was revealed through experiments on mitochondria swelling and change of mitochondria $\Delta\Psi_m$, which are related to the protective action of GTPs on cell apoptosis. The slow response of mitochondria under stress condition such as Ca^{2+} stimulation is beneficial to cell survival, or it will be in solute permeable state causing swelling and outer mambrane rupture, and inevitably leading to cell death in the end.

The mechanism of how GTPs caused the PTP hard to open is still unknown. As its generally recognized role as antioxidant (Ahmad et al., 1997), GTPs may scavenge the surplus ROS so as to make the mitochondria tend to keep the closed state. But on the other hand, GTPs can also be pro-oxidative, may under some condition induce the production of H_2O_2 (Yang et al., 1998). Accelerated partial mitochondria PTP opening due to addition of GTPs may be responsible for this pathway.

The exact composition of PTP is still unknown. It is possible that GTPs affect PTP and Ca^{2+} transport via direct interaction with portentous factors. The fact that its inhibitory effect could be diminished through introducing more Ca^{2+} implies it may use an irreversible way to act with a affecting factor of mitochondria.

Jakun J et al. (1997) reported that EGCG, the main component of GTPs, could bind to proteins such as erokinase to prevent cancer (Jankun

et al., 1997). It is possible that GTPs interact with a certain component of PTP to affect its opening.

Taken together, GTPs have dual roles in affecting mitochondria. One is their highly preventive effects on the PTP opening through inhibition of reactive oxygen intermediate generation or binding to an unknown factor of mitochondria; the other is their acceleration of PTP opening via the prooxidative way.

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