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Mitochondrial Dysfunction Induced by Honokiol

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Abstract Honokiol has shown the ability to induce the apoptosis of several different cancer cell lines. Considering that mitochondria are involved in apoptosis, the aim of the present work was to investigate the effects of honokiol on mitochondria. The effects of honokiol on the permeability of H^+ and K^+, membrane potential, membrane fluidity, respiration and swelling of mitochondria isolated from the rat liver were assessed. The results show that honokiol can significantly induce mitochondrial swelling, decrease membrane potential and affect the respiration of mitochondria. Meanwhile, honokiol does not have a direct effect on the mitochondrial permeability transition pore.

Keywords Honokiol · Mitochondria · Swelling · Respiration · Membrane potential · Apoptosis

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Introduction

Many clinically used cancer chemotherapeutic agents are derived from natural products or semisynthetic compounds of natural origin (Park et al. 2009). Therefore, evaluation of the anticancer properties of natural products has been considered to play an important role in the development of chemotherapeutic agents. Honokiol (CAS 35354-74-6), a small-molecular weight natural product, is an active component extracted from *Cortex Magnoliae officinalis*, a plant used in traditional Chinese and Japanese medicine (Sheu et al. 2008; Tang et al. 2011). Honokiol possesses some special abilities to inhibit angiogenesis and tumor growth. In recent years, novel pharmaceutical techniques have been used to improve the action of honokiol. For example, nanoparticles have been employed to treat honokiol in order to improve its therapeutic effects (Fang et al. 2009).

One of the distinguishing characteristics of cancer cells compared to normal ones is their ability to escape the induction of cell death. Failure to undergo cell death upon appropriate signal is a major obstacle and an unsolved problem in cancer therapeutics (Fulda 2010; Chen et al. 2010). Therefore, improving the therapeutic efficacy and selectivity and overcoming drug resistance are major goals in developing anticancer agents today (Indran et al. 2011).

Mitochondria as a therapeutic target of treatment for cancers have been gaining much attention in recent years. Mitochondria have long been recognized as the energy generators for cells (Modica-Napolitano and Singh 2004). Meanwhile, mitochondria produce reactive oxygen species (ROS), which are involved in the regulation of many

physiological processes but might also be harmful to the cell if produced excessively (Wallace and Starkov 2000). Furthermore, mitochondria are crucial for the regulation of intracellular Ca^{2+} homeostasis (Gogvadze et al. 2008). Permeabilization of the outer and/or inner mitochondrial membranes can trigger a series of catabolic reactions that entail cell death, either by apoptosis or by necrosis (Kroemer and Reed 2000). All these mitochondrial functions are of critical importance to the growth and survival of tumor cells, especially by producing energy and by being involved in proapoptotic protein regulation. Alterations in cellular bioenergetics are an emerging hallmark of cancer (Pathania et al. 2009). A number of additional metabolic alterations associated with mitochondrial function have been observed in cancer cells, including increased gluconeogenesis, reduced pyruvate oxidation, increased lactic acid production, increased glutaminolytic activity and reduced fatty acid oxidation (Modica-Napolitano and Singh 2004). Therefore, mitochondria are regarded as an attractive target for cancer chemotherapy (Okuda et al. 2010; Gogvadze et al. 2009).

Taken together the inhibitory effect of honokiol on cancer cells and the role of targeting mitochondria in cancer therapy, it is important to acquire more information on the interactions of mitochondria with honokiol. However, the mechanisms of action of honokiol on mitochondria have not been fully elucidated yet and are worthy of detailed exploration. Hence, this work aimed to characterize the factors regulating the interaction of mitochondria with honokiol. The effects of honokiol on swelling of the mitochondrial matrix, membrane potential, respiration and proton and potassium permeabilization of isolated rat liver mitochondria were examined. The results provide direct evidence that honokiol affects mitochondrial function. The study may also be helpful in further pharmacological research on honokiol.

Materials and Methods

Chemicals

Cyclosporin A (CsA), oligomycin, rotenone, rhodamine 123 (Rh123) and hematoporphyrin (HP) were purchased from Sigma (St. Louis, MO). Other reagents were of analytical reagent grade. All solutions were prepared with double-distilled water.

Isolation of Mitochondria

Isolation was performed according to the literature (Pon and Schon 2007) with minor modifications. A beaker containing fresh Wistar rat liver washed in solution A (0.22 M mannitol, 0.07 M sucrose, 0.02 M HEPES, 2 mM Tris–HCl [pH 7.4]) was chilled in ice water. The liver was subsequently minced with scissors and washed three times using solution A. The remainder was weighed in a pre-chilled beaker, and about 5 g of mince was suspended in 100 ml of solution A with 0.4 % BSA added. The suspension was homogenized in a Dounce tissue grinder chilled in ice water. The homogenate was centrifuged at 3,000 × *g* for 2 min. The supernatant was decanted. The pellet was resuspended in solution A and centrifuged at 17,500 × *g* for 4 min twice. The resulting deposit was resuspended in 50 ml of solution B (0.22 M mannitol, 0.07 M sucrose, 0.01 M Tris–HCl [pH 7.4], 1 mM EDTA). The Biuret method was then utilized to measure the mitochondrial protein concentration using serum albumin as standard. All of the above operations were performed at 0–4 °C.

Mitochondrial Swelling

A UV–Vis spectrophotometer (SQ-4802; UNICO, Dayton, NJ) was used to measure the absorbance of the mitochondrial suspension at 540 nm over 7 min at 25 °C in order to determine mitochondrial swelling. Mitochondria (0.25 mg protein/ml) were suspended in 2 ml of assay solution (5 mM succinate, 200 mM sucrose, 10 mM Tris–MOPS, 1 mM Pi, 10 μM EGTA–Tris, 2 μM rotenone and 3 μg/ml oligomycin [pH 7.0–7.4]) and treated with different concentrations of honokiol. Different concentrations of CsA (0–30 μM) were also used to inhibit honokiol (100 μM)–induced mitochondrial swelling.

H^+ and K^+ Permeabilization

The method was based on the reported literature (Fernandes et al. 2006). Mitochondrial inner membrane permeabilization of H^+ was detected in K-acetate medium. Mitochondria (0.50 mg protein/ml) were suspended in the assay solution, which contained 135 mM K-acetate, 5 mM HEPES [pH 7.0], 0.1 mM EGTA and 0.2 mM EDTA, supplemented with 2 μM rotenone. All assays were carried out in the presence of 1 μg/ml valinomycin to permeabilize the mitochondrial inner membrane to K^+. With regard to the mitochondrial inner membrane permeabilization of K^+, K-nitrate medium, in which the K-acetate was substituted with KNO_3, was used alternatively. The instrument and experiment parameter were the same as described above, in "Mitochondrial Swelling."

Measurement of Membrane Potential

The assays were performed at 25 °C, with a quartz cell of 1.0 cm path length ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 525$ nm).

Mitochondria (0.50 mg protein/ml) were suspended in 2 ml of assay solution as described above, in "Mitochondrial Swelling." With 100 nM Rh123 as a fluorescence probe, changes of mitochondrial membrane potential ($\Delta\Psi_m$) were detected in terms of fluorescence emission intensity by a fluorescence spectrometer (LS55; Perkin-Elmer, Downers Grove, IL). Rh123 is a cationic fluorophore that is actively accumulated by mitochondria due to the mitochondrial membrane potential. If an agent induces a collapse of the mitochondrial membrane potential, Rh123 will be released into solution and the fluorescence intensity of the solution will recover.

Respiration

Mitochondrial respiratory rates under three different states were measured using a Clark oxygen electrode (Oxygraph; Hatchtech, Dorchester, UK) and recorded by a computer connected to this device. The slope of the oxygen consumption curve was used to evaluate the mitochondrial respiratory rate. All operations were performed at 30 °C with magnetic stirring. Mitochondria (1 mg protein) were added into 1 ml of mitochondria buffer solution, containing 250 mM sucrose, 20 mM KCl, 5 mM K_2HPO_4, 10 mM HEPES, 2 mM $MgCl_2$ and 1 μM rotenone [pH 7.0–7.4]. State 4 was initiated by adding 5 mM succinate into mitochondria buffer solution, and state 3 was initiated by adding 5 mM succinate and 100 μM ADP into mitochondria buffer solution, while the uncoupled respiration state was initiated by addition of 30 μM 2, 4-dinitrophenol (DNP).

Anisotropy

Changes in fluorescence excitation anisotropy (r) of HP bonded to mitochondria were used to evaluate the fluidity of the mitochondrial membranes (Ricchelli et al. 1999, 2005). HP stock solution was prepared in absolute ethanol. The stirred mitochondrial suspension (0.50 mg protein/ml) with HP added (3 μM) was incubated for 2 min before measuring. The values of steady-state fluorescence anisotropy were obtained at 520 nm ($\lambda_{em} = 626$ nm) by measurements of I_{VV} and I_{VH} (i.e., the fluorescence intensities polarized parallel and perpendicular to the vertical plane of polarization of the excitation beam, respectively) with a fluorescence spectrometer (LS55) at 25 °C.

Results

Mitochondrial swelling induced by honokiol was observed. As shown in Fig. 1, the decrease in absorbance at 540 nm suggests that the swelling tendency is proportionate to the testing concentration of honokiol. Under the same circumstances, as shown in Fig. 2, the effects of honokiol on mitochondrial swelling are significantly reduced by CsA. It can be seen from the graph that 1 μM of CsA can protect the mitochondrial membrane from being induced by 100 μM of honokiol. With larger concentrations of CsA, the protective effect is even more obvious.

It is evident that the H^+ permeabilization of the mitochondrial inner membrane synchronizes with the concentration of honokiol (Fig. 3). Specifically, the higher concentration of honokiol results in a greater decrease in absorbance at 540 nm. Likewise, the trend of K^+

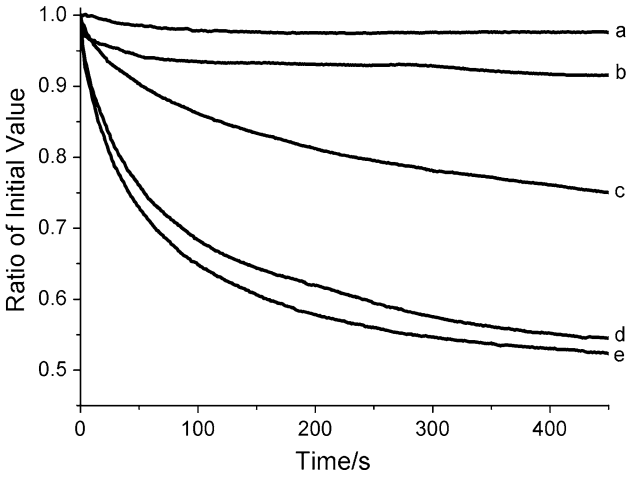

Fig. 1 Honokiol-induced isolated mitochondrial swelling. Honokiol was added in different concentrations: 0 μM (*a*), 25 μM (*b*), 50 μM (*c*), 75 μM (*d*) and 100 μM (*e*). Ordinate (*Ratio of Initial Value*) shows the ratio of real-time absorbance at 540 nm to that at the beginning

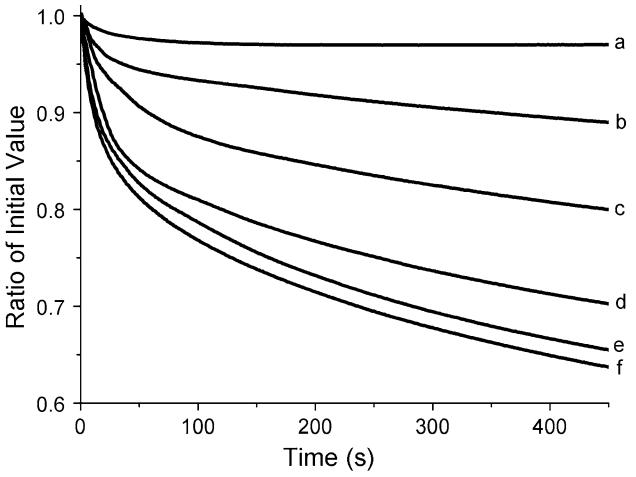

Fig. 2 CsA inhibited honokiol (100 μM)-induced mitochondrial swelling. CsA was added in different concentrations: 0 μM (*f*), 1 μM (*e*), 10 μM (*d*), 20 μM (*c*), and 30 μM (*b*). Especially, mitochondria were untreated with honokiol (*a*). Ordinate value is the same as in Fig. 1

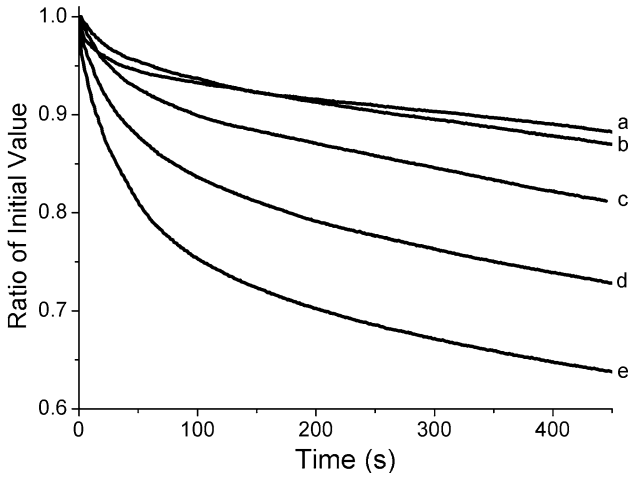

Fig. 3 Effect of honokiol on permeabilization to H^+ by the mitochondrial inner membrane. Honokiol was added in different concentrations: 0 μM (*a*), 25 μM (*b*), 50 μM (*c*), 75 μM (*d*) and 100 μM (*e*). Ordinate value is the same as in Fig. 1

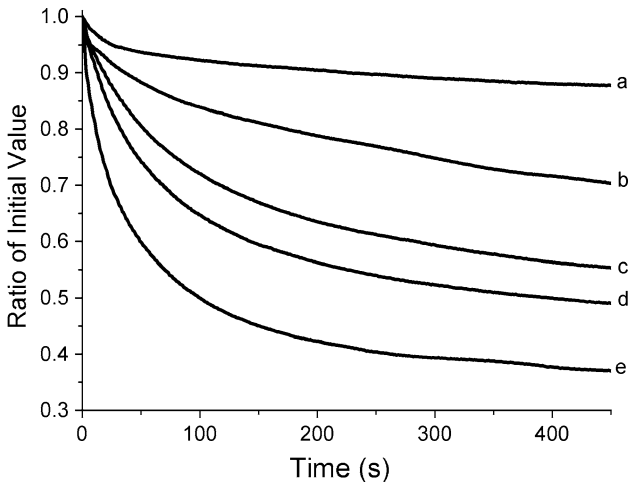

Fig. 4 Effect of honokiol on permeabilization to K^+ by the mitochondrial inner membrane. Honokiol was added in different concentrations: 0 μM (*a*), 25 μM (*b*), 50 μM (*c*), 75 μM (*d*) and 100 μM (*e*). Ordinate value is the same as in Fig. 1

permeabilization of the mitochondrial inner membrane exhibits the same pattern (Fig. 4).

The effects of honokiol on swelling of the isolated mitochondria could also be seen from the changes in membrane potential ($\Delta\Psi_m$) measured by fluorometry. The fluorescent probe Rh123 was adsorbed onto mitochondria due to $\Delta\Psi_m$. Once it was decreased, Rh123 was released into the medium, causing an increase of the fluorescence intensity. As shown in Fig. 5, the rebound of fluorescence intensity (i.e., decrease of $\Delta\Psi_m$) was synchronous with increasing concentrations of honokiol.

As shown in Fig. 6, without the presence of honokiol, the high respiratory rate of state 3 indicated that the

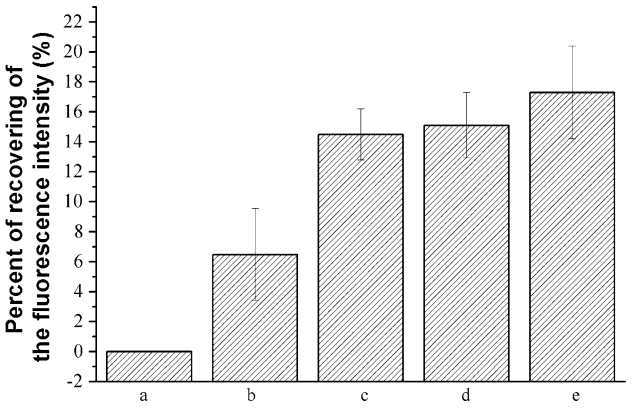

Fig. 5 Honokiol induced a decrease of the membrane potential of isolated mitochondria. Honokiol was added in different concentrations: 0 μM (*a*), 25 μM (*b*), 50 μM (*c*), 75 μM (*d*) and 100 μM (*e*). Data are the mean values of at least three individual experiments of the percent of recovery of fluorescence intensity relative to the decrease of fluoresce intensity without honokiol

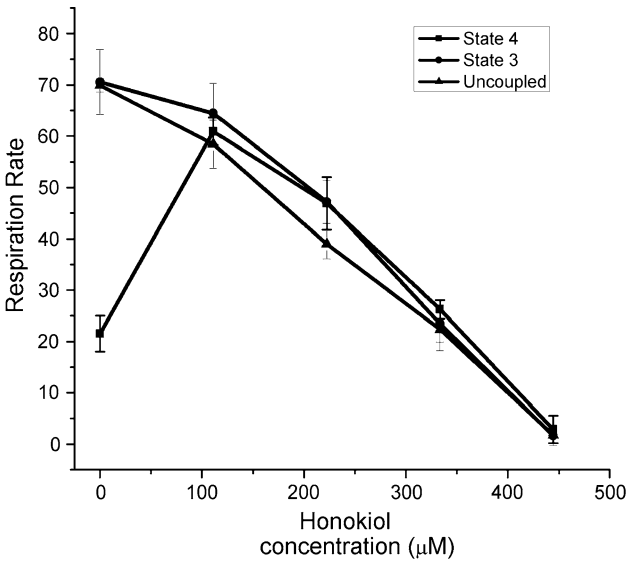

Fig. 6 Effects of honokiol on the respiration of isolated mitochondria. Respiration is represented as nanomoles of O_2 per minute per milligram of protein

respiratory chain and ATP synthesis were intact. On the other hand, the relatively low rate of state 4 shows that the mitochondrial inner membrane was also intact. After the exposure to honokiol, state 4 respiration was stimulated by low concentrations of honokiol and inhibited by high concentrations. The state 3 and DNP-uncoupled respiration demonstrated similar responses under honokiol exposure: the respiratory rate significantly decreased with the addition of honokiol.

With exposure of HP-probed mitochondria to increasing honokiol concentrations (Fig. 7), the fluorescence anisotropy of the assay solution decreased. This indicated a

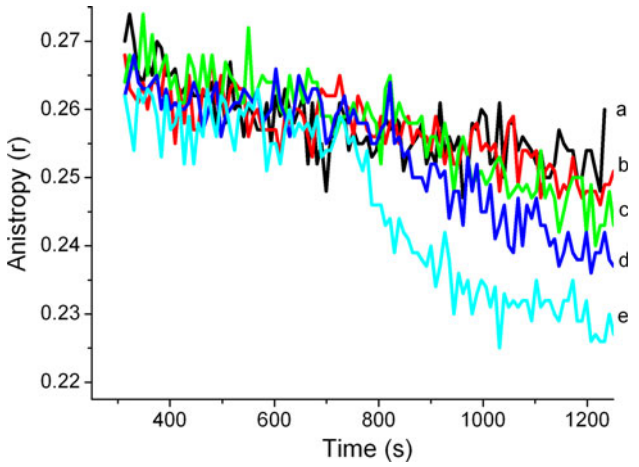

Fig. 7 Honokiol induced an increase of the fluidity of the mitochondrial membrane. Honokiol was added in different concentrations: 0 μM (*a*), 25 μM (*b*), 50 μM (*c*), 75 μM (*d*) and 100 μM (*e*)

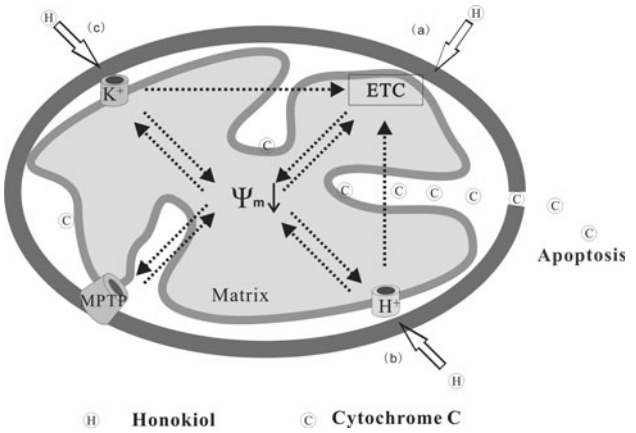

Fig. 8 Proposed mechanism of mitochondrial dysfunction induction by honokiol. Honokiol may inhibit the mitochondrial respiratory chain (*a*) and may increase the permeability to H^+ (*b*) or K^+ (*c*) of the mitochondrial inner membrane

remarkable change in membrane fluidity induced by honokiol.

Discussion

The present study demonstrated that honokiol can induce a small permeabilization of the mitochondrial inner membrane to K^+ (Fig. 4). K^+ is the predominant ion inside the cell (Yu 2003). The basic effects of the mitochondrial K^+ channels include the regulation of mitochondrial matrix volume, matrix pH, mitochondrial respiration and membrane potential ($\Delta\Psi_m$) and the generation of ROS (Szewczyk et al. 2006; Nowikovsky et al. 2009). The decrease of $\Delta\Psi_m$ induced by K^+ flux into the matrix may be responsible for the mitochondrial permeability transition (MPT) and, consequently, mitochondrial swelling, disruption of the outer membrane and release of cytochrome *c*, Smac/DIABLO and apoptosis-inducing factor (Bernardi et al. 1992). Moreover, the mitochondrial inner membrane is generally impermeable to ions other than H^+ (Mitchell 1961; Aon et al. 2006). The increase in K^+ permeability may be responsible for an increased permeability to protons. This is consistent with results showing that honokiol can induce a small permeabilization of H^+ (Fig. 3). The data on K^+ permeabilization (Fig. 4) is consistent with the data on $\Delta\Psi_m$ (Fig. 5) in this article. As originally pointed out by Mitchell, the primary form of energy generated in mitochondria is the so-called electrochemical proton gradient (Mitchell 1961; Wallace and Starkov 2000; Nowikovsky et al. 2009; Szewczyk and Wojtczak 2002).

Many studies have concluded that opening of the mitochondrial permeability transition pore (MPTP) is a key event in cell apoptosis. The permeability transition pore (PTP) is a regulated, high-conductance channel (Nowikovsky et al. 2009). During MPT, the megapores in the mitochondrial inner membrane are opened, which permit solutes of less than 1,500 daltons to cross the inner membrane freely (Scheffler 2001). This will cause a series of negative results. Firstly, it allows protons to cross the inner membrane, causing oxidative phosphorylation to be uncoupled. Secondly, the equilibration of the small-molecular solutes across the inner membrane will lead to matrix swelling and then cause rupture of the outer membrane and the release of proapoptotic proteins (Li et al. 2007; Halestrap et al. 2007; Kroemer and Reed 2000). Thirdly, MPT induces the collapse of $\Delta\Psi_m$. In turn, the PTP can also be modulated by the membrane potential (Nowikovsky et al. 2009). The data on H^+ and K^+ permeabilization (Figs. 3, 4) were consistent with those of $\Delta\Psi_m$ (Fig. 5) in this work. Meanwhile, the swelling experiments showed that honokiol induced a concentration- and time-dependent swelling (Fig. 1). Taken together (swelling, $\Delta\Psi_m$, H^+ and K^+ permeabilization), it seems that honokiol is related to MPT.

In order to confirm the association between honokiol and MPT, the effect of CsA on honokiol-induced swelling was studied. CsA has become the standard tool to test the role of the PTP. Therefore, MPT is categorized as CsA-regulated and unregulated (Okuda et al. 2010). As shown in Fig. 2, honokiol-induced swelling is attenuated by low concentrations of CsA and inhibited by high concentrations. As reported, 1 μM CsA is enough to inhibit the interaction between CypD and the adenine nucleotide translocator (Okuda et al. 2010). Considering the effect of CsA on swelling and only a slight decrease of membrane potential induced by honokiol (Fig. 5), we infer that honokiol is not a classical inducer of MPT. However, honokiol did have a slight influence on MPTP.

As reported, low concentrations of uncoupler can stimulate respiration of state 4 and collapse the mitochondrial membrane potential. On the contrary, higher concentrations can inhibit state 4 and uncouple respiration by impairment of electron transfer and substrate to the respiration chain (Vicente et al. 1998; Floridi et al. 1999; Murphy 2001). The data in Figs. 3 and 4 show that honokiol stimulates proton and potassium permeabilization across the inner membrane, which is consistent with the classical definition of an uncoupler (Wallace and Starkov 2000).

HP preferentially accumulates in protein regions of the inner membrane in mitochondria. Meanwhile, the HP-binding sites take part in membrane permeabilization pore structure or regulation (Ricchelli et al. 1999, 2005). Figure 7 shows the fluorescence anisotropic changes of HP-probed mitochondria affected by honokiol. It indicates that honokiol can obviously induce the increase of the membrane fluidity properties. This represents that honokiol may induce the conformational variation of proteins in the inner membrane.

According to the above discussion, a possible three-pathway mechanism (electron transport chain, H^+ and K^+ permeabilization) of mitochondrial dysfunction induced by honokiol can be proposed and is elucidated briefly in Fig. 8. Permeabilization of the mitochondrial inner membrane to H^+ or K^+ may decrease the potential of the mitochondrial membrane, thus opening the MTP and inhibiting mitochondrial respiration. Correspondingly, opening of the MTP also can collapse the mitochondrial membrane potential, thus permeabilizing the inner membrane to H^+ or K^+. Regarding inhibition of the mitochondrial respiratory chain, they are in a similar situation.

The results of this work indicate that the mitochondrial pathway may be involved in the apoptosis induced by honokiol. But we cannot confirm that the above mechanisms are independent, simultaneous or synergetic interactions based on the current research. However, in order to elucidate the mechanisms of the effect of honokiol on mitochondria, further studies are required to confirm the binding sites (or receptors) for honokiol on mitochondria.

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