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Uncoupling and Antioxidant Effects of Ursolic Acid in Isolated Rat Heart Mitochondria

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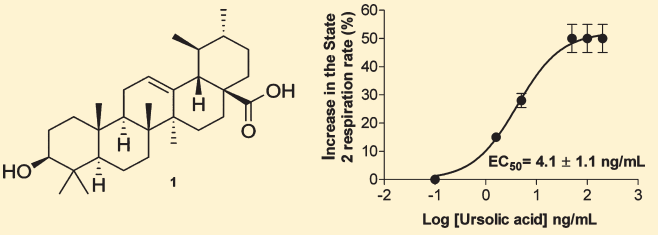
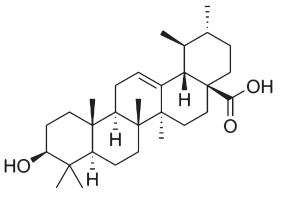
ABSTRACT: Ursolic acid (**1**), a pentacyclic triterpene acid, is one of the major components of certain traditional medicinal plants and possesses a wide range of biological effects, such as anti-inflammatory, antioxidative, and cytotoxic activities. Furthermore, **1**, when present at 1.6−5 ng/mL concentrations in commercial herbal preparations used for patients with cardiac disorders, may also exert pro-cardiac activities. There are several indirect suggestions that the cardioprotective mechanism of ursolic acid could involve the mitochondria; however the mechanism of action is still not known. Therefore, the effects of 0.4−200 ng/mL ursolic acid (**1**) on the functions of isolated rat heart mitochondria oxidizing either pyruvate and malate, succinate, or palmitoyl-L-carnitine plus malate were investigated. It was found that **1** induced a statistically significant uncoupling of oxidative phosphorylation. A statistically significant decrease in H_2O_2 production in the mitochondria was observed after incubation with 5 ng/mL **1**. This effect was comparable to the effectiveness of the classical uncoupler carbonyl cyanide 3-chlorophenylhydrazone. Since mild mitochondrial uncoupling has been proposed as one of the mechanisms of cardioprotection, the present results indicate that ursolic acid (**1**) has potential use as a cardioprotective compound.



Ursolic acid (**1**), a natural pentacyclic triterpenoid carboxylic acid, is a component of certain traditional medicine herbs and ornamental species and is also found in fruits, such as apples, prunes, cranberries, and blueberries.[1,2] It is known that **1** exerts a wide range of biological activities, such as anti-inflammatory, cytotoxic, and hepatoprotective effects.[2,3] Some of these protective effects of **1** are determined by its strong antioxidative properties.[4−7] Moreover, the mechanism of the cytotoxic activity of **1** in cancer cells depends on the disease model and cell culture type, encompassing various intracellular signaling pathways that lead to either improved survival or apoptosis.[8−13]



Ursolic acid (**1**) may be present at 1.6−5 ng/mL concentrations in commercial herbal preparations [e.g., *Crataegus laevigata* DC. (Rosaceae), *Leonurus cardiaca* L. (Lamiaceae)] prescribed in Europe as an auxiliary treatment for patients with cardiac disorders. There are several indirect suggestions based on the mechanism of action of various phenolic compounds from herbal preparations that the cardioprotective mechanism of **1** may also involve these organelles, since they are well-recognized intracellular players regulating cellular energy turnover under normal and pathological conditions in humans.[14−17] The main physiological function of the mitochondria is production of ATP as well as the control of cell survival and death.[18,19]

The mechanism of action of ursolic acid (**1**) on heart mitochondria, to the best of our knowledge, has not been investigated previously. Therefore, the aim of this study was to investigate the direct effect of **1** on the oxidative phosphorylation

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Table 1. Effect of Ursolic Acid (1) on Rat Heart Mitochondrial Respiratory Parameters

	pyruvate (6 mM) and malate (6 mM)		succinate (12 mM) and amytal sodium (2 mM)		palmitoyl-L-carnitine (9 μM) and malate (0.24 mM)	
	− 1	+ 1	− 1	+ 1	− 1	+ 1
V_0[a]	52 ± 7[e]	61 ± 2	238 ± 39	238 ± 28	85 ± 4	82 ± 2
1 (1.6 ng/mL)		70 ± 3*[f]		279 ± 37*		90 ± 2*
1 (5 ng/mL)		78 ± 5*		299 ± 38*		92 ± 2*
V_{ADP}[b]	389 ± 40	374 ± 42	606 ± 108	599 ± 93	436 ± 54	403 ± 50
V_{ADP+C}/V_{ADP}[c]	1.6 ± 0.2	1.5 ± 0.1	1.8 ± 0.1	1.5 ± 0.1	1.3 ± 0.1	1.2 ± 0.1
RCI[d]	8.0 ± 1.2	4.8 ± 0.3*	2.5 ± 0.1	2.0 ± 0.1*	5.2 ± 0.4	4.4 ± 0.6

[a] V_0 = respiration rate in the presence of substrates. [b] V_{ADP} = respiration rate in the presence of 1 mM ADP. [c] V_{ADP+C}/V_{ADP} = effect of cytochrome *c* (times), where V_{ADP+C} = respiration rate in the presence of 32 μM cytochrome *c*. [d] RCI = respiratory control index. [e] Respiratory rates are given as nmol O/min/mg protein. [f] *$p < 0.05$ vs control, $n = 3, 4$.

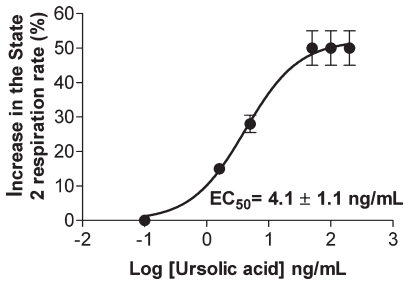

Figure 1. Concentration-dependent effect of ursolic acid (**1**) on mitochondrial State 2 respiration rates. Substrates: 6 mM pyruvate and 6 mM malate, $n = 4$. Missing error bars are smaller than the symbol size.

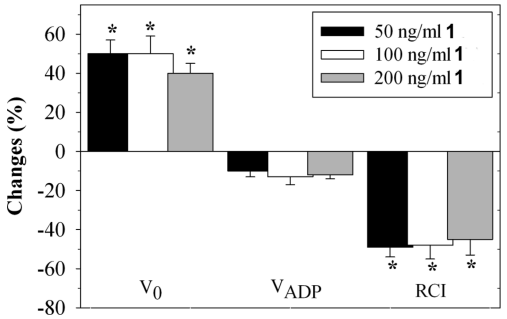

Figure 2. Effect of different concentrations of ursolic acid (**1**) on mitochondrial State 2 and State 3 respiration rates. Chemicals were added in the following order: V_0, 6 mM pyruvate and 6 mM malate (State 2); V_{ADP}, 1 mM ADP (State 3); RCI, respiratory control index. *$p < 0.05$ vs control, $n = 4$.

in isolated rat heart mitochondria in order to determine the possible mechanism of cardioprotection by this compound.

In this study the effects of different concentrations of ursolic acid (**1**) were investigated on the functions of isolated rat heart mitochondria oxidizing either pyruvate and malate, succinate, or palmitoyl-L-carnitine plus malate. All concentrations of **1** used in the first series of experiments have not exceeded the concentrations (1.6−5 ng/mL) found in commercial herbal preparations from *C. laevigata* and *L. cardiaca*. Thus, the present results revealed that at very low concentrations, 0.4, 0.8, and 1.2 ng/mL, ursolic acid (**1**) had no effect on the basal respiration rate (V_0) with all used substrates (data not shown). The State 2 respiration rate with the mitochondrial respiratory chain Complex I-dependent substrates pyruvate and malate increased by about 15% with 1.6 ng/mL ursolic acid and by 28% with 5 ng/mL ursolic acid (Table 1).

Thus, ursolic acid (**1**) in a concentration-dependent manner (EC_{50} of 4.1 ± 1.1 ng/mL) induced statistically significant uncoupling of oxidative phosphorylation (Figure 1).

A very similar and statistically significant uncoupling effect of **1** was also observed with succinate (Complex II substrate). It was demonstrated that the State 2 respiration rate increased by 17% after addition of 1.6 ng/mL **1** and by 26% after 5 ng/mL **1**. Interestingly, the effect of **1** on the State 2 respiration rate with palmitoyl-L-carnitine, the main respiratory substrate of the heart mitochondria, was less pronounced. Nevertheless, a statistically significant increase by 10−12% in V_0 after addition of 1.6 and 5 ng/mL of **1** was observed. Of note, the maximal State 3 respiration rate in the presence of 5 ng/mL **1** was unchanged with all substrates used. The respiratory control index (RCI) values obtained at the same concentration of **1** were significantly lower, by 40% (with pyruvate and malate) and by 20% (with succinate), as compared to the controls. The intactness of the outer mitochondrial membrane was assessed by a cytochrome *c* test, in which the stimulation of V_{ADP} by exogenous cytochrome *c* is measured. Cytochrome *c* increased the State 3 respiration rate to a similar degree in both the control mitochondria and mitochondria treated with **1** (Table 1). Thus, ursolic acid (**1**) had no effect on the integrity of the mitochondrial outer membrane.

At higher concentrations of 50, 100, and 200 ng/mL (Figure 2), ursolic acid (**1**) increased the State 2 respiration rate by 40−50%. Moreover, such high concentrations had no statistically significant effect on the State 3 respiration rate. However, the RCI decreased significantly, by 45−49%, due to an increase in the State 2 respiration rate, i.e., an uncoupling of oxidation from phosphorylation (Figure 2).

In the last series of experiments, the influence of ursolic acid (**1**) was investigated on the H_2O_2 production in mitochondria and the antioxidant activity of this compound. The fluorimetric measurements demonstrated (Figure 3) that only 5 ng/mL **1** significantly decreased H_2O_2 production in mitochondria, by 36−56% ($n = 3$, $p < 0.05$). Furthermore, **1** was shown to be an effective scavenger of H_2O_2 radicals in in vitro studies. Ursolic acid (**1**) at concentrations 1.6 and 5 ng/mL decreased the H_2O_2 fluorescence intensity by 49.5 ± 6% and 55.6 ± 8%, respectively ($p < 0.05$, $n = 3$).

Thus, the present investigation has revealed dose-dependent changes in the State 2 respiration rate with several different respiratory substrates. Since mitochondrial respiration rate in

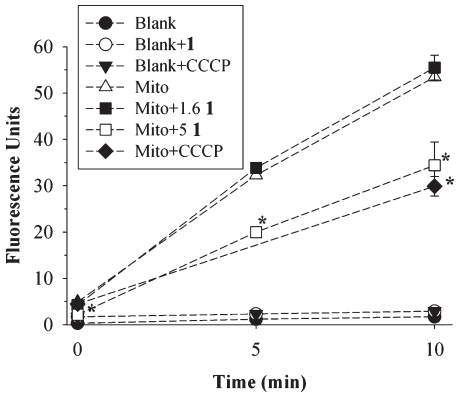

Figure 3. Effect of different concentrations of ursolic acid (**1**) on H_2O_2 generation in normal and uncoupled mitochondria. Blank, pyruvate and malate (6 mM each) plus antimycin A (16 ng/mL), Amplex Red (5 μM), and horseradish peroxidase (2 U/mL); Blank+**1**, as in Blank plus 5 ng/mL **1**; Blank+CCCP, as in Blank plus 1 μM of carbonyl cyanide 3-chlorophenylhydrazone (CCCP); Mito, 0.25 mg/mL of mitochondria plus all other chemicals as in Blank; Mito+CCCP, as in Mito plus 1 μM CCCP; Mito+1.6 **1** and Mito+5 **1**, as in Mito plus 1.6 ng/mL or 5 ng/mL of **1**, respectively. For details, see the Experimental Section. *$p < 0.05$ vs control (Mito), $n = 3$. Missing error bars are smaller than the symbol size.

State 2 to a great extent depends on the passive proton flux through the mitochondrial inner membrane,[20] increase in the State 2 respiration rate shows the uncoupling effect of the investigated compound. Ursolic acid (**1**) at relatively low concentrations (0.4 to 1.2 ng/mL) did not affect the basal respiratory rate, whereas at pharmacological concentrations (1.6 to 5 ng/mL) an uncoupling of oxidation from phosphorylation occurred. The uncoupling effect was dependent on the concentration of ursolic acid (EC_{50} 4.1 $\pm$ 1.1 ng/mL, Figure 1) and increased using higher concentrations of this compound (Figure 2). Of note, neither the maximal capacity of heart mitochondria to sythesize ATP (expressed as the State 3 respiration rate) nor the integrity of the mitochondrial outer membrane (assessed by a stimulatory effect of exogenous cytochrome *c*) was affected at different concentrations of **1**. The uncoupling of oxidative phosphorylation by ursolic acid indicates an increase in proton leak through the inner mitochondrial membrane. This fact is of special importance, since it is well known that mild uncoupling of mitochondrial respiration attenuates the generation of reactive oxygen species (ROS).[21−24] Such a situation could be beneficial under pathological conditions associated with oxidative stress in the heart, such as during hypoxia or ischemia-reperfusion. Indeed, it was found that a statistically significant decrease in H_2O_2 production in mitochondria after incubation with 5 ng/mL **1** occurred (Figure 3). This effect was comparable to the effectiveness of the classical uncoupler CCCP. However, 1.6 ng/mL **1** did not reduce H_2O_2 production, even though at such a low concentration the mitochondrial State 2 respiration rate with pyruvate and malate was increased by 15% (Table 1 and Figure 3). These results suggest that there should be a certain degree of "mild" uncoupling resulting in a significant decrease of mitochondrial ROS level.

Furthermore, it was shown recently that mild mitochondrial uncoupling is a highly effective in vivo antioxidant strategy.[25] Treatment of healthy mice with low doses of the protonophore 2,4-dinitrophenol (DNP) enhanced tissue respiratory rates, decreased ROS levels, and reduced tissue DNA and protein oxidation. Importantly, DNP-treated animals also presented enhanced longevity.[25] Similarly, ursolic acid (**1**) demonstrates effective antioxidative activities. It was shown that administration of **1** to rats at a dose of 20 mg/kg reduced ethanol-induced oxidative stress in the heart.[26] This was achieved by decreasing lipid peroxidation products, increasing the activities of ROS scavenging enzymes, and increasing the levels of nonenzymatic antioxidants. In another experiment, administration of 60 mg/kg **1** to rats protected the heart against oxidative damage by scavenging free radicals and, therefore, reducing lipid peroxidation level in isoproterenol-induced myocardial ischemia.[27]

Moreover, an increasing amount of experimental data has suggested the organ- and tissue-protective activities of ursolic acid (**1**) through the mitochondrial pathway and even the direct effect of **1** on mitochondria themselves. It was revealed that the chloroform extract of *Terminalia catappa* L. (Combretaceae) leaves and its component **1** were effective against carbon tetrachloride-induced acute liver damage and D-galactosamine-induced hepatocyte injury.[6] In addition, Ca^{2+}-induced mitochondrial swelling was inhibited in a dose-dependent manner by 50−500 μM **1**. Moreover, the same concentrations of this compound showed strong superoxide-anion and hydroxyl-radical scavenging activities.

Another study confirmed that 20, 50, and 100 μg/mL ursolic acid (**1**) protected mouse liver mitochondria against Ca^{2+}-induced mitochondrial swelling, dissipation of mitochondrial membrane potential, and Ca^{2+} release from the mitochondrial matrix, as well as release of apoptosis-inducing factor and cytochrome *c* from mitochondrial intermembrane space to the cytoplasm.[28]

Finally, a recent study on the effect of a crude extract of *Crataegus monogyna* DC. (Rosaceae) fruit on isolated rat heart mitochondrial functions has shown the potential of some herbal preparations in cardioprotection.[16] It was demonstrated that this extract significantly decreased H_2O_2 production in mitochondria. Moreover, this extract (0.278−13.9 μg/mL of total phenolic compounds) and several pure flavonoid constituents (12.6 ng/mL quercetin, 4.9 ng/mL quercitrin, 449 ng/mL epicatechin, 293 ng/mL hyperoside, and 7 ng/mL procyanidin) stimulated mitochondrial State 2 respiration by 11−34%. Another two identified phenolic compounds had different effects on mitochondrial respiration; namely, the flavonoid rutin (26 ng/mL) increased State 2 respiration rate by 70%, whereas chlorogenic acid (30 ng/mL) had no effect.[16] However, it has to be emphasized that ursolic acid, as a constituent of *Crataegus monogyna* DC. (Rosaceae), could also contribute to the uncoupling activity of this crude extract.

In summary, the direct effect of pentacyclic triterpenoid ursolic acid (**1**) on isolated rat heart mitochondria has been demonstrated. The results have shown that **1** in a dose-dependent manner induces uncoupling of oxidative phosphorylation in the heart mitochondria without affecting State 3 respiration rate. Moreover, **1** significantly suppressed the H_2O_2 production in isolated mitochondria. Since the uncoupling of mitochondrial oxidation from phosphorylation promotes preconditioning-like cardioprotection in the heart,[29,30] the present results imply that mild mitochondrial uncoupling induced by pharmacological concentrations of **1** as found in some herbal preparations could be beneficial in cardioprotection. However, further investigations on the cardiac mitochondrial functions after prolonged treatment with ursolic acid (**1**) alone or in combination with other phenolic

compounds are needed to confirm this triterpenoid as a cardioprotective compound.

EXPERIMENTAL SECTION

Chemicals. Ursolic acid (**1**, purity 90%, as determined by HPLC) and Amplex Red were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals used in this work were of analytical grade from commercial sources.

Preparation of Rat Heart Mitochondria. Approval of the Lithuanian Ethical Committee for Laboratory Animal Use was obtained before commencement of the experiments. Hearts of male Wistar rats weighing 250−300 g were excised and rinsed in ice-cold 0.9% KCl solution. Subsequently, the tissue was cut into small pieces and homogenized in a buffer containing 160 mM KCl, 10 mM NaCl, 20 mM Tris/HCl, 5 mM EGTA, and 1 mg/mL BSA (pH 7.7). The homogenate was centrifuged at 750*g* for 5 min, and the supernatant obtained was recentrifuged at 6800*g* for 10 min. Each mitochondrial pellet was resuspended in buffer containing 180 mM KCl, 20 mM Tris/HCl, and 3 mM EGTA (pH 7.35) to approximately 50 mg/mL protein and kept on ice. The mitochondrial protein concentration was determined by the biuret method using bovine serum albumin as a standard.

Measurements of Mitochondrial Respiratory Rates. Oxygen uptake rates were recorded at 37 °C by means of the Clark-type electrode system in a solution containing 20 mM imidazole, 20 mM taurine, 0.5 mM dithiothreitol, 1.6 mM $MgCl_2$, 100 mM MES, 3 mM KH_2PO_4, 3 mM CaK_2EGTA, and 7.1 mM K_2EGTA (free Ca^{2+} concentration, 0.1 μM) (pH 7.1 adjusted with KOH at 37 °C). Different respiratory substrates were used: (a) 6 mM pyruvate and 6 mM malate, (b) 12 mM succinate (plus 2 mM amytal sodium), and (c) 9 μM palmitoyl-L-carnitine plus 0.24 mM malate. The solubility of oxygen was estimated to be 422 nmol O/mL. Mitochondrial respiration rates were expressed as nmol O/min/mg protein. The final mitochondrial protein concentration in all experiments was 0.5 mg/mL.

The mitochondrial State 2 respiration rate (V_0) was registered after the addition of mitochondria and substrates: pyruvate and malate, succinate, or palmitoyl-L-carnitine plus malate. Then ADP was added, and the maximal State 3 respiration rate (V_{ADP}) was measured. It reflects the maximal capacity of mitochondria to synthesize ATP. The intactness of the outer mitochondrial membrane was assessed by the addition of exogenous cytochrome *c* in State 3. The calculated respiratory control index, i.e., the ratio between the State 3 and State 2 respiration rates (V_{ADP}/V_0), together with the changes in V_0, was used to define the effectiveness of ADP phosphorylation in mitochondria.

Measurement of Mitochondrial H_2O_2 Generation. The generation of reactive oxygen species (ROS) was estimated as the release of H_2O_2 from isolated rat heart mitochondria (0.25 mg/mL), as determined fluorimetrically using a Thermo Scientific fluorometer. Mitochondria were incubated for 7 min at 37 °C in the same medium as used for the mitochondrial respiration but supplemented with pyruvate and malate (6 mM each) plus antimycin A (16 ng/mL) (control sample) and with 1.6 ng/mL or 5 ng/mL ursolic acid (**1**). The same incubations were also peformed in the presence of 1 μM of uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) but without **1**. After incubation, Amplex Red (5 μM) and horseradish peroxidase (2 U/mL) were added, and fluorescence (excitation at 544 nm, emission at 590 nm) was measured. Amplex Red fluorescence response was calibrated by adding known amounts of H_2O_2.

Evaluation of Antioxidant Activity of Ursolic Acid (1). The antioxidant activity of **1** in neutralizing H_2O_2 was determined fluorimetrically. A 5 μL aliquot of a 3% H_2O_2 solution was mixed with 200 μL of the same medium as used for mitochondrial respiration studies, and 1.6 ng/mL or 5 ng/mL concentrations of **1** were added. Amplex Red (5 μM) and horseradish peroxidase (2 U/mL) were added after 7 min of incubation, and fluorescence (excitation at 544 nm, emission at 590 nm) was measured. In parallel, the level of fluorescence was measured in a control sample without **1**. The percentage difference in fluorescence intensity between the control and the ursolic acid-containing samples was representative of the efficiency of the antioxidant activity of **1**.

Statistical Analysis. Data are presented as means ± SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by Dunnett's post test using the software package Statistica 1999, v. 5.5 (StatSoft Inc.). A value of $p < 0.05$ was taken as the level of significance.

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