The Effect of α-Tocopheryl Succinate on Succinate Respiration in Rat Liver Mitochondria

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Short title:

Effect of α-Tocopheryl Succinate on Rat Liver Mitochondria

Summary

We compared the effect of α-tocopheryl succinate (TOS) on succinate-dependent respiration in rat liver mitochondria, homogenate and permeabilized hepatocytes in both a coupled and uncoupled state. In isolated mitochondria, a significant inhibitory effect was observed at a concentration of 5 µM, in liver homogenate at 25 µM and in permeabilized hepatocytes at 50 µM. The inhibitory effect of TOS on succinate respiration in an uncoupled state was less pronounced than in a coupled state in all the experimental models tested. When the concentration dependence of the TOS inhibitory effect was tested, the most sensitive in both states were isolated mitochondria; the most resistant were permeabilized hepatocytes.

Key words

Tocopheryl succinate; Complex II; Liver; Mitochondria; Homogenate; Hepatocytes

Introduction

α-Tocopheryl succinate (TOS) is a succinyl ester of α-tocopherol, a lipid soluble antioxidant of the vitamin E group (Zingg, 2007). The succinate is linked to the hydroxyl oxygen of the chromanol ring of α-tocopherol by an ester bond, resulting in the loss of α-tocopherol's antioxidant properties (Angulo-Molina *et al.*, 2014). Surprisingly, new and unique anticancer effects of TOS have been recently described by various authors (Jha *et al.*, 1999; Neuzil *et al.*, 2006b; Weber *et al.*, 2002). It has been shown under experimental conditions that TOS induces apoptosis and inhibits cancer cell proliferation both *in vitro* (Jha *et al.*, 1999; Neuzil *et al.*, 2006a) and *in vivo* (Dong *et al.*, 2011; Stapelberg *et al.*, 2005; Zhao *et al.*, 2009). These new features of TOS were described both in cell cultures and animal models with implanted tumors, but not in non-malignant cells. Some studies even declare that TOS could be beneficial to normal human cells and help reduce oxidative stress caused by toxic agents. It was supposed that TOS may be split inside non-malignant cells, with original α-tocopherol working as an antioxidant in lipid membranes (Fariss *et al.*, 2001; Zhang *et al.*, 2001). The exact mechanism of TOS action and how TOS inhibits cancer cell proliferation has not yet been fully elucidated. There is some evidence suggesting that the apoptotic pathway is induced by suppressing the function of the transcription factor nuclear factor κB (Bellezza *et al.*, 2012). It has also been shown repeatedly that the proapoptotic effect of TOS is associated with an increase in mitochondrial permeabilization (Prochazka *et al.*, 2010) and greater production of reactive oxygen species (ROS) with consequent mitochondrial and cellular damage and apoptosis induction (Fukuzawa *et al.*, 2004; Kogure *et al.*, 2004). A possible molecular mechanism has also been recently described. Dong *et al.* (2008) postulate that TOS selectively binds to the ubiquinone binding subunit of mitochondrial respiratory Complex II. This inhibition of Complex II blocks the electron flux to ubiquinone and consequently leads to the escape of electrons from the respiratory chain. These electrons interact with oxygen and generate a high amount of ROS. Increased ROS production afterwards damages mitochondrial structures and leads to the subsequent release of cytochrome *c* to the cytosol (Kluckova *et al.*, 2013; Ralph *et al.*, 2011). Furthermore, it has been suggested that more components of the respiratory chain are affected by TOS and that the TOS inhibitory effect may differ depending on cell type. Some authors indicate that, like Complex II, Complex III also seems to be affected, and to a lesser extent even Complex I (Gruber *et al.*, 2014). Dos Santos *et al.* (2012) revealed that TOS inhibits preferentially Complex I in acute promyelotic leukemia cells, while the activity of Complex II was nearly unchanged. Moreover, Rauchova *et al.* (2014) described the inhibitory effect of TOS on the flavoprotein dependent enzyme glycerol-3-phosphate dehydrogenase in brown adipose tissue mitochondria. Different effects of TOS on succinate-dependent mitochondrial respiration of non-malignant cells have also been described (Gogvadze *et al.*, 2010).

The aim of our study was to assess the effect of TOS on succinate driven mitochondrial respiration in rat liver in three different models: isolated permeabilized hepatocytes; isolated liver mitochondria; and liver tissue homogenate. This study is supposed to provide more detailed information about the TOS influence on succinate oxidation in non-malignant liver tissue. For even a better description of TOS action we decided to measure oxygen consumption in two different respiratory states – a physiological coupled state (oxidative phosphorylation with saturation amounts of succinate, cytochrome *c* and ADP) and uncoupled state in the presence of FCCP.

Materials and Methods:

Chemicals

α-Tocopherol succinate (TOS), succinate (SUC), cytochrome *c* (CYT), adenosine diphosphate (ADP), rotenone (ROT) and carbonylcyanide-p-trifluoromethoxy-phenylhydrazone (FCCP) and all other chemicals were all of analytical grade and were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Male Albino Wistar rats (Biotest Konarovice, Czech Republic) with body weights of 250 ± 30 g were used. The animals had free access to standard laboratory diet (DOS 2B Velaz, Czech Republic) and tap water. All animals received care according to the guidelines set by the Animal-Welfare Body of the Charles University, Prague, Czech Republic, and the International Guiding Principles for Biomedical Research Involving Animals.

Isolation of rat hepatocytes

Hepatocytes were isolated from rat liver by two step collagenase perfusion (Berry *et al.* 1991). The viability of freshly isolated hepatocytes was more than 90 % as confirmed by a trypan blue exclusion test. The hepatocyte count in the samples was assessed using Cellometer (Nexcelom Bioscience LLC, USA).

Isolation of rat liver mitochondria

Liver mitochondria were isolated by differential centrifugation as described by Bustamante (Bustamante *et al.*, 1977) in a medium containing 220 mM mannitol, 70 mM sucrose, 2 mM HEPES, and 0.5 mg/ml bovine serum albumin (BSA), pH 7.2. The tissue homogenate was centrifuged for 10 min at 800 x g, and the supernatant was centrifuged 10 min at 8000 x g.

Sedimented mitochondria were washed twice by centrifugation for 10 min at 10000 x g in the isolation medium without BSA and the final sediment was also resuspended in the isolation medium without BSA. Fresh mitochondria were prepared for each experiment and used within 5 hours.

Determination of protein content

Protein content in isolated mitochondria and liver homogenate was determined by the Bradford method (Bradford, 1976) using BSA as a standard.

Oxygen consumption

Oxygen consumption was measured at 37^{o}C with a High Resolution Oxygraph 2K (Oroboros, Austria) in 2 ml of incubation medium containing 80 mM KCl, 10 mM Tris-HCl, 5 mM K-phosphate, 3 mM $MgCl_2$, 1 mM EDTA, pH 7.2. The oxygraphic curves presented are the first negative derivatives of oxygen tension changes in the incubation medium (Gnaiger *et al.*, 1995). Oroboros software Datlab 5 was used for calculating oxygen uptake rates and for presenting the oxygraphic curve. Oxygen uptake in isolated mitochondria, liver homogenates and isolated hepatocytes are expressed as either pmol/s/mg protein or pmol/s/million cells.

Respiratory protocols

At the beginning of each measurement isolated mitochondria, liver homogenate or isolated hepatocytes were added. Digitonin (4.1 µM) was added for hepatocyte permeabilization. Specific Complex I inhibitor rotenone (0.5 µM) and afterwards succinate (10 mM) was added to gain specific electron flow dependent on succinate oxidation (Gnaiger 2014). The same mitochondria/homogenate/hepatocytes were used for control and TOS influenced measurements in every experiment to gain the TOS specific effect in comparison to control. Furthermore, the maximal respiratory rate (MAX) of each sample was determined by the addition of cytochrome *c* (10 µM) and ADP (3.5 mM) for the coupled state (state 3 as described by Chance and Williams, 1955) or titration with the protonophore FCCP (0.25 µM steps) to obtain the uncoupled state (state 3u). Figure 1 illustrates the experimental conditions for evaluating the inhibitory effect of TOS on Complex II respiration in rat liver homogenate. After reaching the maximal respiration in both states we added TOS to observe its influence and note the values of the oxygen consumption rates after fifteen minutes. Since TOS was dissolved in ethanol (EtOH), we added an equivalent amount of EtOH to the control samples. When the concentration of oxygen in the chamber dropped below 40 nmol/ml, we reoxygenated the chamber by opening it as recommended by manufacturer.

Statistical Analysis

Experiments were repeated at least six times using different isolations of rat mitochondria, hepatocytes and liver homogenates. The results are expressed as a mean percentage of control respiration ± SD. After testing the normality, the statistical significance was analyzed using the one-way ANOVA test followed by Tukey–Kramer's post hoc test (GraphPad Prism 6 for Windows, GraphPad Software, USA); $p<0.05$ was considered to be statistically significant.

Results:

The representative respirometry curves of liver homogenates after the addition of rotenone, succinate, cytochrome *c* and ADP or FCCP are shown in Figure 1, where the black line represents the control measurement with ethanol and the gray line depicts the measurement with TOS. The mean absolute oxygen consumption rates of control mitochondria/homogenate after 15 minutes incubation in the coupled state were 1549 and 402 pmol O/s/mg protein, respectively; in the uncoupled state 3244 and 1210 pmol O/s/mg protein, respectively. The mean absolute oxygen consumption rates of control hepatocytes after 15 minutes incubation in the coupled state were 1548 pmol O/s/million cells and in the uncoupled state 2228 pmol O/s/million cells, respectively. In Tables 1, 2 and 3 we summarize the data obtained from the three preparations used. Table 1 shows the dose dependent inhibitory effect of TOS on isolated mitochondria in both the coupled and uncoupled state of respiration. 5 µmol/l ($p<0.05$) was the lowest concentration of TOS causing a significant decrease in succinate-dependent respiration in the coupled state; for the uncoupled state the concentration was 25 µmol/l ($p<0.001$). In the subsequent experiment with liver homogenates a significant TOS inhibitory effect was found at 25 µM TOS in the coupled state and at 100 µM in the uncoupled state (Table 2). In permeabilized hepatocytes we found an even lower sensitivity to TOS inhibitory action (Table 3). The lowest concentration which caused significant inhibition in this model was 50 µmol/l ($p<0.01$) in the coupled state and 150 µmol/l ($p<0.05$) for the uncoupled state.

Since 50 µM TOS exerts an inhibitory effect in all three samples we used this concentration to illustrate the varying levels of TOS action in individual models. This concentration of TOS has also been the one most often used in the *in vitro* experiments of other authors. Figure 2 depicts the different influence of TOS action on succinate-stimulated respiration in the suspensions of cells, mitochondria and homogenates. The most pronounced inhibitory effect of TOS in the coupled state was 54% in the case of isolated mitochondria, 38% in liver homogenates and 20% in permeabilized hepatocytes (see Figure 2). A significant inhibitory effect of 50 µM TOS in the uncoupled state was observed only in isolated mitochondria (37%; $p<0.001$). In liver homogenate and permeabilized hepatocytes a significant inhibitory effect of TOS on the uncoupled respiration was found at a higher concentration. In all models the impact of adding TOS was considerably lower in the uncoupled state in comparison to coupled state.

Discussion

In our experiments we focused on one previously described phenomenon of the TOS molecule to inhibit the mitochondrial Complex II (Dong *et al.,* 2008). Since there is a great number of articles describing this feature on cancer cells and since the inhibition rate of succinate respiration seems to differ in various cell lines, we decided to study the TOS effect on succinate respiration in non-cancerous rat liver mitochondria. For attain a better description of TOS action we performed this experiment on three different models in two respiratory states. Other authors have also tested the effect of TOS on non-cancerous succinate-dependent respiration. Gruber *et al.* (2014) used submitochondrial particles (SMP) isolated from bovine heart and succeeded in establishing the half maximal inhibitory concentration for TOS on Complex II activity at 42µM. SMP, another model for studying mitochondria, is generally considered very similar to uncoupled mitochondria and consume oxygen in a comparable ADP independent way. The study Dong *et al.* (2008) used also SMP isolated from *Paracoccus denitrificans* and rat liver mitochondria when investigating TOS action, and the results achieved are in accordance with our observations.

From our results it is clear that, in all models, TOS action on succinate-dependent respiration was more pronounced in a coupled state in comparison to an uncoupled state. Since we used a similar protocol and exactly the same conditions for both measurements (with the exception of ADP and FCCP for the coupled and uncoupled state, respectively), these results lead us to assumption that in case of rat liver mitochondria oxidative phosphorylation may be also affected by TOS action, as has been previously suggested (Gogvadze *et al.,* 2010). However, this assumption needs further investigation.

According to our results the model most sensitive to TOS action is the isolated mitochondria. Given the chemical characteristic of TOS as a lipophilic element, it is to be expected that the higher the amount of lipid material in the tested sample the weaker the inhibitory effect of TOS on succinate oxidation on the inner site of the inner mitochondrial membrane. In homogenate and hepatocytes TOS will unequally diffuse to lipid membranes of various organelles and less will remain to block the succinate dehydrogenase in the mitochondria, especially when we have a model of isolated mitochondria by itself with the highest proportion of mitochondria in the biological sample and we target the TOS directly to it, the inhibitory effect measured by respirometry may enhance. Recently, a specially adjusted molecule of TOS called MitoVES was developed (Dong *et al.*, 2011). This molecule facilitates TOS transport to the mitochondria. It was shown that MitoVES has a stronger effect on inhibiting the proliferation and induction of apoptosis in tumor tissue. Another explanation for the altering of the inhibitory action in permeabilized cells and tissue homogenates is the presence of intracellular esterases. These esterases, as suggested earlier (Neuzil and Massa, 2005), are located in the cytosol of hepatocytes and hydrolyze the ester bond between succinate and α-tocopherol. In cases such as these, TOS will provide the antioxidant α-tocopherol and the succinate; thus the inhibition of succinate oxidation should be limited. However, if the concentration of TOS is increased, the inhibition can be present by reaching the maximal capacity of these enzymes. In the case of liver homogenate the esterases are diluted with other components of shredded tissue and may not be as active as in permeabilized cells. We assume that this mechanism could offer clarification for the reduction of TOS action in these two models.

In conclusion, the results of the current study indicate that isolated mitochondria are more sensitive to TOS action than homogenate and hepatocytes, as doses required for the same level of inhibition in the latter two are much higher. We find it particularly noteworthy that there is a more pronounced inhibitory effect of TOS on oxygen consumption in a coupled state than in an uncoupled state. This leads us to the suggestion that ATP synthase activity may also be partly inhibited by TOS, since it serves as a rate limiting step of respiration in a coupled state.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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Figure legends

Figure 1: Experimental conditions for detecting the inhibitory effect of TOS. Respiration of rat liver homogenate in the presence of succinate, cytochrome *c*, and ADP (A) or FCCP (B) for the coupled state or uncoupled state. Respiration was influenced by the addition of TOS or ethanol in case of control measurement. The black curve indicates control respiration; the gray curve indicates TOS influenced respiration (c_{TOS}= 100 µmol/l for panel A; c_{TOS}= 200 µmol/l for panel B). REOX stands for re-oxygenation of the chamber.

Figure 2: Comparison of the inhibitory effect of 50µM TOS on the rate of succinate-dependent oxygen consumption in isolated rat liver mitochondria (MITO), homogenate (HOM) and digitonin-permeabilized hepatocytes (HEPA) in the coupled (Fig. 2A) and uncoupled (Fig. 2B) state.

(** $p<0.01$; *** $p<0.001$).

Table 1. The inhibitory effect of TOS on succinate respiration in rat liver mitochondria

Group	Coupled	Uncoupled
Control	100	100
TOS 5µM	75.7 ± 8.9*	95.6 ± 5.1
TOS 10µM	66.2 ± 8.5***	89.6 ± 9.2
TOS 25µM	52.3 ± 9.2***	66.8 ± 9.0***
TOS 50µM	45.7 ± 8.3***	62.8 ± 16.1***

The values indicate the relative rate of succinate-dependent respiration (mean ± SD) 15 min after addition of TOS expressed in % of control values without TOS.
(n=6; * p<0.05; ** p<0.01; *** p<0.001)

Table 2. The inhibitory effect of TOS on succinate respiration in liver homogenate

Group	Coupled	Uncoupled
Control	100	100
TOS 25µM	79.6 ± 10.3*	95.4 ± 4.6
TOS 50µM	61.6 ± 12.4***	89.8 ± 4.8
TOS 100µM	53.3 ± 13.1***	83.5 ± 9.4**
TOS 200µM	47.6 ± 11.0***	63.8 ± 9.5***

The values indicate the relative rate of succinate-dependent respiration (mean ± SD) 15 min after addition of TOS expressed in % of control values without TOS.
(n=6; * p<0.05; ** p<0.01; *** p<0.001)

Table 3. The inhibitory effect of TOS on succinate respiration in permeabilized hepatocytes

Group	Coupled	Uncoupled
Control	100	100
TOS 50µM	79.9 ± 12.0**	89.0 ± 9.1
TOS 100µM	70.8 ± 9.7***	82.3 ± 11.4
TOS 150µM	63.9 ± 9.3***	79.2 ± 11.3*
TOS 200µM	61.9 ± 7.2***	70.7 ± 17.9***

The values indicate the relative rate of succinate-dependent respiration (mean ± SD) 15 min after addition of TOS expressed in % of control values without TOS.
(n=6; * $p<0.05$; ** $p<0.01$; *** $p<0.001$)

Figure 1

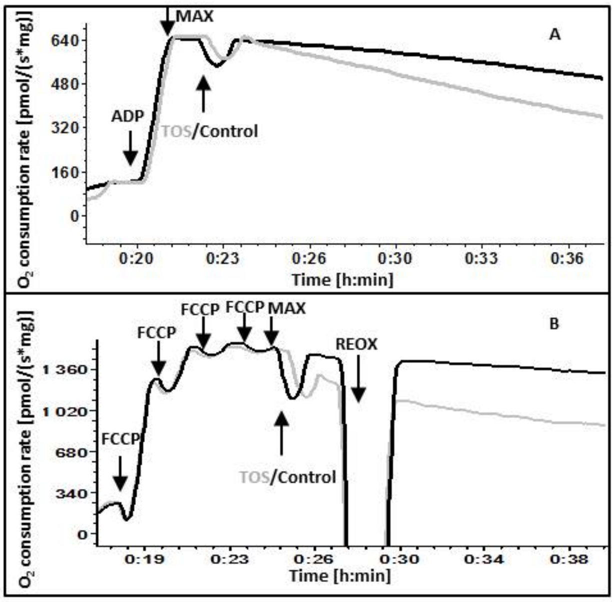

Figure 2