Estrogens prevent calcium-induced release of cytochrome *c* from heart mitochondria

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Abstract We investigated the effect of estrogens on heart mitochondrial functions and whether estrogens can prevent calcium-induced release of cytochrome *c* from mitochondria. 10 nM–10 μM 17β-estradiol or 4-hydroxytamoxifen did not affect mitochondrial respiration rate and membrane potential in state 3 and state 4. Higher concentrations of both agents decreased state 3 respiration rate and membrane potential. 100 nM 17β-estradiol and 4-hydroxytamoxifen blocked high calcium-induced cytochrome *c* release from mitochondria but not mitochondrial swelling. Thus, at physiological concentrations estrogens do not affect mitochondrial respiratory functions but protect heart mitochondria from high calcium-induced release of cytochrome *c*.

Key words: Heart; Mitochondria; Estrogen; Cytochrome *c*; Respiration

1. Introduction

Mitochondrial damage is a crucial event in the pathogenesis of ischemic cell injury and especially in promoting cell death by apoptosis [1–3]. Recent evidence suggests that apoptosis in ischemic/reperfused heart can be triggered by cytochrome *c* release from mitochondria leading to activation of caspases [4,5]. Elevation of intracellular calcium has been implicated as a causative damaging factor during heart ischemia/reperfusion, and calcium-triggered opening of mitochondrial permeability transition pore (MPT) has been shown to cause loss of cytochrome *c* from mitochondria [6]. Estrogens appear to preserve heart function in experimental models of ischemia-reperfusion [7,8] and to inhibit apoptosis in cardiomyocytes [9], however the underlying cardioprotective mechanisms remain poorly understood. There is evidence that the protective effects of estrogens are not restricted to binding to estrogen receptors and subsequent changes in gene expression. Transcription-independent actions of estrogens in myocardial ischemia and reperfusion have been suggested to be due to stimulation of NO production, inhibition of myocardial calcium accumulation or due to the antioxidant properties of estrogens [8,10]. It has also been shown that 17β-estradiol preserves mitochondrial structure and function during ischemia/reperfusion [8], however the mechanism is unclear.

Recently it has been shown that tamoxifen, a synthetic antiestrogenic agent widely used in treatment of breast cancer [11], at low concentration inhibits MPT [12,13], which may be implicated in ischemia-reperfusion injury and apoptosis [14,15]. 4-Hydroxytamoxifen is a metabolite of tamoxifen with higher affinity for estrogen receptor but lacking the ability to induce apoptosis. Both tamoxifen and 4-hydroxytamoxifen are lipophilic molecules with high partitioning into membranes [16], thus it is possible that their pharmacological effects are mediated by interactions with mitochondrial membranes.

In the present study, we aimed to elucidate the effect of 17β-estradiol and 4-hydroxytamoxifen on heart mitochondrial functions and whether at physiological concentrations they can inhibit Ca^{2+}-induced release of cytochrome *c* from mitochondria.

2. Materials and methods

Mitochondria from hearts of female Wistar rats were isolated by the method of differential centrifugation as described in [17]. Mitochondrial respiration rate was measured with a Clarke-type oxygen electrode at 37°C in 1 ml incubation buffer containing 110 mM KCl, 2.24 mM $MgCl_2$, 10 mM Tris–HCl, 5 mM nitrilotriacetic acid (NTA), 10 mM mannitol, 5 mM KH_2PO_4, 4 IU/ml creatine kinase, 50 mM creatine, 1 mM dithiothreitol and respiration substrate 1 mM pyruvate+1 mM malate (pH 7.2). Mitochondrial state 3 respiration rate was achieved by adding 1 mM ATP. 0.1 mM $CaCl_2$ was added to the medium in the experiments with high calcium concentrations and free Ca^{2+} was calculated as described in [18].

Mitochondrial membrane potential was measured using a tetraphenylphosphonium (TPP^+)-selective electrode (as described in [17]) in the experiments with estradiol or spectrophotometrically using 10 μM safranine in the experiments with 4-hydroxytamoxifen (to avoid binding of 4-hydroxytamoxifen to TPP^+-selective electrode membrane). Changes in absorption were recorded with a dual-wavelength Hitachi-557 spectrophotometer at wavelengths 484 and 522 nm. The safranine signal was calibrated (in mV) in experiments where the TPP^+ uptake was also measured in a separate vessel but at the same time with a TPP^+ electrode.

In the experiments on cytochrome *c* release, isolated heart mitochondria (1 mg/ml) were incubated for 5 min under aerobic conditions in a stirred vessel in a buffer containing 110 mM KCl, 10 mM mannitol, 5 mM NTA, 5 mM KH_2PO_4, 2.24 mM $MgCl_2$, 50 mM creatine, 1 mM pyruvate, 1 mM malate, 20 μM ATP, 5 μM free Ca^{2+}, pH 7.2, 37°C, in the presence or absence of 100 nM 17β-estradiol, 100 nM 4-hydroxytamoxifen or 0.2 μM cyclosporin A. In some experiments 20 mM KH_2PO_4 (instead of 5 mM) was used to induce maximal mitochondrial swelling. After incubation mitochondria were centrifuged at 13 000 rpm × 3 min in an Eppendorf centrifuge and supernatants were used for spectrophotometric measurements of cytochrome *c* as described in [17,19]. Total cytochrome *c* content was determined in mitochondria solubilized with 1% (w/v) Triton X-100.

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Mitochondrial swelling was measured spectrophotometrically as decrease in absorbance at 540 nm.

Chemicals used in this study were: KCl, Tris–HCl – from Roth, NTA, pyruvate – from Merck, KH_2PO_4, malate – from Serva, all other reagents – from Sigma.

3. Results

We investigated whether estrogens at physiological concentrations can prevent calcium-induced release of cytochrome *c* from mitochondria. After 5 min incubation of mitochondria in the medium containing 5 μM free Ca^{2+}, there was substantial increase in cytochrome *c* level in the supernatants compared with control mitochondrial incubated without Ca^{2+} (Fig. 1A). The release of cytochrome *c* was prevented by 0.2 μM cyclosporin A (Fig. 1A) indicating that it was related to MPT. In the presence of 100 nM 17β-estradiol or 4-hydroxytamoxifen Ca^{2+}-induced release of cytochrome *c* from mitochondria was substantially reduced (Fig. 1). Although some protective effect of estrogens was seen in the absence of added ATP, statistically significant protection by estrogens was observed in the presence of low, 20 μM, concentrations of ATP, which in our experimental conditions should be converted to ADP by mitochondrial creatine kinase in the presence of creatine. β-Estradiol also significantly reduced cytochrome *c* release induced by calcium and high phosphate (20 mM), similar to the effect of cyclosporin A (Fig. 1B). As can be seen from Fig. 2, the release of cytochrome *c* was accompanied by cyclosporin A-sensitive mitochondrial swelling, indicating MPT. However, swelling was not extensive, being only about 10% of that induced by Ca^{2+} and high (20 mM) phosphate concentration (Fig. 2). Not that high-amplitude swelling induced by Ca^{2+} and 20 mM phosphate was also prevented by cyclosporin A. However, Ca^{2+}-induced swelling was insensitive to 17β-estradiol or 4-hydroxytamoxifen (Fig. 2), suggesting that these agents did not prevent Ca^{2+} accumulation in mitochondria. Further support for this was obtained in experiments where mitochondrial respiration rate was measured. Incubation of mitochondria in medium with 5 μM free Ca^{2+} resulted in a time-dependent (due to accumulation of Ca^{2+} in mitochondria) decrease in state 3 respiration rate and after 5 min incubation the steady-state respiratory rate was 217 ± 28 natom O/min/mg in Ca^{2+}-treated mitochondria compared to 284 ± 25 natom O/min/mg in untreated control mitochondria ($P < 0.05$). 100 nM 17β-estradiol and 4-hydroxytamoxifen did not prevent Ca^{2+}-induced inhibition of state 3 respiration rate: 194 ± 19 and 193 ± 21 natom O/min/mg in the presence of estradiol and 4-hydroxytamoxifen, respectively, suggesting that estrogens at low concentration did not inhibit Ca^{2+} uptake by mitochondria.

Next we tested whether the protective effect of estrogens could be related to estrogen-induced changes in mitochondrial respiratory rate or membrane potential. As can be seen from Fig. 3, 10 nM–10 μM of estradiol had no effect on mitochondrial respiration and membrane potential. Higher concentrations of estradiol (50–100 μM) decreased state 3 respiration and membrane potential by about 30–50% (Fig. 3A). State 4 respiration rate was not affected by 17β-estradiol, however membrane potential was significantly inhibited by high concentrations of estradiol (Fig. 3B). Similar results were obtained with 4-hydroxytamoxifen: 10 nM–10 μM of 4-hydroxytamoxifen had no effect on mitochondrial respiration and membrane potential in state 3 and state 4, however at 50 μM concentration it inhibited both investigated parameters (Fig. 4). These data show that in the range of physiological, nanomolar concentrations 17β-estradiol (as well as 4-hydroxytamoxifen) does not affect mitochondrial respiratory functions; inhibition occurs only at high, non-physiological concentrations.

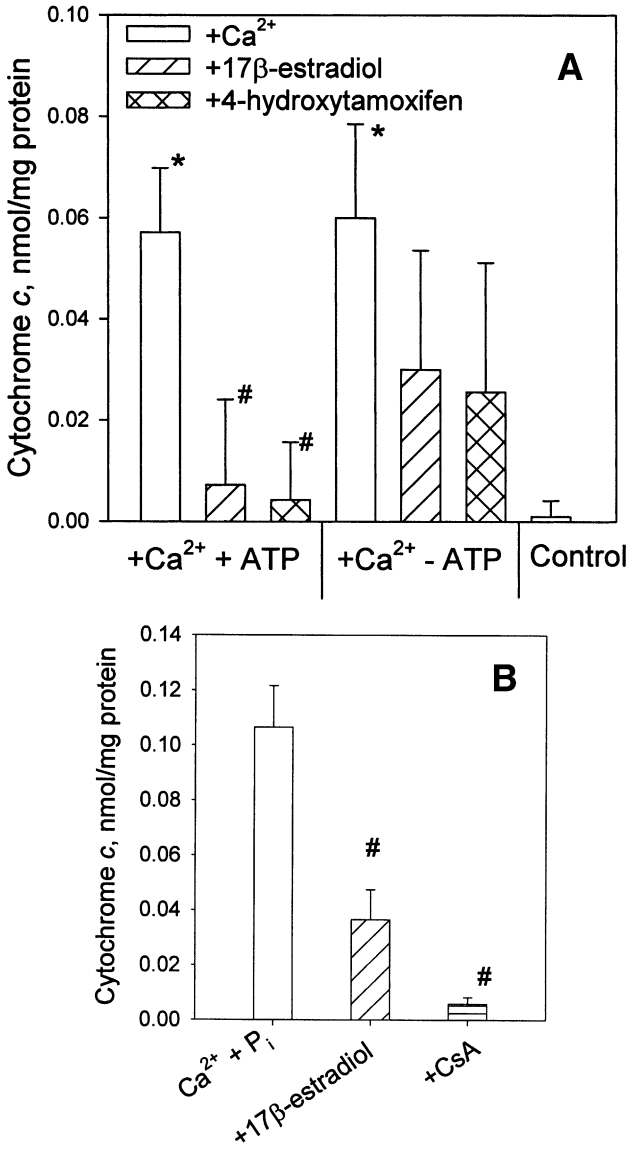

Fig. 1. Effect of estrogens on the amount of cytochrome *c* released from mitochondria. A: Mitochondria (1 mg/ml protein) were incubated at 37°C for 5 min in the medium containing 110 mM KCl, 10 mM Tris–HCl, 10 mM mannitol, 5 mM NTA, 5 mM KH_2PO_4, 1 mM free Mg^{2+}, 50 mM creatine, 1 mM pyruvate and 1 mM malate, 20 μM ATP, 5 μM free Ca^{2+}. Where indicated, 100 nM 17β-estradiol, 100 nM 4-hydroxytamoxifen, were added or ATP excluded. Control – mitochondria incubated in the same medium but without calcium. Mitochondria were removed by centrifugation, the supernatants were used for measurement of released cytochrome *c*. The total mitochondrial cytochrome *c* content was 0.379 ± 0.022 nmol/mg protein. B: Mitochondria were incubated under similar conditions as in (A) (in the presence of 20 μM ATP), but the incubation medium contained 20 mM KH_2PO_4. 0.2 μM cyclosporin A was added where indicated. * – statistically significant difference compared to control, # – compared to 5 μM Ca^{2+}, $P < 0.05$ ($n = 5$–8).

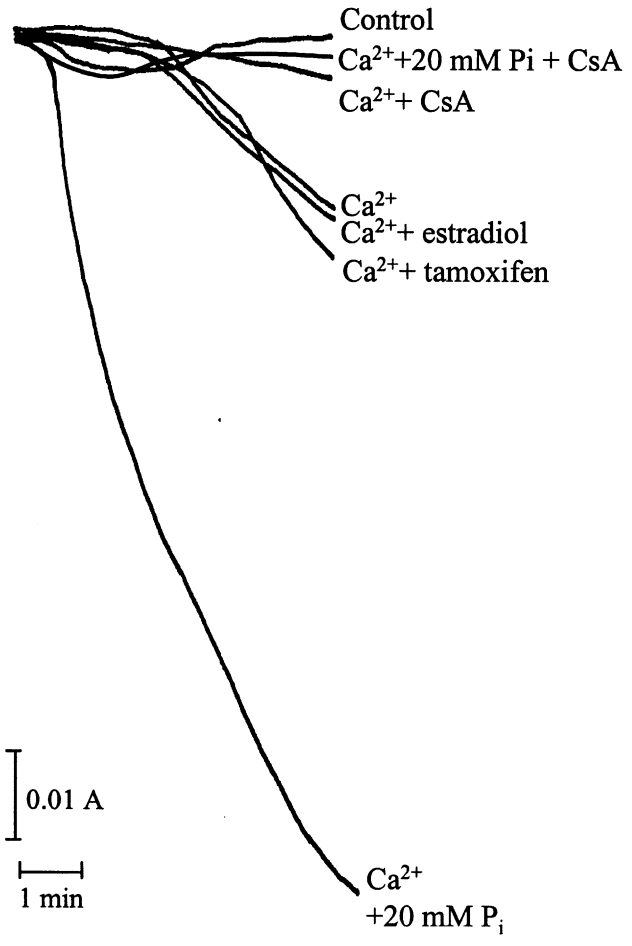

Fig. 2. Effect of estrogens on Ca^{2+}-induced mitochondrial swelling. Mitochondria (0.5 mg/ml) were incubated under the same conditions (with 20 μM ATP) as in Fig. 1 for 5 min. Where indicated 100 nM 17β-estradiol, 100 nM 4-hydroxytamoxifen, 0.2 μM cyclosporin A or 20 mM phosphate were added. Representative traces of five experiments are shown.

4. Discussion

The present study for the first time reports that at physiological, nanomolar concentrations 17β-estradiol and 4-hydroxytamoxifen prevent calcium-induced release of cytochrome *c* from heart mitochondria. It has been previously shown that tamoxifen, and estrogen receptor agonist/antagonist, like cyclosporin A, inhibits induction of MPT in rat liver mitochondria [12] and cultured neurons [13]. We hypothesized that similar effect might be exerted by a metabolite of tamoxifen, 4-hydroxytamoxifen, and 17β-estradiol, thus we studied the effect of these agents on MPT-related release of cytochrome *c* from mitochondria. Calcium-induced release of cytochrome *c* from isolated mitochondria is known to be related to opening of MPT [6,17,20]. We observed that at nanomolar concentrations both 4-hydroxytamoxifen and 17β-estradiol prevented calcium-induced loss of cytochrome *c* from heart mitochondria. The protective effect of estrogens was greatly enhanced in the presence of low micromolar concentrations of adenine nucleotides, which suggests possible involvement of the ATP/ADP translocator, a component of MPT. However, in contrast to cyclosporin A, estrogens did not inhibit Ca^{2+}-induced mitochondrial swelling. This suggests that: (i) the release of cytochrome *c* from mitochondria was not related to mitochondrial swelling; and (ii) the protective actions of estrogens are not due to blocking swelling. We do not think that the protective effect of estrogens is due to inhibition of Ca^{2+} uptake by mitochondria because we found that Ca^{2+} inhibited mitochondrial respiration to the same extend in the presence or absence of 17β-estradiol and 4-hydroxytamoxifen. Moreover, estrogens did not prevent Ca^{2+}-induced mitochondrial swelling. Consistent with this, it has previously been shown that estradiol does not inhibit the uptake of Ca^{2+} by rat brain mitochondria [21]. It has been suggested that estrogens might stimulate Ca^{2+} efflux through the Na^+/Ca^{2+} antiporter [21], however such an effect is unlikely in our experimental conditions where Na^+-free mitochondrial incubation buffers (and reagents) were used.

High concentrations of estrogens have a damaging effect on heart mitochondrial functions. We show that 50–100 μM estrogens strongly inhibit mitochondrial respiration and membrane potential presumably due to decreased activity of the

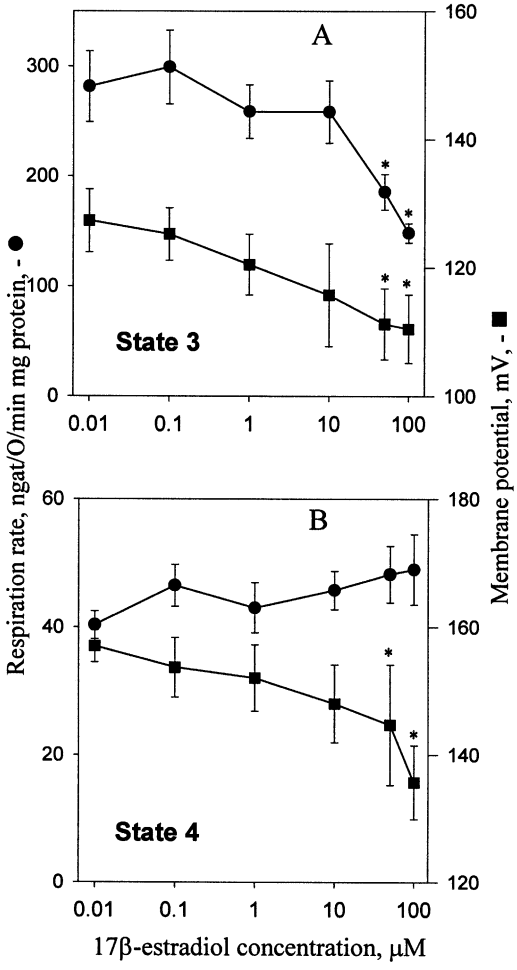

Fig. 3. The effect of 17β-estradiol on heart mitochondrial respiration rate and membrane potential in state 3 (A) and state 4 (B). Isolated female rat heart mitochondria were incubated with 10 nM–100 μM of 17β-estradiol for 5 min at 37°C. Means ± standard errors of four separate experiments are presented. State 3 respiration rate in control without 17β-estradiol was 295 ± 23 natom O/min/mg protein, membrane potential 129 ± 4 mV, in state 4 – respectively 43 ± 2 and 157 ± 2.5. $^*P < 0.05$ compared to control,. ● – respiration rate, ■ – membrane potential.

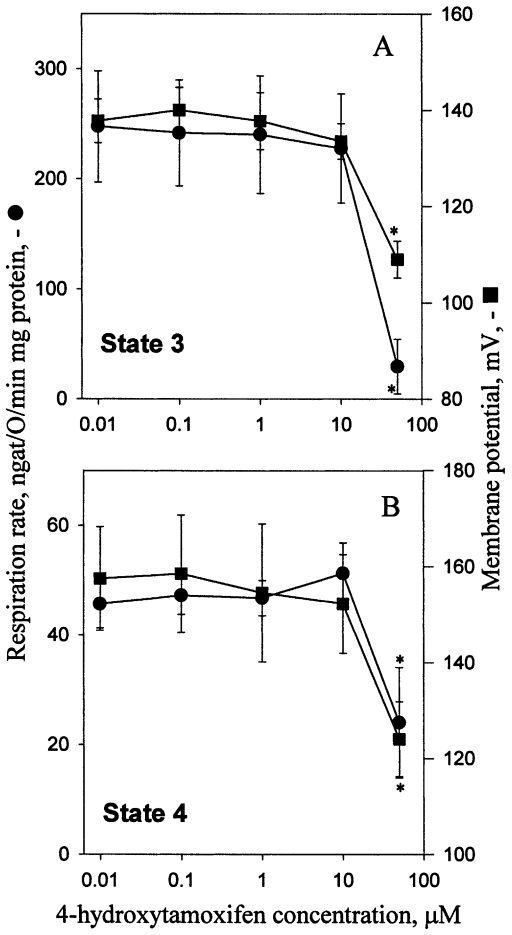

Fig. 4. The effect of 4-hydroxytamoxifen on heart mitochondrial respiration rate and membrane potential in state 3 (A) and state 4 (B). Experimental conditions were as in Fig. 3. Means ± standard errors of four separate experiments are presented. State 3 respiration rate in control without 4-hydroxytamoxifen was 278 ± 28 natom O/min/mg protein, membrane potential 144 ± 3 mV, in state 4 – respectively 45 ± 2 and 158 ± 24. * – $P < 0.05$ compared to control. ● – respiration rate, ■ – membrane potential.

respiratory chain. The inhibition of the respiratory chain may be due to non-specific binding of estrogens to hydrophobic regions of the mitochondrial membranes [16,22], which may change protein–lipid interactions, disturb electron transport through the inner mitochondrial membrane and reduce membrane potential. Tamoxifen and 4-hydroxytamoxifen have been shown to inhibit electron transport at the levels of complex III and complex IV [23]. Rat brain mitochondrial F_0F_1-ATPase has been also shown to be a target for estradiol and related estrogenic compounds [24], therefore, state 3 respiration with high concentrations of estradiol may be inhibited due to inhibition of the ATPase.

In conclusion, the present study provides evidence that at physiological concentrations, which do not inhibit mitochondrial functions, estrogens can protect heart mitochondria from the loss of cytochrome *c* induced by high calcium, and this might be one of the possible mechanisms by which estrogens preserve myocardial cell viability after ischemia/reperfusion.

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