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Estradiol prevents ischemia-induced release of cytochrome c from heart mitochondria

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It has been shown that estrogens play a cardioprotective role in global myocardial ischemia-reperfusion in female rats, however, the mechanism is unclear. We have previously reported that 17β-estradiol can protect heart mitochondria from the loss of cytochrome c induced by high calcium [4]. In this study we aimed to elucidate whether estradiol can prevent release of cytochrome c from mitochondria and subsequent inhibition of respiration after 30 min stop-flow ischemia in Langendorff-perfused rat female hearts. We found that pre-perfusion of hearts with 100 nM of 17β-estradiol (high physiological concentration) partially prevented the loss of cytochrome c from mitochondria and subsequent inhibition of mitochondrial respiration. These data suggest that the cardioprotective effect of estrogens against ischemic damage might be partly related to their direct effect on mitochondria.

Key words: heart, mitochondria, estrogen, cytochrome c, respiration

INTRODUCTION

Myocardial ischemia is the most widespread disease in the world. When the blood flow is interrupted, the concentration of oxygen falls to very low levels and causes structural and functional disorders of cells and, finally, cell death. It is well known that premenopausal women have much lower risk of ischemic heart disease, although the mechanism remains unclear. Estrogens appear to preserve heart function in experimental models of ischemia-reperfusion [1, 2]. There is evidence that protective effects of estrogens are not restricted to binding to estrogen receptors but they might be direct. Protective effects of estrogens during myocardial ischemia and reperfusion have been suggested to be due to the antioxidant properties, stimulation of nitric oxide production, inhibition of myocardial calcium accumulation [2]. Furthermore, they can prevent oxidative stress- and other stimuli-induced apoptosis in cardiomyocyte culture [3]. It also has been shown that estradiol preserves mitochondrial structure and function during ischemia/reperfusion, however, the mechanism is unclear [2]. Our previous studies have shown that estrogens can protect isolated heart mitochondria from the loss of cytochrome c induced by high calcium [4], which is thought to be one of the damaging factors during heart ischemia/reperfusion [5, 6]. It was also shown that cytochrome c release from mitochondria is one of the earliest mitochondrial events during ischemia [12, 13], which causes inhibition of respiration, activation of caspases and nuclear apoptosis [7, 8]. Thus, in the present study, we sought to elucidate whether perfusion of the heart with 17β-estradiol can prevent cytochrome c release from mitochondria and mitochondrial respiratory inhibition induced by a 30-min stop-flow ischemia in rat female hearts.

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MATERIALS AND METHODS

1. Experimental model

Hearts from female Wistar rats were used in experiments. The hearts were perfused on a Langendorff perfusion system with Krebs–Henseleit solution (11 mM glucose, 118 mM NaCl, 25 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.7 mM $MgSO_4$ and 0.7 mM Na pyruvate, pH 7.2 at 37 °C), with or without 100 nM of 17β-estradiol. After 15 min of perfusion, stop-flow ischemia was induced for 30 min. *In vitro* ischemia was induced by autolysis as described previously [9].

2. Preparation of cytosolic and mitochondrial fractions of myocardial tissue

Hearts were cut into small pieces and homogenised in the medium (5 ml/1 g of tissue) containing 180 mM KCl, 20 mM Tris HCl, 1 mM EGTA, pH 7.3 at a temperature of 2 °C. Cytosolic and mito-

Table 1. **Pre-perfusion with estradiol prevents ischemia-induced release of cytochrome c from mitochondria to cytosol**

Content of cytochromes	Control	Estradiol + control	30 min ischemia	Estradiol + 30 min ischemia
Mitochondrial cytochrome c content, nmol/mg prot.	0.451 ± 0.015	0.466 ± 0.051	0.349 ± 0.017*	0.408 ± 0.009#
Mitochondrial cytochrome a content, nmol/mg prot.	0.319 ± 0.019	0.303 ± 0.018	0.272 ± 0.011	0.291 ± 0.009
Cytosolic cytochrome c content, μg/mg prot.	2.31 ± 0.24	2.5 ± 0.14	3.08 ± 0.19*	2.59 ± 0.06#

Hearts were perfused with/without 100 nM of estradiol, then a 30 min stop-flow ischemia was induced, after which mitochondria were isolated. Control hearts were perfused for the same time with buffer ± estradiol only. Mitochondrial content of cytochromes was measured spectrophotometrically, and cytosolic cytochrome c – using ELISA kit as described in Methods. Means ± standart errors of 4–10 separate experiments are presented. * $p < 0.05$, compared with control, # $p < 0.05$, compared with 30 min ischemia.

chondrial fractions were separated by differential centrifugation (5 min ´ 1000 g, 10 min ´ 9000 g). The post-mitochondrial supernatant was additionally centrifuged for 30 min at 100000 g and the resulting supernatant (S_{100}) was used for determination of cytochrome c content in the cytosol. Total cytosolic and mitochondrial protein was measured by a modified Biuret method [10].

3. Measurement of respiration rate.

Mitochondrial respiration rate was measured with a Clarke-type oxygen electrode at 37 °C in 1 ml of incubation buffer containing 110 mM KCl, 2.24 mM $MgCl_2$, 10 mM Tris HCl, 5 mM KH_2PO_4, 4 IU/ml creatine kinase, 50 mM creatine, and respiration substrate 1 mM pyruvate + 1 mM malate (pH 7.2). Mitochondrial state 3 respiration rate was achieved by adding 1 mM ATP. In some experiments mitochondrial respiration was measured in the presence of 30 μM exogenous cytochrome *c*.

4. Measurement of cytochrome c and a in mitochondria.

Total cytochrome c and cytochrome a content was determined in mitochondria solubilized with 1% Triton X-100 (w/v). Sodium hydrosulphite-reduced minus hydrogen-peroxide-oxidized absorption difference spectra were recorded with a Hitachi-557 spectrophotometer and the content of mitochondrial cytochromes was calculated as described in [11].

5. Determination of cytochrome c content in cytosol.

Cytochrome c content in cytosolic fractions was detected using Quantikinine M rat/mouse Immunoassay ELISA kit (R & D Systems). Cytosolic fraction proteins were dissolved in 0.5% Triton X-100 and the further procedures were performed according to manufacturer's protocol.

6. Statistical analysis.

Data are expressed as means ± S.E. of at least three separate experiments. Statistical comparison between experimental groups was performed using Student's t test.

RESULTS AND DISCUSSION

We investigated whether 17β-estradiol can prevent the release of cytochrome c from mitochondria and a subsequent inhibition of respiration after a 30-min ischemia in Langendorff-perfused rat female hearts. After 30 min of ischemia the mitochondrial level of cytochrome c decreased by 23% *versus* the control level (Table 1). However, in mitochondria from ischemic hearts pre-perfused with 100 nM estradiol the content of cytochrome c was not significantly different from control mitochondria. Ischemia and estradiol had no significant effect on the mitochondrial content of cytochrome a, an integral component of the inner membrane. We also measured cytochrome c concentration in cytosolic fraction after pre-perfusion with estradiol and a 30-min ischemia (Table 1). Cytochrome c concentration in cytosolic extracts from 30 min ischemic hearts increased by 33% compared to control, while in cytosolic extracts from ischemic + estradiol hearts there was no significant increase in cytochrome c concentration compared to control level. Together these results show that estradiol prevents a 30-min ischemia-induced release of cytochrome c from mitochondria to the cytosol.

Next we tested whether perfusion of the heart with estradiol has any effect on mitochondrial respiration.

Table 2. **Pre-perfusion with estradiol decreases the inhibition of mitochondrial respiration rate in state 3 after 30 min ischemia**

Type of respiration rate	Control	Estradiol + control	30 min ischemia	Estradiol + 30 min ischemia
State 3 respiration rate, ngat O/min mg prot.	360 ± 23	322 ± 20	192 ± 10*	273 ± 16#
Cytochrome c stimulated state 3 respiration rate, ngat O/min mg prot.	536 ± 39	488 ± 37	349 ± 35*	449 ± 25#

After 15 min of perfusion and subsequent ischemia mitochondria were isolated and respiration rate was measured. 1 mM pyruvate + 1 mM malate was used as respiratory substrates. Mitochondrial state 3 respiration rate was achieved by adding 1 mM ATP. Where indicated, 30 mM exogenous cytochrome c was added. Means ± standart errors of 4–10 separate experiments are presented. * $p < 0.05$, compared with control, # $p < 0.05$, compared with 30 min ischemia

Mitochondrial state 3 respiration rate after 30 min of ischemia was lower by 46% compared to control mitochondria oxidizing pyruvate and malate (Table 2). However, in mitochondria from ischemic hearts preperfused with estradiol, state 3 respiration rate was higher by 23% if compared to respiration of ischemia-damaged mitochondria. Exogenous cytochrome c added to mitochondrial incubation medium stimulated the respiratory rate by 110% after 30 min of ischemia, and this effect was decreased to 70% in mitochondria from ischemia + estradiol hearts. Exogenous cytochrome c stimulates respiratory rate only if there is deficiency of endogenous cytochrome c and if the mitochondrial outer membrane is damaged and permeable to cytochrome c. Thus, these data suggest that inhibition of respiration after ischemia is partially due to the loss of cytochrome c and to the fact that estradiol increases a 30-min ischemia-inhibited respiratory rate partially due to the protection of mitochondria from the loss of cytochrome c. Part of mitochondria might be damaged during the isolation procedure, and that possibly was the reason why the added cytochrome c had an ~30% effect on respiration of control mitochondria.

Our data provide some evidence that the cardioprotective effect of estrogens against ischemic damage might be related to their direct or indirect effect on mitochondria. The data presented here are consistent with the results of other investigators who showed that perfusion with estradiol increased mitochondrial functional activity and prevented structural disorders after ischemia/reperfusion [2]. The same authors demonstrated that estradiol reduced accumulation of cytosolic calcium after ischemia/reperfusion. High calcium during ischemia/reperfusion may open a mitochondrial permeability transition pore [14], which induces the release of cytochrome c and inhibits ATP synthesis [7] may also activate phospholipases that cause damage to membranes [15], or may increase the generation of reactive oxygen species [16]. Reactive oxygen species can damage mitochondrial membranes and may also activate permeability transition pore opening [17]. It has been demonstrated that estradiol has antioxidant properties [18, 19], therefore the protective effect of estradiol after ischemia may be due to its antioxidant action. We have previously shown that the release of cytochrome c during ischemia is related to the mitochondrial permeability transition [7, 8] and that estradiol may protect isolated mitochondria from the MPT-related release of cytochrome c [4], therefore the action of estradiol on mitochondria during ischemia might be related to MPT.

In conclusion, the present study provides evidence that at physiological concentrations estradiol partially protects heart mitochondria from the release of cytochrome c and subsequent inhibition of respiration, and this might be one of the possible mechanisms by which estrogens preserve myocardial cell viability in females after ischemia/reperfusion.

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ESTRADIOLIS APSAUGO ÐIRDIES MITOCHONDRIJAS NUO CITOCHROMO C NETEKTIES PO 30 MIN. TRUKUSIOS ISCHEMIJOS

S a n t r a u k a

Nustatyta, kad estrogenai apsaugo ðirdies làsteles nuo ischemijos/reperfuzijos metu atsirandanèios paþaidos, taèiau mechanizmai nëra iki galo iðtirti. Ankstesniais tyrimais iðsiaiðkinome, kad estrogenai apsaugo izoliuotas ðirdies mitochondrijas nuo kalcio jonø sukelto citochromo c iðëjimo ið mitochondrijø. Ðiame darbe tyrëme, ar estradiolis apsaugo mitochondrijas nuo citochromo c netekties ir kvëpavimo greièio inhibicijos po 30 min. trukmës ischemijos, perfuzuodami þiurkiø pateliø ðirdis Langendorfo bûdu. Nustatëme, kad po 100 nM 17b-estradiolio (aukðta fiziologinë koncentracija) pre-perfuzijos ir 30 min. trukusios ischemijos ið dalies sustabdomas citochromo c iðëjimas ið mitochondrijø á citozolá, taip pat padidëja mitochondrijø kvëpavimo greitis treèioje metabolinëje bûsenoje oksiduojant piruvatà ir malatà. Tai gali bûti vienas ið estradiolio apsauginiø mechanizmø, veikianèiø miokardo làstelëse ischemijos metu.