BIOLOGIJA. 2006. Nr. 3. P. 88–91

Estradiol protects female and male rat heart mitochondria from ischemic damage

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We investigated whether estradiol can prevent the ischemia-induced release of cytochrome *c* from mitochondria and inhibition of mitochondrial respiration in Langendorff-perfused female and male rat hearts. We found that pre-perfusion of hearts with 100 nM 17β-estradiol significantly reduced 60 min ischemia-induced loss of cytochrome c from mitochondria in both female and male hearts. Estradiol also reduced inhibition of mitochondrial state 3 respiration rate oxidizing pyruvate and malate caused by 60 min ischemia in hearts from both animal genders. Therefore, prevention of loss of cytochrome *c* from mitochondria by 17β-estradiol may be one of the possible mechanisms by which estrogens preserve myocardial cell viability during ischemia in both genders.

Key words*:* estrogens, heart, ischemia, mitochondria, respiration, cytochrome *c*

INTRODUCTION

The incidence of cardiovascular disease differs significantly between men and women, in part because of differences in steroid hormones. A variety of experimental models established estrogens as critical mediators of cardioprotection. Estrogens have been demonstrated to reduce the extent of irreversible myocardial injury, ventricular arrhythmias, infarct size after ischemia / reperfusion [1–3]. A number of mechanisms have been proposed to explain the protective actions of estradiol in the heart, including classical genomic or non-genomic responses. Estrogens act primarily through nuclear estrogen receptors (ERα and ERβ) which regulate gene expression leading to the control of a variety of cellular functions [4]. Estrogens can also exert "non-genomic", transcription-independent effects on cellular functions [5]. These non-transcriptional actions of estrogens in myocardial ischemia / reperfusion have been suggested to be due to stimulation of NO production, inhibition of myocardial calcium accumulation, preservation of mitochondrial structure and function [2, 6] and antioxidant action [7, 8]. Interestingly, estrogens improve not only female but also male cardiac function after ischemia / reperfusion [3, 9].

Studies indicate that estrogens exert beneficial effects on the cardiovascular system, partly through their action on mitochondria [2, 9]. However, the mechanism of how estrogens preserve mitochondrial structure and function remains poorly understood. Our previous studies have shown that estradiol protects isolated heart mitochondria from the loss of cytochrome *c* induced by high calcium [10], which is thought to be one of the damaging factors during ischemia / reperfusion [11, 12]. Cytochrome *c* release from mitochondria is one of the earliest mitochondrial events during ischemia [13, 14], which causes inhibition of ATP synthesis and apoptosis [15, 16]. Thus, in the present study, we aimed to elucidate whether perfusion of the heart with 17β-estradiol can prevent cytochrome *c* release from mitochondria to cytosol and the subsequent mitochondrial respiratory inhibition induced by 60 min stop-flow ischemia in Langendorff-perfused rat female and male hearts.

MATERIALS AND METHODS

The procedures used in this study are approved by the European Convention for the protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (License No. 0006).

Hearts from 2–3-month-old female and male Wistar rats were used in the 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, 1.2 mM $CaCl_2$, 1.7 mM $MgSO_4$ and 0.7 mM Na pyruvate, pH 7.2 at 37 °C) for 15 min ± 100 nM 17β-estradiol. Control hearts were perfused for the same time but without

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estradiol. After 15 min of perfusion stop-flow ischemia was induced for 60 min.

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 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 which in the presence of creatine kinase and creatine is converted into ADP. In some experiments mitochondrial respiration was measured in the presence of 30 μM exogenous cytochrome *c*.

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 spectra difference was recorded with a Hitachi-557 spectrophotometer. Cytochrome *c* content was estimated by using the absorption difference at the wavelength pair 550 / 535 nm and $\varepsilon = 14.5$ $mM^{-1}cm^{-1}$ (at the wavelength 550 nm); for cytochrome *a*– wavelength pair 605/630 nm and $\varepsilon = 10.4$ $mM^{-1}cm^{-1}$ (at the wavelength 605 nm) as described in [17].

Data are expressed as means ± S.E. of at least five separate experiments. An analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used for comparisons between experimental groups. A value of $p < 0.05$ was considered statistically significant.

RESULTS

We investigated whether a high physiological concentration of 17β-estradiol (100 nM) can prevent the release of cytochrome *c* from mitochondria to the cytosol after 60 min of ischemia in perfused rat female and male hearts. In female rat hearts, as can be seen in Table 1, the content of cytochrome *c* in mitochondria decreased by 26% after 60 min of ischemia as compared to the control level. However, in mitochondria isolated from ischemic hearts pre-perfused with 100 nM estradiol, the content of cytochrome *c* was not significantly different from control or control + estradiol mitochondria. Similar results were obtained in male heart mitochondria (Table 1). The amount of cytochrome *c* after 60 min of ischemia decreased by 24% as compared to the control level, and estradiol blocked the release of this protein from mitochondria during ischemia. Estradiol did not change the amount of cytochrome *c* in both female and male control mitochondria. Ischemia or estradiol itself had no significant effect on the mitochondrial content of cytochrome *a,* an integral component of the inner membrane in both female and male heart mitochondria (Table 1), indicating that there was no general degradation of mitochondrial proteins and the release was specific to cytochrome *c*.

Next we have tested whether perfusion of the hearts with estradiol can prevent ischemia-induced inhibition of mitochondrial respiration. As can be seen from Table 2, mitochondrial state 3 respiration rate after 60 min of ischemia was lower by 63% in female and 69% in male mitochondria as compared to control mitochondria oxidizing pyruvate and malate. However, in mitochondria from 60 min ischemic hearts pre-perfused with estradiol, state 3 respiration rate was lower by 32% in female and by 49% in male mitochondria as compared to the respiration of control mitochondria.

We also tested the effect of cytochrome *c* on the respiration rate of ischemia-damaged mitochondria and compared this effect with the respiration of estradiol-

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

Content of cytochromes	Control	Estradiol + control	60 min ischemia	Estradiol + 60 min ischemia
Mitochondrial (female) cytochrome *c* content, nmol/mg protein	0.440 ± 0.015	0.466 ± 0.051	0.327 ± 0.017*	0.408 ± 0.022#
Mitochondrial (male) cytochrome *c* content, nmol/mg protein	0.484 ± 0.019	0.479 ± 0.015	0.369 ± 0.021*	0.463 ± 0.022#
Mitochondrial (female) cytochrome a content, nmol/mg protein	0.297 ± 0.013	0.303 ± 0.018	0.262 ± 0.026	0.264 ± 0.013
Mitochondrial (male) cytochrome a content, nmol/mg protein	0.346 ± 0.035	0.332 ± 0.039	0.308 ± 0.022	0.357 ± 0.009

Hearts were perfused with Krebs–Henseleit solution +/– estradiol for 15 min, then 60 min stop-flow ischemia was induced, after which mitochondria were isolated. The control hearts were perfused for the same time with Krebs–Henseleit solution +/– estradiol only. Mitochondrial content of cytochromes was measured spectrophotometrically as described in Methods. Means ± standart errors of 5–11 separate experiments are presented.

* – $p < 0.05$, compared with control, # – $p < 0.05$ compared with 60 min ischemia.

Table 2. **Pre-perfusion with estradiol increases the mitochondrial respiration rate in state 3 after 60 min ischemia in female and male hearts**

Type of respiration rate	Control	Estradiol + control	60 min ischemia	Estradiol + 60 min ischemia
State 3 respiration rate, ngat O/min mg protein – female	360 ± 20	322 ± 20	132 ± 21*	245 ± 31#
Cytochrome *c* stimulated state 3 respiration rate, ngat O/min mg protein – female	548 ± 37*	506 ± 46*^	355 ± 33*^	483 ± 41#^
State 3 respiration rate, ngat O/min mg protein – male	522 ± 79	531 ± 39	162 ± 27*	265 ± 34#*
Cytochrome *c* stimulated state 3 respiration rate, ngat O/min mg protein – male	730 ± 69	756 ± 45^	367 ± 49^	502 ± 46#^

Hearts were perfused with Krebs–Henseleit solution +/– estradiol for 15 min, then 60 min stop-flow ischemia was induced, after which mitochondria were isolated and respiration rate was measured. The control hearts were perfused for the same time with Krebs–Henseleit solution +/– estradiol only. 1 mM pyruvate + 1 mM malate was used as a respiratory substrate. Where indicated, 30 μM exogenous cytochrome c was added. Means ± standart errors of 5–11 separate experiments are presented. * – $p < 0.05$ compared with control, # – $p<0.05$ compared with 60 min ischemia, ^ – $p < 0.05$ compared with the same sample without cytochrome c.

treated ischemia-damaged mitochondria. Exogenous cytochrome *c* stimulates respiratory rate only if there is a deficiency of endogenous cytochrome *c* and if the mitochondrial outer membrane is damaged and permeable to cytochrome *c*. Exogenous cytochrome *c* added to the incubation medium stimulated respiratory rate by 169% in female and by 127% in male mitochondria after 60 min of ischemia as compared with respiration of ischemia-damaged mitochondria without cytochrome *c* added (Table 2). However, when hearts were perfused with estradiol, cytochrome *c* stimulated respiration only by 97% and 89% in female and male mitochondria from 60 min ischemia, respectively. Part of mitochondria might be damaged during isolation, which may be the reason why the added cytochrome *c* stimulated, by 52% in female and by 40% in male, the respiration of control mitochondria. Thus, our data suggest that inhibition of respiration after ischemia is partially caused by the loss of cytochrome *c* from mitochondria and that estradiol partially prevents ischemia-induced respiratory inhibition, partly by protecting mitochondria from the loss of cytochrome *c*.

DISCUSSION

In the present study, we have demonstrated that estradiol preserves heart mitochondria from the loss of cytochrome *c* and subsequent inhibition of respiration after ischemia in both animal genders. Myocardial ischemia causes impairment of mitochondrial function, termination of mitochondrial ATP synthesis and, finally, cellular energy deficiency. One of the earliest events during ischemia is the loss of cytochrome *c*, a protein of the mitochondrial respiratory chain. Some authors have demonstrated that estradiol-treated hearts showed a higher total activity of dehydrogenases (including mitochondrial enzymes, measured by MTT (dimethylthiazoldiphenyltetrazolium bromide) assay) after ischemia-reperfusion [2, 9]. Similarly, a recent study has reported that estradiol reduced cytochrome *c* translocation and minimized the hippocampal damage caused by transient global ischemia in rat [18].

The classical target of estrogens is the nucleus, however recent studies indicate that exogenously added estrogens are also transported to mitochondria [19]. Therefore it is likely that heart perfusion with estradiol may also lead to accumulation, at least partial, of this hormone in mitochondria where it can act on membranes directly or indirectly through estrogen receptors which have recently been identified in mitochondria [20, 21]. In isolated mitochondria, exogenously added estradiol has been shown to inhibit permeability transition pore (PTP)-related release of cytochrome *c* from mitochondria induced by high calcium [10]. High calcium during ischemia / reperfusion may open the mitochondrial PTP pore [22] which induces the release of cytochrome *c* and inhibits ATP synthesis [15, 16]. Some authors have shown that estradiol inhibits calcium accumulation in cytosol during ischemia / reperfusion [2, 8], so it might be that estradiol reduces the possibility to open the PTP induced by high calcium during ischemia. In addition, estradiol inhibits oxidative stress [8], therefore the protective effect of estradiol after ischemia might be induced by its antioxidant action on mitochondrial membrane integrity.

In conclusion, the present study provides evidence that, at high physiological concentrations, estradiol protects female and male 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 during ischemia / reperfusion.

ACKNOWLEDGEMENTS

This work was supported by the Lithuanian State Science and Studies Foundation.

Received 20 February 2006
Accepted 29 June 2006

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ESTRADIOLIO APSAUGA NUO IŠEMIJOS SUKELTOS ŽIURKIŲ PATELIŲ IR PATINŲ MITOCHONDRIJŲ PAŽAIDOS

S a n t r a u k a

Šiame darbe tyrėme, ar estradiolis apsaugo mitochondrijas nuo išemijos sukelto citochromo c praradimo ir kvėpavimo greičio slopinimo perfuzuojant žiurkių patelių ir patinėlių širdis Langendorfo būdu. Nustatėme, kad po perfuzijos su 100 nM 17β-estradiolio ir 60 min. išemijos labai sumažėjo mitochondrijų citochromo c praradimas tiek patelių, tiek patinėlių širdyse. Taip pat estradiolis sumažino 60 min. išemijos nulemtą mitochondrijų kvėpavimo greičio slopinimą oksiduojant piruvatą ir malatą abiejų lyčių gyvūnėliuose. Gauti rezultatai rodo, kad 17β-estradiolis sustabdo citochromo c išėjimą iš mitochondrijų, ir tai gali būti vienas iš estradiolio apsauginių mechanizmų, veikiančių miokardo ląstelėse išemijos metu.