Low Concentrations of 17β-Estradiol Protect Single Cardiac Cells Against Metabolic Stress-Induced Ca\(^{2+}\) Loading

Sofija Jovanović, DVM, Ph.D.*,† Aleksandar Jovanović, MD, Ph.D.,*† Win K. Shen, MD, FACC,* Andre Terzic, MD, Ph.D.*

Rochester, Minnesota, and Dundee, Scotland

**OBJECTIVES**

The main objective of the present study was to determine whether low physiological levels of estrogen directly protect cardiac cells against metabolic stress.

**BACKGROUND**

The beneficial effect of estrogens on the cardiovascular system has been traditionally ascribed to decrease in peripheral vascular resistance and to an antiatherogenic action. Whether physiological concentrations of 17β-estradiol (E2) are also able to protect cardiomyocytes against metabolic insult directly is unknown.

**METHODS**

Isolated ventricular cardiomyocytes were loaded with the Ca\(^{2+}\)-sensitive fluorescent dye Fluo-3 and imaged by a digital epifluorescence imaging system. In cardiac cells preincubated with hormones and/or drugs for 8 h, metabolic stress was induced by addition and removal of 2,4-dinitrophenol (DNP).

**RESULTS**

In cardiomyocytes, a 3-min-long exposure to chemical hypoxia, followed by reoxygenation, produced intracellular Ca\(^{2+}\) loading independently of gender (female: 729 ± 88 nmol/liter; male: 778 ± 97 nmol/liter). Pretreatment with E2 (10 nmol/liter) significantly reduced the magnitude of hypoxia/reoxygenation-induced Ca\(^{2+}\) loading in female (E2-treated: 298 ± 39 nmol/liter; untreated: 729 ± 88 nmol/liter), but not in male (E2-treated: 1029 ± 177 nmol/liter; untreated: 778 ± 97 nmol/liter) cardiac cells. The protective action of E2 was not mimicked by the inactive estrogen stereoisomer, 10 nmol/liter 17α estradiol (17α estradiol-treated: 886 ± 122 nmol/liter; untreated: 729 ± 88 nmol/liter), and was abolished by tamoxifen (1 μmol/liter), which acts as an antagonist of E2 on estrogen receptors (E2 plus tamoxifen-treated: 702 ± 98 nmol/liter; untreated: 729 ± 88 nmol/liter).

**CONCLUSIONS**

In a gender-dependent manner, E2 directly protects cardiac cells against hypoxia-reoxygenation injury through an estrogen receptor–mediated mechanism. Such property of E2 may contribute to cardioprotection in the female gender. (J Am Coll Cardiol 2000;36: 948–52) © 2000 by the American College of Cardiology

Gender-specific differences in the incidence of cardiovascular disease were recognized half a century ago. While the risk of heart disease in men increases constantly with age, premenopausal women have a significantly lower risk, which, however, increases rapidly after menopause to levels comparable to male counterparts (1). Estrogen substitution in postmenopausal women reduces cardiovascular mortality by 30% to 50%, suggesting that estrogens are protective (2). In fact, the antiatherogenic action of estrogens on the lipid profile and arterial wall has been well documented (3). More recently, however, experiments performed in whole heart preparations have revealed that estrogens may also decrease infarct size and protect against ischemia–reperfusion injury without changing hemodynamic parameters (4–7). These findings have implied that estrogens can directly interact with cardiomyocytes. This is supported by the finding that 17β-estradiol (E2), applied in high pharmacological concentrations, inhibits hyperkalemia-induced Ca\(^{2+}\) loading in cardiomyocytes through a “Ca\(^{2+}\) antagonistic action” (8). However, whether low physiological concentrations of E2 protect cardiomyocytes against metabolic insult is unknown.

We report that E2 protects cardiomyocytes against hypoxia-reoxygenation, and we present evidence that gender plays a crucial role in the cytoprotective action of estrogens.

**METHODS**

**Single ventricular cardiomyocytes.** Ventricular cardiomyocytes were enzymatically dissociated from pentobarbital-anesthetized, sexually mature female and male guinea pigs (8,9). Hearts were perfused (at 37°C) with medium 199, Ca\(^{2+}\)-EGTA-buffered low-Ca\(^{2+}\) medium, and low-Ca\(^{2+}\) medium containing pronase E (8 mg per 100 ml), proteinase K (1.7 mg per 100 ml), bovine albumin (0.1 g per 100 ml), and 200 μmol/liter CaCl\(_2\). Ventricles were cut into fragments, and single cells were isolated by stirring tissue in pronase E, proteinase K and collagenase (5 mg per 10 ml). The investigation conformed with the “Guide for the Care and Use of Laboratory Animals” (NIH publication 85-23, revised 1985).
**Digital epifluorescent imaging.** Rod-shaped cardiomyocytes with clear striations and smooth surface were imaged by digital epifluorescent microscopy (8,9). Cells were loaded (for 30 min) with the esterified form of the Ca$^{2+}$-sensitive fluorescent probe Fluo-3 acethoxymethyl ester (5 μmol/liter Fluo-3, dissolved in dimethyl sulfoxide plus pluronic acid; Molecular Probes) and superfused with Tyrode solution (in mmol/liter: 136.5 NaCl; 5.4 KCl; 1.8 CaCl$_2$; 0.53 MgCl$_2$; 5.5 glucose; 5.5 HEPES-NaOH; pH 7.4). Cardiomyocytes were imaged using a digital epifluorescent imaging system coupled to an inverted microscope (Zeiss Axiovert-135 TV) with a ×40 oil-immersion objective lens. A 100-W mercury lamp served as a source of light to excite Fluo-3 at 488 nm. An excitation dichroic mirror with a cutoff of 510 nm, and a long pass emission filter with a cutoff of 520 nm, were used to detect Fluo-3 fluorescence using an intensified charge coupled device camera. Fluorescence was digitized using an imaging software (Attoflor RatioVision, Atto Instruments). An estimate of cytosolic Ca$^{2+}$ concentration was calculated according to the equation: \([\text{Ca}^{2+}]_c = K_d(F - F_{\text{min}}/F_{\text{max}} - F)\), where \(F_{\text{min}}\) and \(F_{\text{max}}\) are minimal and maximal fluorescence intensity, \(K_d\) dissociation constant of the Fluo-3-Ca$^{2+}$ complex (422 nmol/liter) and \(F\) intensity of fluorescence. To obtain \(F_{\text{min}}\) and \(F_{\text{max}}\) values, cells were exposed to 100 μmol/liter ionomycin either in the absence of Ca$^{2+}$ (extracellular Ca$^{2+}$ was removed and 3 mmol/liter EGTA added to the extracellular solution) or in the presence of saturating concentrations of Ca$^{2+}$ (10 mmol/liter CaCl$_2$), respectively (8,9).

**Chemical hypoxia-reoxygenation injury.** Cardiomyocytes, superfused with Tyrode solution, were exposed to 2 mmol/liter 2,4-dinitrophenol (DNP), a metabolic poison that inhibits mitochondrial oxidative phosphorylation. Following 3-min treatment, DNP was removed (~10 s was required for removal of DNP), and cells reexposed to Tyrode solution (9–11). We have previously established that this protocol induces Ca$^{2+}$ overload in cardiomyocytes followed by an irreversible cellular hypercontracture and cell death, with the intracellular concentration of Ca$^{2+}$ reflecting accurately the condition of a cardiac cell (9). Such chemical hypoxia-reoxygenation protocol was applied to cells previously incubated for 8 h in the absence (control) or presence of E2 with or without the antiestrogen tamoxifen, or in the presence of 17α-estradiol, an inactive E2 stereoisomer. Estrogens and tamoxifen were dissolved in alcohol, and Fluo-3-AM was dissolved in dimethyl sulfoxide plus pluronic acid. The final concentration of solvents was kept to less than 0.1%. At this concentration, solvents did not affect intracellular Ca$^{2+}$ levels (8).

**Statistical analysis.** Data are presented as mean ± SEM, with n representing number of imaged fields. Mean values were compared by the two-way repeated measures analysis of variance (ANOVA) using SigmaStat program (Jandel Scientific). A p value <0.05 was considered statistically significant.

**RESULTS**

**Hypoxia-reoxygenation induces Ca$^{2+}$ loading in both female and male cardiomyocytes.** Resting cardiomyocytes from both male and female hearts had a low cytosolic Ca$^{2+}$ concentration (98 ± 11 nmol/liter; n = 13–14; Fig. 1). A 3-min-long exposure to chemical hypoxia induced by the mitochondrial uncoupler DNP, followed by reoxygenation evoked by rapid removal of DNP, produced significant Ca$^{2+}$ loading independently of gender (female: 729 ± 88 nmol/liter, n = 14; male: 778 ± 97 nmol/liter, n = 13, Fig. 1). The gender difference in the intracellular Ca$^{2+}$ concentration was not statistically significant (p = 0.110). However, the difference in the Ca$^{2+}$ concentration under control conditions and following hypoxia-reoxygenation was statistically significant (p < 0.001). No statistically significant interaction existed between gender and cellular response to the metabolic insult (p = 0.846).

![Figure 1](image-url)  
*Figure 1.* Hypoxia-reoxygenation induces Ca$^{2+}$ loading in both female and male cardiomyocytes. Time-course (left and central panels) of fluorescence in Fluo-3 loaded female and male cardiomyocytes exposed to hypoxia-reoxygenation, with average concentrations of intracellular Ca$^{2+}$ prior and following hypoxia-reoxygenation (right panel). Bars represent mean ± SEM (n = 13–14). Hypoxia-reoxygenation was induced by application (3 min at 37°C), and removal of 2 mmol/liter DNP.
The 17β-estradiol (E2) prevents hypoxia-reoxygenation–induced Ca²⁺ loading in female, but not male, cardiomyocytes. In female cardiac cells, pretreatment with 10 nmol/liter E2 significantly reduced the magnitude of hypoxia-reoxygenation–induced Ca²⁺ loading (untreated: 729 ± 68 nmol/liter, n = 14; E2-treated: 298 ± 39 nmol/liter, n = 7; Figs. 1 and 2), without affecting the basal levels of Ca²⁺ (see Figs. 1 and 2). The difference between E2-treated and untreated cells was statistically significant (p = 0.001). The interaction between metabolic status of the cell and treatment was also statistically significant (p = 0.001). The gender-dependent difference in response to the treatment with E2 was statistically significant (p = 0.001).

Tamoxifen blocks the protective action of E2. The protective effect of E2 (10 nmol/liter) was abolished by tamoxifen (1 μmol/liter), a partial agonist of estrogen receptors. Following hypoxia-reoxygenation, in the presence of both E2 (10 nmol/liter) and tamoxifen (1 μmol/liter), cytosolic Ca²⁺ levels increased to 702 ± 98 nmol/liter in female cardiomyocytes (n = 5; E2-treated: 298 ± 39 nmol/liter, n = 7, Fig. 3). The difference between E2-treated and E2 + tamoxifen–treated cells was statistically significant (p = 0.001).

Figure 2. The 17β-estradiol (E2) prevents decreases hypoxia-reoxygenation–induced Ca²⁺ loading in female, but not male, cardiomyocytes. Epifluorescent digital images of Fluo-3AM loaded, and 10 nmol/liter pretreated cardiac cells from female (A) and male (B) hearts prior to and following hypoxia-reoxygenation (H/R). Horizontal bar corresponds to 30 μm. Time-course of averaged Fluo-3 fluorescence in cells presented in A (A1) and B (B1). AU: arbitrary units. (C) Average concentration of intracellular Ca²⁺ at rest, and following hypoxia-reoxygenation. Bars represent mean ± SEM (n = 6–7).

Figure 3. Tamoxifen blocks the protective action of E2. Epifluorescent digital images of Fluo-3AM loaded cardiac cells from female hearts, pretreated with 10 nmol/liter E2 plus 1 μmol/liter tamoxifen, prior to and following hypoxia-reoxygenation (H/R). Horizontal bar corresponds to 30 μm. (A1) Time-course of averaged Fluo-3 fluorescence in cell presented in A. AU = arbitrary units. (B) Average concentration of intracellular Ca²⁺ at rest and following hypoxia-reoxygenation. Bars represent mean ± SEM (n = 5).
Vulnerable to such an insult. Reoxygenation confirms that cardiomyocytes are highly vulnerable to metabolic insult leading to cell injury (9–11). In cardiomyocytes, used here, basal levels of cytosolic Ca\(^{2+}\) were low and similar to those previously reported for healthy cells (8,9). The significant increase in intracellular Ca\(^{2+}\) following hypoxia-reoxygenation confirms that cardiomyocytes are highly vulnerable to such an insult.

Female gender as a protector against ischemic heart disease has been associated with high levels of circulating estrogens (2,3). The beneficial effect of estrogens has been traditionally ascribed to decrease in peripheral vascular resistance and to an antiatherogenic action (3,13), although more recently additional effects on the myocardium have also been proposed (4–7). In the present study, exposure of cardiomyocytes to nanomolar levels of E2 protected female, but not male, cardiomyocytes against hypoxia-reoxygenation, providing evidence at the cellular level for gender-dependent cardioprotective properties of estrogens.

Our findings that exposure of cardiomyocytes to 17α-estradiol, an inactive E2 stereoisomer, or to E2 in the presence of the antiestrogen tamoxifen, did not protect against hypoxia-reoxygenation further suggests that activation of estrogen receptors is necessary for the prevention of Ca\(^{2+}\) overload. In this regard, the present findings strongly support previous studies, done at the whole heart level, that have indicated a protective action of estrogens on the myocardium itself (4–7). Moreover, the present study also supports the notion that low blood levels of estrogens may have direct cardioprotective properties in females (6). Using single cardiomyocytes, a pure myocardial preparation free of neuronal and vascular elements, we provide first direct evidence that physiological levels of E2 indeed confer resistance toward hypoxia-reoxygenation injury in a gender-dependent manner.

In recent years, it has been reported that estrogen supplementation may be beneficial in males under some conditions of metabolic stress (14). Conversely, disruptive mutation of the estrogen receptor gene in man has been associated with premature coronary artery disease (15). The present study indicates that gender difference in myocardial response to metabolic insult is not solely the consequence of different circulating E2 levels, but rather to a more profound difference in the interaction between cardiomyocytes and E2. In principle, estrogen receptors have been found in cardiac cells in both males and females (16). However, it should be mentioned that estrogen receptors are apparently more functional and present at higher density in female than in male cardiomyocytes, which may explain the gender-dependence of E2-mediated cytoprotection observed in the present study (17,18). In some studies, it has been demonstrated that estrogens may be protective in a gender-independent manner (4,5). Such findings have been obtained by achieving higher blood levels of E2, which further supports our conclusion that gender-dependent difference in the response to low E2 concentration may be due to differences in the density and function of estrogen receptors. Taken together, it is apparent that activation of estrogen receptors in a cardiac cell may protect this cell type against metabolic stress, thus effectively preventing deleterious Ca\(^{2+}\) overload under hypoxia-reoxygenation injury.

Both the role and function of estrogen receptors in the heart are still largely unknown. It is possible that E2 may regulate the expression of genes that encode proteins implicated in endogenous cardioprotection (19–21). In agreement with such a possibility are recent findings that expres-
sion of the cardiac calcium channel, an ion conductance central in cardiac excitation-contraction coupling, is regulated by activation of estrogen receptors (16). Moreover, it has been recently demonstrated that estrogens up-regulate the expression of protein kinase C, a signaling cascade protein known to protect cardiomyocytes against Ca2+ overload and associated cellular injury (22,23). Therefore, it is possible that E2-induced expression of genes encoding cytoprotective proteins protects cardiac cells against metabolic stress.

Conclusion and significance. This study demonstrates, for the first time, that E2 directly protects, in a gender-dependent manner, cardiac cells against hypoxia-reoxygenation injury through an estrogen receptor-mediated mechanism. These findings may provide further support for the therapeutic use of estrogens and contribute to an understanding of the resistant phenotype associated with female gender.

Study limitations. To determine the direct effect of E2 on intracellular Ca2+ concentration during hypoxia-reoxygenation, it was necessary to image isolated cardiomyocytes. Such approach provided a direct visualization of the protective effect of E2 at the single-cell level. However, it should be considered that, in intact myocardium, additional cardiac, as well as extracardiac, mechanisms could further modulate the action of E2.

Reprint requests and correspondence: Dr. Aleksandar Jovanović, Tayside Institute of Child Health, Ninewells Hospital and Medical School, University of Dundee, Dundee, DD1 9SY, Scotland, United Kingdom.

REFERENCES