Cardioprotection by chronic estrogen or superoxide dismutase mimetic treatment in the aged female rat

Yi Xu,1 Stephen J. Armstrong,1 Ivan A. Arenas,1 Daniel J. Pehowich,2 and Sandra T. Davidge1

1Departments of Obstetrics/Gynecology and Physiology and 2Department of Dentistry, Perinatal Research Centre, University of Alberta, Edmonton, Alberta, Canada T6G 2S2

Submitted 15 January 2004; accepted in final form 23 February 2004

Xu, Yi, Stephen J. Armstrong, Ivan A. Arenas, Daniel J. Pehowich, and Sandra T. Davidge. Cardioprotection by chronic estrogen or superoxide dismutase mimetic treatment in the aged female rat. Am J Physiol Heart Circ Physiol 287: H165–H171, 2004. — Aging and estrogen deficiency increase the risk for developing cardiovascular disease (CVD). Oxidative stress has also been implicated in the pathophysiology of CVD and in ischemia-reperfusion (I/R) injury. We tested the hypothesis that chronic in vivo estrogen treatment or superoxide inhibition with the SOD mimetic EUK-8 improves cardiac functional recovery after I/R in the aged female rat. Sprague-Dawley rats (12–14 mo) were used as follows: intact (n = 6), ovariectomized + placebo (OVX, n = 6), OVX + EUK-8 (EUK-8, 3 mg/kg, n = 6), and OVX + estrogen (1.5 mg/pellet, 60 days release, n = 6). Perfused isolated hearts were subjected to global ischemia (25 min) followed by reperfusion (40 min). Functional recovery after I/R and myocardial protein expression of NADPH oxidase (p22, p67, and gp91phox), inducible nitric oxide synthase (NOS), endothelial NOS, and SOD1, as well as nitrotyrosine levels (as a marker for peroxynitrite), were assessed. Compared with OVX, EUK-8 and estrogen markedly improved functional recovery after I/R, which was associated with a decrease in NADPH oxidase expression and nitrotyrosine staining. However, estrogen increased inducible NOS expression, whereas EUK-8 had little effect. There were no significant changes in endothelial NOS and SOD1 expression among the groups. These results indicate that EUK-8 and estrogen improved cardiac recovery after I/R. Given the controversy surrounding hormone replacement therapy, EUK-8 may be an alternative to estrogen in protecting those at risk for myocardial ischemia in the aging population.

EXPERIMENTAL and clinical evidence has shown that aging and estrogen deficiency increase the risk for developing cardiovascular disease. Coronary heart disease is the leading cause of mortality and a major source of disability for adult women in most industrialized nations, with the risk being higher when women approach menopause (25, 34). Aging and menopause are associated with oxidative stress, which has been implicated in the pathophysiology of cardiovascular disease as well as in the cardiac injury after episodes of ischemia-reperfusion (I/R).

Estrogen has been shown to affect responses to I/R in the heart (5, 40) as well as other tissues (24, 31). Infusion of 17β-estradiol into the coronary artery before ischemia and during reperfusion decreased infarct size and reperfusion-induced ventricular arrhythmias in male canine hearts (23). Moreover, in young female ovariectomized rats subjected to I/R, estrogen replacement has been associated with improved functional recovery (5, 40). Furthermore, postmenopausal women taking hormone replacement therapy have lower mortality due to postmyocardial infarction than women without estrogen replacement (28). However, other studies have shown little or no beneficial effect (29); indeed, recent clinical trials have produced conflicting results concerning the long-term cardioprotective effects of estrogen (20, 21).

Despite all these observations indicating that estrogen can affect the cardiac response to I/R, the mechanisms involved are not completely understood. Furthermore, most of these studies have been performed in young animals. Aging has been shown to cause alterations in the endogenous mechanisms of cardioprotection, with a reduced tolerance to myocardial ischemia (16). Therefore, it is important to understand whether these observations apply to the aged female heart and whether estrogen is beneficial in the aging state.

Some observations suggest that estrogen effects on nitric oxide (NO) production could play an important role in modulating cardiac responses to I/R (23). NO is an important cardioprotective molecule, and it is essential for normal heart function. However, NO is detrimental if it combines with superoxide anion (O2−) to form peroxynitrite (ONOΟ−), which is one manifestation of oxidative stress. Moreover, ONOO− rapidly decomposes to highly reactive oxidant species, leading to tissue injury. Increased formation of ONOO− and O2− is cytotoxic to the myocardium and contributes to I/R injury in isolated rat hearts (39) and humans (11). Many of these studies showed a correlation between endogenous ONOO− formation and deterioration of cardiac function due to poor ventricular recovery, increased infarct size, and metabolic alterations (7, 39). Therefore, a critical balance among cellular concentrations of NO, O2−, and superoxide dismutase (SOD), the enzyme that scavenges O2−, determines the formation of ONOO−.

SOD/catalase mimetics have been developed to take advantage of these enzymes’ abilities to dismutate O2− to H2O2 and, subsequently, form H2O and O2. EUK-8 belongs to the salen-manganese group of these drugs and has been shown to be beneficial in reducing oxidative stress in astrocytes (27) as well as after I/R injury in the brain (4). In agreement with this oxidative stress hypothesis in I/R injury, acute in vitro treatment with this SOD mimetic during reperfusion improved cardiac functional recovery in young male rats (26). However, whether chronic in vivo treatment with EUK-8 improves cardiac function or protects against I/R insult in aging animals is not known. This is important, because the aging process in the...
female rat (relevant to the postmenopausal woman) could contribute to enhanced oxidative stress and ONOO− production in the heart. The overall hypothesis of this study was that estrogen replacement or treatment with an SOD mimetic in the aging female rat improves cardiac recovery after an I/R insult, in part, through reduction in the formation of the reactive oxygen species ONOO−.

**MATERIALS AND METHODS**

**Animal model.** Female Sprague-Dawley rats were obtained from Charles River and aged (12–14 mo) at the University of Alberta. Ovariectomies were performed 1 mo before experimentation (11–13 mo) to control for variable estrogen levels, which occur as rats approach reproductive senescence. Because any endogenous estrogen could reduce cardiac dysfunction after I/R, this presented a confounding variable, which was controlled for by ovariectomy. At the time of ovariectomy, rats received a 17β-estradiol pellet (E2, 1.5 mg/pellet, 60-day release; Innovative Research of America; n = 6) or a placebo pellet (Innovative Research of America; n = 6) subcutaneously or were injected with an SOD mimetic (EUK-8, 3 mg·kg−1·day−1·ip, n = 6; Calbiochem, La Jolla, CA). The estrogen dose took into account the larger size of the aged rats and was calculated on the basis of our previous studies (3, 9). When we adjusted for weight and the larger size of the aged rats and was calculated on the basis of our previous studies (3, 9). When we adjusted for weight and the larger size of the aged rats and was calculated on the basis of our previous studies (3, 9).

**Heart perfusion.** Hearts were excised, placed in ice-cold buffer, and perfused via the Langendorff technique with Krebs-Henseleit solution prebound to 3% BSA (fraction V), 0.5 mmol/l lactate, and 100 mU/l insulin. After treatments, there was no significant difference in body weight among all groups (Table 1). Uterine weights and the uterine weight-to-body weight ratio (a biological marker of estrogen status) were enhanced in the E2 treatment group compared with all other groups (Table 1). Left ventricular (LV) weight and LV weight-to-body weight ratio were higher in the ovariectomized group than in intact animals, suggesting LV hypertrophy in these animals (Table 1). However, E2 and EUK-8 treatment of ovariectomized animals reduced LV weight and LV weight-to-body weight ratio compared with the ovariectomized group treated with a placebo (Table 1). Right ventricular weight and right ventricular weight-to-body weight ratio were not different among the groups (Table 1).

**RESULTS**

**Effect of EUK-8 and estrogen on body, uterine, left ventricular, and right ventricular weight.** After treatments, there was no significant difference in body weight among all groups (Table 1). Uterine weights and the uterine weight-to-body weight ratio (a biological marker of estrogen status) were enhanced in the E2 treatment group compared with all other groups (Table 1). Left ventricular (LV) weight and LV weight-to-body weight ratio were higher in the ovariectomized group than in intact animals, suggesting LV hypertrophy in these animals (Table 1). However, E2 and EUK-8 treatment of ovariectomized animals reduced LV weight and LV weight-to-body weight ratio compared with the ovariectomized group treated with a placebo (Table 1). Right ventricular weight and right ventricular weight-to-body weight ratio were not different among the groups (Table 1).

**Effects of EUK-8 and estrogen on the recovery of cardiac function after I/R.** The baseline values of cardiac work (Fig. 1A) and cardiac output (Table 2) were significantly lower in the ovariectomized placebo-treated group than in the ovariectomized placebo-treated group.

---

**Table 1. Characteristics of animal model**

<table>
<thead>
<tr>
<th></th>
<th>Intact (n = 6)</th>
<th>OVX (n = 6)</th>
<th>E2 (n = 6)</th>
<th>EUK (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>525.2±1.3*</td>
<td>470.7±15.4*</td>
<td>420.1±26.5*</td>
<td>452.3±22.7*</td>
</tr>
<tr>
<td>Uterine wt, g</td>
<td>0.69±0.06*</td>
<td>0.54±0.04*</td>
<td>1.15±0.14†</td>
<td>0.45±0.07*</td>
</tr>
<tr>
<td>Uterine wt/body wt, mg/g</td>
<td>1.43±0.02*</td>
<td>1.16±0.01†</td>
<td>2.73±0.04†</td>
<td>1.00±0.02†</td>
</tr>
<tr>
<td>LV wt, mg</td>
<td>989±94‡</td>
<td>1088±50‡</td>
<td>848±43‡</td>
<td>821±31‡</td>
</tr>
<tr>
<td>RV wt, mg</td>
<td>298±23*</td>
<td>248±16*</td>
<td>236±13*</td>
<td>256±23*</td>
</tr>
<tr>
<td>LV wt/body wt, mg/g</td>
<td>2.26±0.07†</td>
<td>2.49±0.01†</td>
<td>2.03±0.07†</td>
<td>2.02±0.09†</td>
</tr>
<tr>
<td>RV wt/body wt, mg/g</td>
<td>0.57±0.04*</td>
<td>0.50±0.03*</td>
<td>0.62±0.04†</td>
<td>0.56±0.03*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of rats; LV, left ventricle; RV, right ventricle; OVX, ovariectomized rats that received a placebo pellet for 1 mo; E2, ovariectomized rats that received an estrogen pellet (1.5 mg/pellet, 60-day release) for 1 mo, EUK: ovariectomized rats that received the SOD mimetic EUK-8 (3 mg·kg−1·day−1) for 1 mo. Different symbols (*, †) indicate values that are significantly different (P < 0.05); each group is compared with all other groups by one-way ANOVA.

**Experimental protocol.** In all groups, hearts were perfused for a 50-min stabilization period, subjected to global ischemia for 25 min, and then reperfused for 40 min. A clamp precooled in liquid nitrogen was used to freeze the hearts, which were then stored at −70°C before use.

**Immunofluorescence.** After I/R, frozen heart biopsies were cut in 8-μm sections, mounted on glass slides at −30°C, and stored at −80°C. Sections were immunostained using polyclonal nitrotyrosine (a marker for ONOO−) antibodies (1:100; Upstate Biotechnology). Primary antibody incubation occurred at 4°C overnight. Incubation with the secondary antibody took place for 30 min in the dark. Slides were sealed with coverslips, and the Vectashield H-1200 mounting kit (Vector Laboratories) was used for immunofluorescence. SigmaScan was used to analyze fluorescence intensity data.

**Western blot analysis.** Immunoblottings were performed as described previously (36). Primary rabbit polyclonal antibodies (1:1,000) were used for endothelial NOS (eNOS) and inducible NOS (iNOS). Goat polyclonal antibodies (1:100) were used for the NAD(P)H oxidase subunits and the cytosolic Cu/Zn SOD, SOD1. Antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Imaging was conducted using a Fluor-S Multilaminar (Bio-Rad).

**Data analysis.** Values are means ± SE and expressed as a percentage of the control. One-way ANOVA with Tukey’s test was used to determine statistical differences among groups. P < 0.05 was considered significant.
expression of gp91phox and p67phox was significantly reduced compared with the ovariectomized group (Fig. 2, A and B). There was no significant change in the expression of p22phox and SOD1 in any group (Fig. 2, C and D).

Effects on iNOS and eNOS expression and nitrotyrosine levels. LV expression of iNOS protein was enhanced only in the E2-treated group (Fig. 3A). eNOS levels were unchanged in all the groups (Fig. 3B). LVs from the intact and ovariectomized groups exhibited positive staining for nitrotyrosine (Fig. 4, A and B). E2 and EUK-8 treatment reduced the degree of immunostaining for nitrotyrosine in the reperfused LV (Fig. 4, C and D).

**DISCUSSION**

The potential for a SOD mimetic to be a cardioprotective agent in the aged female rat is well supported by our data. Oxidative stress has been implicated in the reperfusion injury after myocardial infarction, and aging, as well as estrogen deficiency, exacerbates this process. Chronic EUK-8 treatment in aged, ovariectomized rats was found to restore postischemic function to 70% of the preischemic baseline compared with only 22% in the placebo-treated ovariectomized animals. As well, EUK-8 was shown to markedly reduce nitrotyrosine levels (marker for ONOO−) and decrease the expression of gp91phox and p67phox (NADPH oxidase subunits) compared with ovariectomized controls, indicative of a decrease in oxidative stress. Hence, EUK-8 may be a feasible cardioprotective alternative to estrogen in aging postmenopausal women.

Although the role of 17β-estradiol as cardioprotective has been established in various isolated heart models (23, 40), recent controversy in the field of estrogen replacement has cast doubt on whether the hormone is solely beneficial. The Heart and Estrogen/Progestin Replacement Study revealed no benefit of hormone replacement therapy in women who had a previous cardiovascular event (13). Moreover, the

**Table 2. Hemodynamic parameters during baseline and reperfusion periods of ischemia-reperfusion protocol**

<table>
<thead>
<tr>
<th></th>
<th>Intact (n = 6)</th>
<th>OVX (n = 6)</th>
<th>E2 (n = 6)</th>
<th>EUK (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, beats/min</td>
<td>216±9*</td>
<td>204±6*</td>
<td>224±11*</td>
<td>227±13*</td>
</tr>
<tr>
<td>Reperfusion</td>
<td>192±9*</td>
<td>191±9*</td>
<td>211±12*</td>
<td>203±10*</td>
</tr>
<tr>
<td>PSP, mmHg</td>
<td>118±9*</td>
<td>113±4*</td>
<td>120±3*</td>
<td>118±10*</td>
</tr>
<tr>
<td>Reperfusion</td>
<td>76±4*‡</td>
<td>53±8‡</td>
<td>95±2‡</td>
<td>104±5‡</td>
</tr>
<tr>
<td>DP, mmHg</td>
<td>28±5*</td>
<td>24±2*</td>
<td>23±3*</td>
<td>24±3*</td>
</tr>
<tr>
<td>Reperfusion</td>
<td>28±3*</td>
<td>26±1*</td>
<td>25±3*</td>
<td>27±2*</td>
</tr>
<tr>
<td>CO, ml·min⁻¹·g⁻¹</td>
<td>45±2*‡</td>
<td>37±1*</td>
<td>47±3‡</td>
<td>48±2‡</td>
</tr>
<tr>
<td>Reperfusion</td>
<td>26±3*†</td>
<td>16±4*‡</td>
<td>33±4‡</td>
<td>36±2‡</td>
</tr>
<tr>
<td>CF, ml·min⁻¹·g⁻¹</td>
<td>24±3*</td>
<td>20±2*</td>
<td>24±2*</td>
<td>23±2*</td>
</tr>
<tr>
<td>Reperfusion</td>
<td>13±3*</td>
<td>11±2*</td>
<td>23±2†</td>
<td>22±2†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of rats. HR, heart rate; PSP, peak systolic pressure; DP, developed pressure; CO, cardiac output; CF, coronary flow; baseline, after 50 min of aerobic perfusion; reperfusion, after 40 min of recovery following ischemia. See Table 1 for a description of treatment groups. Different symbols (*, †, ‡, and §) indicate values that are significantly different from all other groups (P < 0.05).
Womens Health Initiative reported a small increase in the number of myocardial infarctions in women taking hormone replacement therapy (15). In this study, we found that 17β-estradiol restored cardiac function to 55% of the aerobic baseline after I/R. Similar to the effects of EUK-8, estrogen treatment reduced nitrotyrosine intensity as well as gp91phox and p67phox protein levels. However, estrogen replacement was also found to enhance iNOS levels in the aged hearts, which was not observed with EUK-8 treatment.

In the intact and ovariectomized placebo groups, cardiac work was significantly depressed after I/R, with functional recovery of only 41% and 22% of the baseline value, respectively. Preischemic baseline function was reduced in the ovariectomized group; this may have been due to hypertrophy and remodeling of the LV (Table 1). We previously showed that ovariectomy enhances LV hypertrophy in the aged female rat (36). In this study, reduced baseline function may be predictive of postischemic recovery; however, the other groups had similar baseline function but differed in their recovery. Intact aged animals exhibited slightly reduced detrimental effects of aging as evidenced by an enhanced postischemic recovery compared with the ovariectomized group. This further supports a beneficial role for endogenously produced ovarian steroids, even in animals that are approaching reproductive senescence. For the most part, however, the aged, intact animals mirrored the ovariectomized animals by exhibiting reduced cardiac function after I/R, likely because of reduced ovarian function in the aged animals.

Ovariectomized rats presented slightly enlarged hypertrophic hearts compared with E2- and EUK-8-treated animals, which had significantly lower heart weights and heart weight-to-body weight ratios (Table 1). These observations agree with our previous study in which estrogen reduced LV hypertrophy of aged female rat hearts (36). Similarly, others found that manganese(III)tetrakis(1-methyl-4-pyridinyl)porphyrin pentachloride (another SOD mimetic) or EUK-8 given to norepinephrine-stimulated cardiomyocytes reduces cellular hypertrophy (1), suggesting that oxidative stress could be involved in the pathogenesis of LV hypertrophy associated with aging and estrogen deficiency.

Although estrogen and SOD have been shown to protect hearts from I/R injury in young animals (17, 22, 23), less is known about their effects on the aging heart. To our knowledge, the present study is the first to use a SOD mimic (EUK-8) chronically in an aging animal model. A previous study used EUK-8 acutely in an isolated iron-overloaded working heart model (26). EUK-8 given in the perfusion medium (50 μM) was able to maintain systolic and diastolic pressure, as well as LV developed pressure, after 15 min of ischemia and 15 min of reperfusion (26). Moreover, EUK-8 significantly reduced damage to the mitochondria and sarcomeres of the hearts (26). Similarly, it has been shown that EUK-8 can reduce the incidence of repfusional arrhythmias in isolated rat hearts subjected to 10 min of regional ischemia (32). Overall, these observations indicate a beneficial cardiac effect of EUK-8 in I/R. In addition, the antioxidant properties of EUK-8...
and estrogen may also contribute to their protective effects against endothelial dysfunction associated with I/R. Indeed, 17β-estradiol has been shown to enhance the expression of antioxidant enzymes in vascular smooth muscle cells (30) and reduce expression of prooxidant enzymes in endothelial cells (10). This is in agreement with our LV protein data showing reduced NADPH oxidase subunit expression with 17β-estradiol treatment. To relate this to vascular function, our data show that EUK-8 and estrogen treatment improved coronary artery flow that was otherwise reduced after I/R.

At the cellular level, EUK-8 is known to dismutate O₂⁻ to H₂O₂ and then further catalyze its breakdown to H₂O and O₂ (27). Moreover, it is capable of nitrosative species breakdown such as reducing ONOO⁻ to NO₂⁻ or oxidizing NO to NO₃⁻ (27). We speculate that in our aging model the presence of ONOO⁻ is enhanced as a result of elevated levels of oxidative stress. ONOO⁻ has been shown to contribute to I/R injury in the heart (14, 33). Given exogenously, or produced endogenously, it appears to be detrimental to postschismic recovery. As such, one of the actions of EUK-8 could be not only to reduce the enhanced superoxide levels present during reperfusion, but also to eliminate or transform ONOO⁻. Indeed, we observed a marked reduction in nitrotyrosine levels in the EUK-8-treated hearts compared with the ovariectomized controls. Although the chemical formation of ONOO⁻ is more than twice as fast as the dismutation reaction between SOD and O₂⁻, EUK-8’s ability to break down both reactive oxygen species helps explain the observed reduction in nitrotyrosine formation. Furthermore, EUK-8’s actions extended to the alteration of protein levels of the NADPH oxidase subunits p67phox and gp91phox. Hence, the drug effect appears to be twofold: to directly catalyze the breakdown of reactive oxygen species and, whether indirectly or directly, to reduce protein expression of the NADPH oxidase enzyme. In this regard, it has been shown that, in the kidney, EUK-8 may affect NADPH oxidase activity through modulation of transforming growth factor expression (35). EUK-8 did not, however, alter eNOS, iNOS, or Cu/Zn SOD expression compared with controls. By contrast, the E₂-treated group showed enhanced iNOS expression. This was contrary to our previous observations in young female rats that underwent low-flow ischemia for 60 min and 30 min of reperfusion (9). Interestingly, in that study, it was demonstrated that 17β-estradiol increases calcium-independent NOS activity, but not expression, in young rats under these conditions (9). iNOS may contribute to late preconditioning effects in the ischemic heart, implicating its upregulation as a protective mechanism (6). However, iNOS has also been shown to be detrimental in the ischemic heart (19). Yet the
upregulation of iNOS in the E2-treated group in this study was associated with an improved recovery after I/R. Indeed, recent work revealed that gene transfer of iNOS using intramyocardial injection of an adenovirus reduced infarct size in the mouse heart (18). Moreover, in a canine study of myocardial I/R injury, estrogen was found to increase NO production and limit infarct size (23). Hence, iNOS could play a predominant role in the protection from I/R injury mediated by estrogen. However, studies of younger animals do not account for the ONOO− formation that is seen in models of aging (8). Similar to EUK-8, estrogen treatment significantly reduced cardiac nitrotyrosine levels and also decreased p67phox and gp91phox expression. As well, estrogen itself is known to have antioxidant properties, which could play a role in reducing superoxide and/or ONOO− levels (12). Taken together, EUK-8 and estrogen appear to share some common actions, although EUK-8 had no effect on iNOS expression, probably because it is more target specific.

In summary, our data indicate that the SOD mimetic and 17β-estradiol are capable of improving posts ischemic cardiac function in the aged female rat heart. This improvement was associated with a decrease in the expression of the NADPH oxidase subunits p67phox and gp91phox and a reduction in the amount of nitrotyrosine (a marker for ONOO−). Given the recent controversy in the hormone replacement therapy field, EUK-8 may be an alternative to estrogen to protect those at risk for myocardial ischemia in the aging population.

ACKNOWLEDGMENTS

We thank Jennifer Ngo for assistance with immunofluorescence.

GRANTS

This work was supported by a grant from the Canadian Institute of Health Research. S. T. Davidse is the Canadian Chair in Women’s Cardiovascular Health and a Senior Scholar of the Alberta Heritage Foundation for Medical Research.

REFERENCES


