Estradiol abolishes reduction in cell death by the opioid agonist Met^5^-enkephalin after oxygen glucose deprivation in isolated cardiomyocytes from both sexes

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Merkel MJ, Liu L, Cao Z, Packwood W, Hurn PD, Van Winkle DM. Estradiol abolishes reduction in cell death after oxygen-glucose deprivation (OGD) by opioid agonist ME in a sex-independent manner. Am J Physiol Heart Circ Physiol 295: H409–H415, 2008. First published May 23, 2008; doi:10.1152/ajpheart.01018.2007.—There is evidence for differences in the response to treatment of cardiovascular disease in men and women. In addition, there are conflicting results regarding the effectiveness of pharmacologically induced protection or ischemic preconditioning in females. We investigated whether the ability of Met^5^-enkephalin (ME) to reduce cell death after oxygen-glucose deprivation (OGD) is influenced by the presence of 17β-estradiol (E2) in a nitric oxide (NO)- and estrogen receptor-dependent manner. On postnatal day 7, murine cardiomyocytes from wild-type or in inducible NO synthase (iNOS) knockout mice were separated by sex, isolated by collagenase digestion, cultured for 24 h, and subjected to 90 min OGD and 180 min reoxygenation at 37°C (n = 4 to 5 replicates). Cell cultures were incubated in E2 for 15 min or 24 h before OGD. ME was used to increase cell survival. Cell death was assessed by propidium iodide. More than 300 cells were examined for each treatment. Data are presented as means ± SE. As a result, in both sexes, ME-induced cell survival was lost in the presence of E2, and the ability of ME to improve cell survival was restored after treatment with the estrogen receptor antagonist ICI-182780. Furthermore, iNOS was necessary for ME to increase cell survival following OGD in vitro. We conclude that ME-induced reduction in cell death is abolished by E2 in a sex-independent manner via activation of estrogen receptors, and this interaction is dependent on iNOS.

viability; sex hormone; hypoxia

There is increasing evidence that men and women respond differently to treatment for cardiovascular disease. According to recent studies, being female is an independent risk factor for adverse outcomes following conventional coronary artery bypass grafting, and women reportedly suffer a higher mortality rate following myocardial infarction (1, 32, 40). At the same time, premenopausal women experience a genuine protection against ischemia-reperfusion (I/R) injury, which is in part due to the female sex steroid estrogen. Experimental findings demonstrate that estrogen induces protection against I/R injury in vivo and reduces cell death in vitro (10, 20, 22, 30, 37). Estrogen-induced protection shares common pathways of cellular protection with known pharmacological preconditioning agents, such as opioids, bradykinin, and volatile anesthetics, as well as with ischemic preconditioning (26, 37). Pretreatment with these agents or with brief episodes of repeated ischemia activates a cascade of cellular signals involving phosphatidylinositol 3-kinase pathway (PI3K/Akt), nitric oxide (NO) synthase (NOS), and the opening of ATP-sensitive K^-channels in males (7, 11, 27, 28). Our laboratory has shown that Met^5^-enkephalin (ME), a pentapeptide that activates the 6-opioid receptor, induces protection against I/R injury in males in vitro and in vivo (8, 20). In contrast, there are conflicting results regarding the ability of pharmacological or ischemic preconditioning to induce protection in females (22, 34, 35, 39, 43). This apparent discrepancy remains unresolved.

We hypothesized that the cytoprotective properties of ME are influenced by estrogen [17β-estradiol (E2)] in an estrogen receptor (ER)- and NO-dependent manner. To test this hypothesis, we simulated I/R using oxygen-glucose deprivation (OGD) in sex-specific, isolated murine cardiomyocyte cultures in the absence and presence of both ME and E2. Selective inhibitors for ER or NOS were used and cardiomyocytes from inducible NOS (iNOS) knockout mice of both sexes.

Materials and Methods

The Institutional Animal Care and Use Committee at Portland Veterans Affairs (VA) Medical Center approved this study, and all animals received treatment in compliance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Research, National Research Council; National Academy Press, 1996). Because dietary phytoestrogens have been reported to elicit protection against cerebral and myocardial ischemia (23, 44), we used cardiomyocytes from animals whose dams were fed a phytoestrogen-free diet (No. 2014, Harlan Teklad, Madison, WI). All study animals were allowed access to food and water ad libitum until induction of anesthesia. Pups used in this study were bred in the Portland VA, veterinary medical unit from C57BL/6J breeders or from homozygous iNOS^-/- knockout breeders.

Cell isolation and culture. Ventricular cardiomyocytes from neonatal mice [postnatal day (PND) 7 to 8] were prepared as recently published (6). Briefly, neonatal mice were sexed by measuring the anogenital distance; pups with an intermediate anogenital distance were excluded (16). Pups were then anesthetized with pentobarbital sodium (50 mg/kg ip), and the hearts were removed aseptically. The ventricles from three to five hearts from same-sex mice were pooled, and the cardiomyocytes were isolated and cultured. Ventricles were kept on ice in Hanks’ balanced salt solution (HBSS) without Ca^2+ and Mg^2+.
Mg\(^2+\) containing (in g/l) 0.4 KCl, 0.06 KH\(_2\)PO\(_4\), 8.0 NaCl, and 0.05 Na\(_2\)HPO\(_4\) (pH 7.4). The ventricles were then washed three times with HBSS and minced into small fragments. Cells were dissociated at 25°C for 15 min with 0.625% (wt/vol) trypsin in HBSS without Ca\(^{2+}\) and Mg\(^{2+}\) (pH 7.4). Cells released after the first digestion were discarded; cells from subsequent digestions were added to an equal volume of cold HBSS with Ca\(^{2+}\) and Mg\(^{2+}\) containing (in g/l) 0.14 CaCl\(_2\), 0.4 KCl, 0.06 KH\(_2\)PO\(_4\), 0.047 MgCl\(_2\), 0.049 MgSO\(_4\), 8.0 NaCl, 0.35 NaHCO\(_3\), 0.05 Na\(_2\)HPO\(_4\), and 1.0 D-glucose (pH 7.4) until all cardiac cells were isolated. The resulting mixture was centrifuged for 8 min at 200 g, and the cells were resuspended in FBS-medium 199 (M199) supplemented with estrogen-free 15% FBS (vol/vol, Colombo), 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 μM cytosine arabinoside (to inhibit fibroblast attachment and proliferation). To exclude nonmuscle cells, the isolated cells were first plated in tissue culture dishes at 37°C for 1 h under a water-saturated atmosphere of 5% CO\(_2\)-95% O\(_2\). Suspended cardiomyocytes were then collected and plated at a density of 1.0 × 10\(^5\) cells/cm\(^2\) and incubated under the same conditions as above for 24 h.

**Oxygen-glucose deprivation.** To simulate ischemia, glucose-free medium (MEM-HBSS) was preequilibrated in 100% N\(_2\) at 37°C for 2 h. Oxygenated medium was removed from the cardiomyocyte cultures, and the N\(_2\)-preequilibrated glucose-free medium was immediately added. Cultures were promptly placed in a custom-made Plexiglas hypoxia chamber and exposed to 100% N\(_2\) for 1.5 h at 37°C. For reoxygenation, the glucose-free medium was replaced with M199, and the cells were reoxygenated in 21% O\(_2\)-5% CO\(_2\)-74% room air for 3 h at 37°C.

**Drugs.** The agents used in this study were E\(_2\) (10 nM and 1 μM), ICI-182780 (ICI; 0.5 μM), N\(^\omega\)-nitro-l-arginine methyl ester hydrochloride (L-NAME; 100 μM), and ME (100 μM). E\(_2\), ICI, and L-NAME were all obtained from Sigma (St. Louis, MO). E\(_2\) is the principal mammalian estrogen and signals through its cognate receptor to induce gene transcription or a rapid, nontranscriptional pathway in the cardiovascular system (18). ICI is a nonselective ER antagonist (42). L-NAME, a nonselective NOS inhibitor, blocks all three NOS isoforms. ME, purchased from Peninsula Laboratory (San Carlos, CA) and American Peptide (Sunnyvale, CA), is an endogenously produced pentapeptide that is selective for cloned β-opioid receptors but appears to also activate κ-opioid receptors in vivo (7). A subset of control cells were incubated in ethanol (1:1,000), the solvent for E\(_2\).

ICI was dissolved in DMSO (1:1,000), L-NAME and ME were dissolved in distilled water, and the drugs were then aliquoted and frozen until use. Concentrations were chosen based on our previous studies, reported IC\(_{50}\) values, and literature reports (8). On the day of the experiment, stock solutions were diluted and added directly into cells.

**Experimental protocol.** Sex-specific, isolated cardiomyocytes were incubated in two different concentrations of E\(_2\) (10 nM and 1 μM) for 15 min (short term) before OGD. Cell death was assessed at 60, 120, and 180 min of reoxygenation. All other experiments used 10 nM E\(_2\) or its vehicle for either short term or long term (24 h incubation) before adding ME (100 μM), in the presence or absence of ICI and L-NAME. Sex-specific, isolated cardiomyocytes from iNOS\(^{-/-}\) mice were incubated in ME 15 min before OGD in the presence and absence of E\(_2\) (short-term and long-term incubation). Once added, the agonist and the antagonist remained in the culture medium for the duration of the experiments (See Fig. 1 for experimental time line). Each set of experiments included an oxygenated control group.

**Determination of cell viability.** Cell death was assessed with propidium iodide (5 μM) and quantified using a Zeiss Axiovert 200 fluorescent microscope and MetaMorph imaging system (Universal Scientific Software, Boulder, CO).
Imaging) as recently published (6). Serial measurements of cell viability were performed every 60 min during reoxygenation. More than 300 cells were examined in each sample.

Statistical analysis. Data analysis was performed with a personal computer-based statistical software package (Prism 4.0, GraphPad Software, San Diego, CA). The primary measured end point for all studies was cell death at 180 min of reoxygenation, defined as the uptake of propidium iodide. Cell death was corrected to dead cells in the oxygenated control for each experiment. Differences between experimental groups were plotted for both sexes. All data were analyzed using one- or two-way ANOVA as appropriate, with a Bonferroni post hoc test to compare replicate means. Statistical significance was set at \( P < 0.05 \). Results are expressed as means ± SE.

RESULTS

**E2 improves cell survival after OGD independent of sex.** Cardiomyocytes from male (C_{M}) and female (C_{F}) animals showed similar cell survival after OGD for both E_{2} concentrations tested (Fig. 2). There was no sex difference in cell death in the untreated controls. Ethanol alone (the solvent of E_{2}) had no affect on cell death following OGD (data not shown).

**E_{2} reduces ME-induced degree of cell survival after OGD.** The ability of ME to reduce cell death after OGD was significantly decreased in the presence of E_{2} (15 min and 24 h incubation) in both C_{F} and C_{M} cultures (Fig. 3). However, after 15 min E_{2} incubation, the ME-induced reduction in cell death was significantly less attenuated in C_{M} compared with C_{F}. After 24 h of E_{2} incubation, C_{F} and C_{M} showed a similar degree of loss in the ME-induced reduction of cell death.

**ME-induced reduction in cell death after OGD is restored by ER inhibition in the presence of E_{2}.** The inhibition of ERs restored the ME-induced reduction in cell death after OGD in the presence of E_{2} in C_{F} independent of the E_{2} incubation time and in C_{M} after short-term E_{2} incubation. The improved survival observed in cells treated with E_{2} alone was blocked by ER inhibition in cells from both sexes after long-term E_{2} but not after short-term E_{2} incubation (Fig. 4).

**ME-induced reduction in cell death is only partially blocked by NOS inhibition in the presence of E_{2}.** NOS inhibition after ME treatment had no affect on cell survival after short-term E_{2} incubation compared with E_{2} alone, but increased cell death was observed after long-term E_{2} incubation. This effect was sex independent. However, NOS inhibition after ME treatment in cardiomyocytes derived from animals of both sexes exposed to E_{2} showed an intermediate degree of improved cell survival after OGD compared with E_{2}-free cultures. This was similar to the effect found in E_{2}-free cultures of C_{F}, whereas NOS inhibition completely abolished the ME-induced reduction in cell death after OGD in C_{M}. This sex difference was independent of the timing of NOS inhibition (i.e., before or after ME treatment, data not shown). In the absence of ME, NOS inhibition resulted in increased cell death after short- and long-term E_{2} incubation in C_{F}, but only after long-term E_{2} incubation in C_{M} (Fig. 5).

**E_{2} effect on ME-induced reduction in cell death is iNOS dependent.** OGD-induced cell death was unchanged in ME-treated cardiomyocytes from female iNOS knockout (C_{iNOS^{-/-}}) mice independent of E_{2} incubation time. ME-treated cardiomyocytes from male iNOS^{-/-} animals (C_{M}^{iNOS^{-/-}}) showed improved cell survival after long-term, but not after short-term, E_{2} incubation. OGD-induced cell death was increased in C_{M}^{iNOS^{-/-}} compared with C_{F}^{iNOS^{-/-}} at all conditions tested, but not after 24 h E_{2} incubation (Fig. 6).

DISCUSSION

The principal finding of the study is that in isolated, sex-specific cardiomyocyte cultures, E_{2} abolished ME-induced reduction in cell death after OGD, irrespective of sex. ER inhibition restored the ME-induced increased survival after OGD in cardiomyocytes of both sexes, suggesting that the ability of E_{2} to block ME-induced reduction in cell death is ER mediated. Furthermore, the absence of iNOS significantly reduced ME- or E_{2}-induced reduction in cell death following OGD. These findings suggest that the E_{2}-ME interaction is sex independent, ER mediated, and influenced by an iNOS-dependent mechanism.
Fig. 4. ER inhibition and ME-induced reduction in cell death after OGD. ERs are inhibited with ICI (0.5 μM) followed by E2 (10 nM) and ME (100 μM) treatment and 90 min OGD. The percentage of dead cells after 180 min reoxygenation is represented and corrected to an oxygenated control for each experiment (n = 5 replicates; means ± SE). For comparison, treatment with ME in E2-free medium reduced cell death to 21 ± 2% (cardiomyocytes from males) and 21 ± 1% (cardiomyocytes from females) at 180 min of reoxygenation (see Fig. 5).

Others have reported increased tolerance against myocardial I/R injury in female versus male mice, rats, and rabbits (3, 35, 41, 43), but not in dogs (31). Exogenous estrogen confers cardioprotection against I/R in vivo in females and males of various species (4, 10, 38), but it is unclear whether the protection is dependent on ER-α or -β (5, 14) or is ER independent (10). In addition, ischemic preconditioning is protective in older female mice but not in young females, suggesting the potential for a hormonal influence (39). These previous studies suggest that the capacity to induce protection in the female heart may depend, in part, on the availability of endogenous or exogenous estradiol. More specifically, we found that estradiol limits cardiomyocyte death following OGD but produces an undesirable suppression of ME-induced cell salvage in male and female cardiomyocytes. In contrast, Shinmura et al. (34) reported that neither sex nor age impaired opioid-induced late preconditioning in an isolated rat heart model evaluating functional recovery after global ischemia. These conflicting results may be due to the different models used. The current study was performed in an isolated, sex-specific cultured cardiomyocyte model to specifically investigate the effect of E2 on ME-induced cytoprotection, without the potential confounding influences inherent in an in vivo model (e.g., other cells such as endothelium, hydraulic and mechanic stress, etc.). Although it is premature to definitively extrapolate the current observations to the in vivo situation, the data suggest that circulating estradiol may modulate the magnitude of opioid-induced protection in the in vivo female heart.

Both ER subtypes (ER-α and ER-β) belong to a family of nuclear hormone receptors that are ligand-activated transcription factors which mediate many of the effects of estrogen (24). Nuedling et al. (25) found an ER-mediated increase in iNOS (50 ± 23-fold) and endothelial NOS (eNOS) expression (16 ± 4-fold) in adult and neonatal rat cardiomyocytes after 24 h of E2 incubation (10 nM), which was associated with the translocalization of the ER into the nucleus. Other studies have reported that estradiol can rapidly activate NOS. Five minutes of exposure to E2 resulted in the upregulation of eNOS in vascular endothelial cells, and this effect was blocked by the ER antagonist ICI (21).

A possible explanation for our finding that E2 abolishes ME-induced improvement in cell survival is that NOS expression and/or activity is enhanced by both E2 and ME, resulting in toxic NO levels when E2 and ME are present simultaneously. High levels of NO have been previously reported to injure, rather than protect, myocardium during reperfusion (Ref. 33, and see Ref. 15 for review).

The selective inhibition of iNOS in male rats attenuated the protective effect of a δ-opioid receptor agonist when applied immediately before the ischemic event in vivo (29). Similarly, we found that ME does not improve cell survival after OGD in C57NOS−/−. The negative interaction between E2 and ME resulting in increased cell death after OGD was absent in cardiomyocytes from male and female iNOS−/− animals. In hormone-free conditions, the nonselective inhibition of NOS completely blocked ME-induced reduction in cell death in CM but only partially in CF. After incubation with E2, NOS inhibition in CM resulted in partially blocked ME-induced reduction in cell death following OGD, similar to that seen in CF. This suggests that an additional, NO-independent signal pathway is involved in protecting CF against OGD and that a similar pathway can be activated in CM after incubation in E2.

Our experiments suggest that the intracellular mechanisms responsible for E2 reducing ME-mediated cytoprotection might be sexual dimorphic and could represent the activation of different pathways in both sexes. Further studies are needed to determine sex differences in the signaling pathways involved. Besides an interaction in the NO signaling pathway, as investigated in the present study, there are several other signaling pathways that may serve as potential targets for E2 to negate...
ME-induced cytoprotection. For instance, both E2 and ME are known to activate the PI3K/Akt signaling pathway and ATP-sensitive K⁺ channels (8, 19, 22, 27, 30). Our laboratory recently showed that ME activates Akt isoforms in a sexual dimorphic pattern, with CF primarily activating Akt3 and CM activating Akt1/2 (6). However, it is unknown whether a competitive antagonism exists in these pathways. Alternatively, there may be a sexual dimorphism in E2 receptors, rather than in postreceptor signaling. Although we did not study the functional expression of ERs in our cardiomyocytes, it has been previously shown by Grohe et al. (17) that neonatal rat cardiomyocytes express functional ER-α and -β. However, the sexual dimorphism of the receptor content was not examined. Our observation that the ER antagonist ICI abolished E2-mediated cytoprotection after 24 h of incubation supports the existence of functional ERs in neonatal murine cardiomyocytes but similarly does not address whether there is a sex difference in relative ER-α/ER-β content.

Although we modified culture conditions to eliminate other sources of estrogen, using, for example, estrogen-free bovine serum albumin, the cultured cardiomyocytes undoubtedly were exposed to estrogen to some degree. The developing fetus is exposed to gonadal steroids. In addition, although maternal estrogens are largely bound to plasma proteins and the murine placenta does not produce gonadal steroids during the second half of pregnancy (2, 12), the murine fetal testes produces testosterone with testosterone levels surging at about murine fetal day 16 and at parturition (9, 13). Thus cardiomyocytes from PND 7 to 8 male animals will have been exposed to testosterone surges not experienced by cardiomyocytes from PND 7 to 8 female animals. Whether this affects cardiomyocyte hypoxic tolerance was not addressed in the current study and will require future examination.

Finally, because we used neonatal cardiomyocytes rather than adult cardiomyocytes, we cannot exclude the possibility that adult tissue would behave differently from what we ob-
served in the current study. However, we used PND 7 to 8 murine cardiomyocytes when myocardial hyperplasia is largely completed and when cardiomyocytes are terminally differentiated (36). In addition, eNOS and iNOS expression was similarly upregulated in neonatal (PND 1 to 2) and adult rat cardiomyocytes following 24 h of E2 incubation (25).

In conclusion, the current results demonstrate that ME-induced reduction in cell death after OGD is abolished in the presence of E2 in isolated murine cardiomyocytes. This E2–ME interaction is ER mediated and sex independent. Since E2 can block opioid-induced reduction in cell death following OGD in vitro, this may explain conflicting reports of opioid-induced protection in female animals of undefined hormonal status.

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