Medroxyprogesterone acetate prevents the cardioprotective and anti-inflammatory effects of 17\(\beta\)-estradiol in an in vivo model of myocardial ischemia and reperfusion

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Booth EA, Lucchesi BR. Medroxyprogesterone acetate prevents the cardioprotective and anti-inflammatory effects of 17\(\beta\)-estradiol in an in vivo model of myocardial ischemia and reperfusion. Am J Physiol Heart Circ Physiol 293: H1408–H1415, 2007. First published April 13, 2007; doi:10.1152/ajpheart.00993.2006.—Previous studies demonstrated the protective effects of estrogen administration in models of cardiovascular disease. However, there is a discrepancy between these data and those from the recent clinical trials with hormone replacement therapy in menopausal women. One possible explanation for the divergent results is the addition of progestin to the hormone regimen in the Women’s Health Initiative and the Heart and Estrogen/Progestin Replacement Study trials. The aim of the present study was to examine the effects of a combination of 17\(\beta\)-estradiol (E\(_2\), 20 \(\mu\)g) and medroxyprogesterone acetate (MPA, 80 \(\mu\)g) on infarct size in New Zealand White rabbits. Infarct size as a percentage of the area at risk was significantly reduced by administration of E\(_2\) 30 min before induction of myocardial ischemia compared with vehicle (19.5 ± 3.1 vs. 55.7 ± 2.6\%, \(P < 0.001\)). However, E\(_2\) + MPA failed to elicit a reduction in infarct size (52.5 ± 4.6\%), and MPA had no effect (50.8 ± 2.6\%). E\(_2\) also reduced serum levels of cardiac troponin I, immune complex deposition in myocardial tissue, activation of the complement system, and lipid peroxidation. All these effects were reversed by MPA. The results suggest that MPA antagonizes the infarct-sparing effects of E\(_2\), possibly through modulation of the immune response after ischemia and reperfusion.

medroxyprogesterone acetate; membrane attack complex; C-reactive protein; complement; lipid peroxidation

PREVIOUS PUBLICATIONS HAVE reported the protective effects of estrogens against ischemia-reperfusion injury, atherosclerosis, and arrhythmogenesis (4, 9, 37). However, the premature termination of two large clinical trials has raised serious questions regarding the cardioprotective effects of hormone replacement therapy after menopause (12, 18, 19). One possible explanation for the negative effects of estrogens in the clinical trials is the addition of progestrone to the hormone regimen. Combination therapy is an accepted treatment for hormone replacement therapy after menopause (12, 18, 19). One possible explanation for the divergent results is the addition of progestin to the hormone regimen in the Women’s Health Initiative and the Heart and Estrogen/Progestin Replacement Study trials. The aim of the present study was to examine the effects of a combination of 17\(\beta\)-estradiol (E\(_2\), 20 \(\mu\)g) and medroxyprogesterone acetate (MPA, 80 \(\mu\)g) on infarct size in New Zealand White rabbits. Infarct size as a percentage of the area at risk was significantly reduced by administration of E\(_2\) 30 min before induction of myocardial ischemia compared with vehicle (19.5 ± 3.1 vs. 55.7 ± 2.6\%, \(P < 0.001\)). However, E\(_2\) + MPA failed to elicit a reduction in infarct size (52.5 ± 4.6\%), and MPA had no effect (50.8 ± 2.6\%). E\(_2\) also reduced serum levels of cardiac troponin I, immune complex deposition in myocardial tissue, activation of the complement system, and lipid peroxidation. All these effects were reversed by MPA. The results suggest that MPA antagonizes the infarct-sparing effects of E\(_2\), possibly through modulation of the immune response after ischemia and reperfusion.

Although tissue ischemia of sufficient duration produces irreversible tissue injury and cell death, the extent of conversion from a viable to a nonviable state is increased significantly when the previously ischemic tissue is reperfused. Irreversible tissue injury associated with the restoration of blood flow is referred to as reperfusion injury. Among the multiple factors associated with ischemia-reperfusion injury are activation of the complement cascade (16), generation of reactive oxygen species (21), and emigration of neutrophils to the area at risk (AAR) followed by release of cytotoxic constituents (10). C-reactive protein (CRP), an acute-phase protein, is associated with the pathogenesis of irreversible myocardial injury (8, 42). The proposed mechanism of CRP involvement is through local activation of the complement cascade (39), and increased levels of CRP are reported to correlate with an increase in tissue injury after ischemia and reperfusion (3, 13). Pharmacological inhibition of one or more of the multiple pathways of the inflammatory response has been demonstrated to reduce the extent of irreversible tissue injury after ischemia and reperfusion (15, 36). Previous studies have shown that estrogen has the ability to reduce infarct size in the reperfused myocardium, possibly through modulation of the complement cascade, by reduction of CRP (5), or by an estrogen receptor-mediated anti-inflammatory mechanism (25, 38).

The effects of progestins and progesterone on the cardiovascular system are controversial. The addition of progestin to the hormone treatment regimen has been reported to inhibit estrogen’s protective effects in some, but not all, models of cardiovascular injury (27, 33, 41). Some of the negative effects may be due to a proinflammatory effect of progestin or the ability of progesterin to antagonize estrogen’s anti-inflammatory effects, endothelial function, and/or serum lipids (14, 40, 41). Oral estrogen administration increases plasma levels of the acute-phase CRP, whereas the progesterin MPA attenuates this effect. Because it is possible that elevated CRP is associated with plaque destabilization and rupture, it has been suggested that the proinflammatory effect of estrogen may account for the...
increased number of cardiovascular events during the first years of the Heart and Estrogen/Progestin Replacement Study trial. The increase in CRP was not observed when estrogen was administered transmurally (17), suggesting that this increase may not be an indicator of inflammation but, rather, an effect of first-pass metabolism.

We previously demonstrated that acute estrogen administration alone significantly reduced infarct size and tissue deposition of CRP and the membrane attack complex (MAC) (5). The aim of the present study was to investigate the anti-inflammatory effects of estrogen after myocardial ischemia and reperfusion and determine whether treatment with the combination of estrogen and the synthetic progestrone MPA reverses estrogen's protective effects.

MATERIALS AND METHODS

Guidelines for animal research. The procedures used in this study are in agreement with the guidelines of the University of Michigan Committee on the Use and Care of Animals. The University of Michigan Unit for Laboratory Animal Medicine provides veterinary care. The University of Michigan is accredited by the American Association for Accreditation of Laboratory Animal Care, and the animal care use program conforms to the standards in the National Institutes of Health Guide for the Care and Use of Laboratory Animals [DHHS Publication No. (NIH) 86-23]. Animal protocols were approved by the University of Michigan University Committee on Use and Care of Animals.

Surgical preparation. Male New Zealand White rabbits (2.6–3.2 kg body wt; Covance, Kalamazoo, MI) were anesthetized intramuscularly with xylazine (3.0 mg/kg) + ketamine (35 mg/kg) and then intravenously with pentobarbital sodium (15 mg/kg). After insertion of a cuffed endotracheal tube, the animals were placed on positive-pressure ventilation using room air. The left jugular vein was isolated and cannulated for drug administration. The left carotid artery was isolated and instrumented with a Millar catheter micro-tip pressure transducer (Millar Instrument, Houston, TX) positioned immediately above the aortic valve to monitor aortic blood pressure. A lead II electrocardiogram was monitored throughout the experiment. After a left thoracotomy and pericardiotomy, the left anterior descending coronary artery was identified. A 3-0 silk suture (Genzyme Biosurgery, Cambridge, MA) was passed under the artery and around a short length of polyethylene tubing. Simultaneous downward displacement of the polyethylene tubing during application of upward traction on the suture resulted in occlusion of the coronary artery and cessation of regional myocardial blood flow. Coronary artery occlusion was maintained for 30 min; then reperfusion was initiated by withdrawal of the polyethylene tubing. Regional myocardial ischemia was verified by the presence of a zone of cyanosis in the area of distribution of the occluded vessel and changes in the electrocardiogram consistent with the presence of transmural regional myocardial ischemia (ST-segment elevation).

Experimental protocol. Animals were allowed to stabilize for 15 min before beginning the protocol. Rabbits were randomized equally among four treatment groups: E2 (20 μg/h), E2 + MPA (20 and 80 μg/h, respectively), MPA (80 μg/h), and vehicle (1 ml of 20% DMSO-80% polyethylene glycol). Thirty minutes after drug/vehicle administration, the left anterior descending coronary artery was subjected to 30 min of occlusion followed by 4 h of reperfusion. The hearts from eight animals in each group were stained for determination of infarct size. Tissue from the remaining five hearts in each group was used for Western blot analysis and lipid peroxidation assays.

Determination of infarct size. After 4 h of reperfusion, the heart was removed, the aorta was cannulated, and the coronary vascular bed was perfused on a Langendorff apparatus with oxygenated Krebs-Henseleit buffer at a constant flow of 22–24 ml/min for 10 min to clear the vascular compartment of plasma and blood cellular elements. A 1% solution of triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4, 37°C, 45 ml) was perfused through the heart. TTC demarcates the noninfarcted myocardium within the AAR with a brick-red color, indicating the presence of a formazan precipitate resulting from the reduction of TTC by dehydrogenases in viable myocardial tissue. Irreversibly injured tissue, lacking cytosolic dehydrogenases, is unable to form the formazan precipitate and appears pale yellow. After TTC infusion, the left circumflex coronary artery was ligated at the same site that was ligated during the induction of regional myocardial ischemia. The perfusion pump was stopped, and 3 ml of a 0.25% solution of Evans blue dye was injected slowly through a sidearm port connected to the aortic cannula. The dye was passed through the heart for 10 s to ensure its uniform tissue distribution. The presence of Evans blue dye was used to demarcate the left ventricular tissue that was not subjected to regional ischemia (NLV), as opposed to the risk region (AAR). The heart was removed from the perfusion apparatus and cut into three transverse sections at right angles to the vertical axis. The right ventricle, apex, and atrial tissue were discarded. The unblinded investigator traced both surfaces of each transverse section onto clear acetate sheets. The images were scanned and downloaded into Adobe PhotoShop (Adobe Systems, Seattle, WA). For determination of the NVL, AAR, and infarct region, the number of pixels occupying each area was calculated using Adobe PhotoShop software. Total AAR is expressed as a percentage of the left ventricle and infarct size as a percentage of the AAR.

Biochemical marker of irreversible myocardial injury. Plasma concentrations of cardiac-specific troponin I (cTnI) were determined by ELISA (Life Diagnostics, West Chester, PA). Briefly, serum was prepared from whole blood drawn at baseline and 2 and 4 h after the start of reperfusion: E2 (n = 9), E2 + MPA (n = 9), MPA (n = 9), and vehicle (n = 9). Samples were frozen immediately in liquid nitrogen and stored at −80°C. On the day of the assay, samples were thawed over ice and diluted appropriately with PBS. Protein concentrations were determined by comparison of the optical density of each sample with a standard curve.

Immunofluorescent detection of MAC and CRP. MAC and CRP levels in left ventricular tissue were determined as described previously (5). Briefly, tissue samples used for infarct size determination were fixed in 10% buffered formalin immediately after completion of the experimental protocol. The tissue samples were embedded in paraffin blocks and cut into 2-μm-thick sections, which were mounted on glass slides. Two consecutive sections (mirror images) from a single heart slice were mounted on each slide. The slides were washed three times in xylene to remove the paraffin and rehydrated in an ethanol gradient; they were placed in a boiling solution of a diluted unmasking agent (Vector Laboratories, Burlingame, CA) and then blocked with 5% milk for 45 min. Primary antibodies were incubated at room temperature in a humidity chamber for 45 min. One section per slide was incubated with a chicken anti-rabbit CRP antibody (5 μg/ml final concentration; Immunology Consultants Laboratory, Newberg, OR). The opposing transverse heart section was incubated with a chicken anti-rabbit MAC antibody (1:2,500 final dilution; developed in conjunction with Lampire Biological Laboratories, Pipersville, PA). After three consecutive washes in PBS-1% milk, each section was incubated with a goat anti-chicken biotinylated secondary antibody (1.5 μg/ml final concentration; Vector Laboratories) for 30 min. The slides were washed three times with PBS and then incubated with fluorescein (Fluorescent Streptavidin Kit, Vector Laboratories) for visualization of the proteins. For comparison, digital images were captured using a digital camera (model DKC5000, Sony of America, New York, NY) connected to a fluorescent stereo microscope (model MX FLIII, Leica, Wetzlar, Germany) and the accompanying software (Leica). Images were analyzed using IP Lab software (Scanalytics, Fairfax, VA) to determine mean fluorescence intensity per heart.
section. The sections were normalized to the amount of background on each slide. The mean intensities for five hearts in each treatment group were averaged and compared.

Assessment of complement inhibition. A red blood cell (RBC) lysis assay was used to determine whether the pretreatment was able to inhibit the rabbit complement system. The ex vivo analysis of complement activity is based on the C5b-9-dependent lysis of human RBCs on exposure to rabbit plasma. Complement-mediated RBC hemolysis was assessed by a turbidimetric method described previously (23). Rabbit serum was obtained from whole blood samples drawn from rabbits that were treated with 20 µg of E2 (n = 11), 20 µg of E2 + 80 µg of MPA (n = 11), 80 µg of MPA (n = 11), or vehicle (1 ml of 20% DMSO-80% polyethylene glycol, n = 11). After informed consent was obtained, human whole blood for the isolation of RBCs was obtained by venipuncture of the forearm vein of a healthy male donor who had not been exposed to any medication for the past 7 days. The cells were washed three times in 10 ml of PBS. PBS was then added to a reaction mixture containing 200 µl of 20% (wt/vol) KCl solution. A 100-µl aliquot of the homogenate was added to a reaction mixture containing 200 µl of 8.1% (wt/vol) SDS, 1,500 µl of 20% (wt/vol) acetic acid (pH 3.5), 1,500 µl of 0.8% (wt/vol) thiobarbituric acid, and 700 µl of distilled water. Samples were then heated for 1 h at 95°C and centrifuged at 4,000 rpm for 10 min. The absorbance of the supernatant was measured by spectrophotometry at 515–553 nm.

Materials. Unless otherwise noted, all materials were purchased from Sigma Chemical (St. Louis, MO).

Statistical analysis. Values are means ± SE. Differences between control and experimental groups were determined using Student’s t-test for unpaired comparisons. P < 0.05 was considered to be significant. Statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA).
treated animals but was not significantly different from the plasma cTnI concentration in vehicle-treated animals (Fig. 2).

Immunofluorescence. Immunofluorescence for MAC and CRP was reduced in hearts from E2-, but not E2 + MPA-treated animals. Hearts from vehicle-treated animals demonstrated robust antibody staining with chicken anti-rabbit MAC and chicken anti-rabbit CRP antibodies, indicating the deposition of both proteins in the myocardial region subjected to ischemia and reperfusion. Conversely, in hearts from E2-treated animals, fluorescence and deposition of CRP and MAC were reduced. Fluorescence was similar in heart tissue from E2 + MPA- and MPA-treated rabbits and vehicle-treated rabbits (Fig. 3A). In tissue sections stained for MAC or CRP, the mean intensity of fluorescence was significantly less \( (P < 0.001) \) in E2- than in vehicle-treated animals (Fig. 3B and C). Mean fluorescence intensity of heart sections from animals treated with E2 + MPA or MPA was similar to that from vehicle-treated animals.

Assessment of complement inhibition. E2 treatment reduced mean RBC hemolysis. The RBC hemolysis assay was used to determine the ability of E2 to inhibit the activation of the complement system. Hemolysis of human RBCs was reduced in serum from rabbits pretreated with E2 compared with serum from vehicle-treated animals: 41.6 ± 8.6 vs. 72.3 ± 6.1% \( (P < 0.05) \). As shown in Fig. 4, the inhibitory effect of E2 on RBC lysis was prevented by addition of MPA (66.3 ± 7.3%) or E2 + MPA to the treatment regimen (76.9 ± 5.9%).

Western blot analysis. Hearts from animals treated with E2, but not E2 + MPA, demonstrated a reduction in complement C3 protein. Myocardial tissue from the AAR and NLV of animals treated with vehicle, E2, E2 + MPA, or MPA was subjected to Western blot analysis. Western blot analysis demonstrates bands for C3 in the soluble extract of the myocardial tissue from the AAR of hearts of animals treated with vehicle, E2 + MPA, or MPA and subjected to ischemia and reperfusion (Fig. 5A). In contrast, there was less detectable C3 in heart tissue from animals treated with E2, suggesting that E2 attenuated C3 protein expression. The C3 protein displayed bands at 80 kDa, as reported previously (36). The normalized intensity of bands for samples from five animals is shown in Fig. 5B.

MDA measurements. E2, but not E2 + MPA, reduced lipid peroxidation, as assessed by the measurement of MDA. The thiobarbituric acid reaction was used to quantitate the presence of lipid peroxidation products in the AAR of hearts from animals treated with E2, E2 + MPA, MPA, or vehicle and subjected to ischemia and reperfusion. Acute treatment with E2 significantly decreased the amount of lipid peroxidation in hearts compared with vehicle treatment, as determined by MDA levels: 257.8 ± 83.4 vs. 69.9 ± 18.2 \( \mu M \) MDA/g tissue \( (P < 0.05) \). E2 + MPA or MPA decreased the level of lipid peroxidation compared with vehicle: 186.4 ± 42.0 and 158.4 ± 54.8 \( \mu M \) MDA/g tissue, respectively \( [P > 0.05 \text{ (not significant)]} \). MDA levels significantly increased in animals treated with vehicle, E2 + MPA, or MPA in the AAR compared with the NLV. There was no significant increase in the AAR of E2-treated animals (Fig. 6).

DISCUSSION

Inflammation serves an important role in the pathogenesis of many forms of disease, including myocardial ischemia-reperfusion injury. Treatment with E2 results in anti-inflammatory effects that may be protective in the setting of ischemia-reperfusion injury (7, 26, 31). The most notable finding of the
The present study is that E₂ has a significant effect on CRP expression and complement-mediated injury associated with ischemia and reperfusion. In contrast to other studies, progesterone alone did not demonstrate a robust proinflammatory effect; however, treatment with a combination of progesterone and estrogen reversed the protective effects of E₂ after ischemia.

Previous studies demonstrated the anti-inflammatory capacity of E₂, and our laboratory showed that E₂ modulates the activation of the tissue-associated complement system in the myocardium in response to reperfusion injury (5). The present study expands on these findings by demonstrating that E₂ inhibits complement activation, as determined by a reduction in complement-mediated RBC lysis and C₃ protein expression. C₃ protein plays a central role in the classical and alternative pathways of the complement cascade. The basal tissue and serum concentrations of C₃ are greater than other complement components, and C₃ concentration is amplified on activation of the complement system. Although not statistically significant, E₂ was associated with a trend toward a decrease in tissue protein levels of C₃. Complement activity was assessed via the ex vivo RBC lysis assay. In the present study, there was a significant decrease in RBC lysis in the presence of E₂ that was negated when E₂ was administered in combination with MPA.

Although activation of the complement cascade contributes to the pathogenesis of ischemia-reperfusion injury, individual complement components can trigger signaling pathways that alter cellular responses without causing cell death. Although the effects of C₅a can be detrimental to the tissue, it has been shown to elicit neuroprotective and cardioprotective effects in vivo (24, 35). In vitro pretreatment with sublytic concentrations of complement has been reported to prevent cellular damage from a subsequent exposure to a normally lytic concentration of complement (28, 29). In an in vivo experiment, Tanhehco et al. (35) demonstrated the ability of sublytic levels of complement to reduce infarct size after ischemia and reperfusion. It is possible that although estrogen does not completely inhibit the activation of complement after ischemia and reperfusion, the level of activation is decreased, and the function of the sublytic complement is to exhibit protective cellular responses without precipitating cell death. The addition of
progestin reverses this effect and, thereby, increases complement to levels that cause irreversible tissue injury.

Free radicals are involved in mediating the tissue injury associated with the inflammatory process, including the activation of the complement cascade and tissue expression of CRP (21). Lipid peroxidation represents a direct manifestation of the deleterious effects of free radicals on cell membranes, and initiation of intracellular signaling cascades by free radicals may propagate tissue injury (1, 43). In the present study, we demonstrated a reduction in lipid peroxidation after E2 treatment that was not observed when MPA was added to the treatment protocol. MPA alone caused a slight, but nonsignificant, decrease in lipid peroxidation. A reduction in free radical activity, as demonstrated by reduced lipid peroxidation, may contribute to the reduction in MAC and CRP activation in hearts from E2-treated animals.

The present study clearly demonstrates that E2 protects the heart from ischemia-reperfusion injury, most likely through modulation of the inflammatory response to injury. The results with the synthetic progestrone MPA are not as clear. The addition of MPA to the treatment regimen significantly reverses the protective effects of estrogen, as evaluated by infarct size, serum cTnI levels, complement deposition and activation, and lipid peroxidation. This reversal of estrogen’s action with the addition of progestrone is consistent with previous observations that addition of the progestin MPA, but not norethindrone acetate, inhibited ethinyl estradiol’s infarct-sparing effects. Progesterone has also been shown to counteract estrogen’s protective effects after experimental stroke and cerebrovascular inflammation (34). Treatment with MPA alone caused a nonsignificant reduction in infarct size and cTnI release. The MDA levels suggest a trend toward a reduction in lipid peroxidation with MPA alone, which may suggest a mechanism for the reduction of infarct size and the corresponding reduction of troponin levels. The trend toward an increase in complement-mediated activation after treatment with MPA, although not significant, agrees with the results of Sunday et al. (34) that progesterone exacerbated the inflammatory response in the cerebrovasculature and that this exacerbation opposed the protective anti-inflammatory effects of E2. These proinflammatory effects of MPA may counteract the protective effects on lipid metabolism, vascular reactivity, and atherosclerotic progression. The exact mechanism by which MPA modulates the inflammatory response to injury and negates estrogen’s infarct-reducing effects remains to be determined.

Treatment with the progestin MPA does not fully account for the negative effects observed in the clinical trials. In the Women’s Health Initiative, unopposed estrogen treatment did not increase or decrease the risk of coronary heart disease but did result in an increase in stroke (2,19). There was an increase in cardiovascular events in the first year of the Women’s Health Initiative, which is possibly due to the associated increase in CRP (30). Increases in CRP may be harmful, because CRP has a proatherogenic role: it binds to and activates complement, facilitates LDL uptake by macrophages, and increases plasminogen activator inhibitor-1 expression and activity, thereby inhibiting fibrinolysis. This increase in CRP, which is not seen with transdermal estrogen, may partially be a result of the route of administration.

Fig. 5. Western blot analysis of cytosolic extracts from the AAR (A) and normal left ventricular region (NLV, C) of hearts from rabbits subjected to ischemia and reperfusion. Positions of the molecular mass markers are shown at left. Bands for C3 (at ~80 kDa) appear in the AAR of rabbit hearts treated with vehicle, E2 + MPA, and MPA but are less distinct in the AAR of hearts treated with E2. B and D: relative intensity for AAR and NLV regions in bands normalized to actin.

Fig. 6. Malondialdehyde (MDA) levels in AAR and NLV of hearts from animals treated with vehicle, E2, E2 + MPA, or MPA. Ischemia and reperfusion of the myocardium leads to a considerable increase in MDA levels in the AAR, which is inhibited by E2, but not E2 + MPA or MPA. Values are means ± SE. *P < 0.05 vs. vehicle. **P < 0.01 vs. NLV. ^P < 0.05 vs. NLV.
Furthermore, it is important to note that the end points for the clinical trials and the reported experiments are clearly different. The end points in the clinical trials were coronary events and the ability of hormone replacement to prevent adverse primary or secondary coronary outcomes (19, 32). Specifically, clinical end-point determinations were death due to cardiovascular events or nonfatal myocardial infarction. The trials stopped at this point and did not monitor morbidity or cardiac function after the index events. Our studies do not attempt to show that administration of E2 will prevent an event but, rather, that E2 will reduce the extent of myocardial injury secondary to ischemia and reperfusion, thereby resulting in the salvage of myocardial tissue in the jeopardized ischemic zone or AAR. Therefore, our studies cannot conclude that the clinical trials are incorrect but, rather, that when estrogen (specifically E2) is present, the extent of irreversible tissue injury that occurs on reperfusion is decreased significantly compared with that in an appropriate control group and that the additon of MPA antagonizes this effect.

GRANTS
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