Background. The objective of this study was to examine the effect of estrogen combined with homocysteine on vasomotor function and endothelial integrity in intact porcine coronary arteries.

Materials and methods. Pig coronary artery rings were incubated with estrogen, homocysteine, or estrogen and homocysteine for 24 h. Myographic analysis was performed with thromboxane A2 analog U46619 for contraction and bradykinin or sodium nitroprusside for relaxation. Endothelial nitric oxide synthase (eNOS) levels were determined by immunohistochemistry. Levels of superoxide anion were assessed by lucigenin-enhanced chemiluminescence analysis.

Results. Endothelium-dependent vasorelaxation (bradykinin) for the homocysteine alone group was 62% compared with control ($P < 0.05$), and endothelium-dependent vasorelaxation for the estrogen alone group was 85% compared with control ($P > 0.05$). Endothelium-dependent vasorelaxation for the estrogen-homocysteine combined group was 79% compared with 89% for control ($P > 0.05$). There were no differences in endothelium-independent vasorelaxation (sodium nitroprusside) or in smooth muscle contractility (U46619) between all three groups and control. In addition, the eNOS immunoreactivity was declined in the homocysteine group and had no major change in the estrogen or estrogen plus homocysteine-treated group as compared with controls. The superoxide free radical measurement showed a marked increase in the homocysteine group, no major change from controls in the estrogen group, and a much-lessened effect in the combination of estrogen and homocysteine.

Conclusions. These data demonstrate that combining estrogen with homocysteine significantly blocks the effect of homocysteine on impairing endothelium-dependent vasorelaxation as well as on decreasing eNOS expression and increasing oxidative stress in porcine coronary arteries. This study suggests that estrogen may play a role in preventing homocysteine-mediated endothelial dysfunction and may be of benefit in the hyperhomocysteinemic patient.

Key Words: estrogen; homocysteine; endothelial cells; porcine; coronary artery; eNOS; superoxide anion.

INTRODUCTION

Hyperhomocysteinemia has been linked to premature atherosclerosis and thrombosis. It has been shown that homocysteine significantly decreases endothelium-dependent vasorelaxation and endothelial nitric oxide synthase (eNOS) immunoreactivity, perhaps demonstrating the physiological basis for vascular lesion formation in hyperhomocysteinemia. Homocysteine is a sulfur-containing amino acid metabolized from methionine, one of the essential amino acids. It can be either catalyzed to cystathionine by cystathionine β-synthase with vitamin B6, or remethylated back to methionine by methylene tetrahydrofolate reductase with vitamin B12 as a cofactor and methyltetrahydrofolate as a substrate [1].
Normal plasma concentration of homocysteine ranges from 5 to 15 μM, with hyperhomocysteinemia being defined as any value above 15 μM. Although severe hyperhomocysteinemia (>100 μM) has long been associated with premature atherosclerosis and thrombosis [2, 3], recently mild-to-moderate elevations in homocysteine have been shown to promote the same types of lesions. The severe form of hyperhomocysteinemia is very rare, but the mild form is present in 7% of the general population. More interestingly, 20–30% of patients with coronary and peripheral vascular disease have mild hyperhomocysteinemia [4–7]. The risk of myocardial infarction goes up 3-fold with a mere 12% increase above the upper limit of 15 μM [8].

Although it has been demonstrated that homocysteine impairs this NO-dependent mechanism and thereby induces endothelial dysfunction [1], few studies to date have postulated a drug therapy to prevent or lessen this endothelial damage. Therapy for patients with hyperhomocysteinemia has largely focused on B complex vitamin therapy and risk reduction by modifying other factors associated with atherosclerosis. In unpublished data from our laboratory, estrogen shows antioxidant effect on porcine coronary arteries. Because estrogen is a drug widely used in oral contraceptives and hormone replacement therapy (HRT), we coupled this with the other data compiled in our laboratory to postulate that estrogen may prevent or lessen the effects of homocysteine induced damage on the endothelium. This study could begin the search for investigation-based drug therapy for hyperhomocysteinemia.

MATERIALS AND METHODS

Chemical and Reagents

The culture media used was Dulbecco’s modified Eagle’s medium (Life Technologies, Inc., Grand Island, NY) with 1% antibiotic-antimycotic solution (Mediatech, VA). Water-soluble cyclodextrin-encapsulated estradiol and homocysteine were obtained from Sigma Chemical Co. (St. Louis, MO). The cyclodextrin-encapsulated estradiol was dissolved in deionized water to make 10–5 M estradiol stock solution. Homocysteine was dissolved in deionized water to make 5 mM stock solution. For the contraction and relaxation assays, thromboxane A2 U46619 (10–4 M) and bradykinin (10–7 M) stock solutions were created (Sigma Chemicals). Sodium nitroprusside was used as an endothelium-independent relaxation agent. Fresh solutions were made for each experimental session.

Isometric Tension of Porcine Coronary Arteries

The myograph system used in our laboratory has been previously described [9, 10]. Pig hearts were harvested from 6-month-old farm pigs at a local slaughterhouse. The hearts were harvested less than 10 min after sacrifice, the aortic arches were clamped, and the coronary arteries were retrograde injected with ice-cold phosphate-buffered saline solution. The circumflex coronary artery was harvested from each vessel, and the loose connective tissue was removed. The arteries were then cut into multiple 5-mm rings, which were then incubated in Dulbecco’s modified Eagle’s medium in four groups: controls, homocysteine (50 μM), estrogen (10–7 M), or a combination of homocysteine (50 μM) and estrogen (10–3 M). Cell culture incubation was accomplished at 37°C with 5% CO2 for 24 h. After culture, the coronary rings were suspended between the wires of the organ bath myograph chamber (Danish MyoTechnology Organ Bath 700MO, Aarhus, Denmark) in 6 mL of Krebs’ solution maintained at 37°C and oxygenated with pure oxygen gas. Pretensioning of the vessel rings was accomplished to 30 mN over 60 min. After pretensioning, each vessel was contracted by adding U46619 in each well (10–5 M solution). Each vessel was allowed to fully contract over 1 h, then relaxation dose–response curves were generated by five cumulative additions of the endothelium-dependent vasodilator bradykinin (10–6, 10–7, 10–8, and 10–9 M). Finally, the endothelium-independent vasodilator sodium nitroprusside (10–5 M) was added to fully relax the vessels to baseline. Contractility and percentage of relaxation were calculated based on the tension changes. The data of several coronary artery rings from each heart were averaged and are represented as one data point for statistical analysis.

Immunohistochemistry

After the myographic analyses were performed, the pig coronary artery rings were fixed in 10% neutral buffered formalin overnight and then transferred to 70% alcohol. The specimens were dehydrated using sequentially increasing concentrations of ethanol followed by xylene and embedded in paraffin. Five-micrometer cross sections were cut and stained with hematoxylin and eosin and subjected to immunohistochemistry staining for eNOS using the avidin-biotin complex immunoperoxidase procedure (LSAB kit, Dako Co., Carpinteria, CA). A monoclonal antibody against human eNOS (PharMin gen Corp., San Diego, CA) was used. Two investigators examined all slides from each vessel or ring and representative slides were photographed.

Detection of Superoxide Anion

Levels of superoxide anion produced by endothelial cells were determined using the lucigenin-enhanced chemiluminescence method with Sirius Luminometer and FB12 software (Berthold Detection Systems, Pforzheim, Germany). After 24 h of culturing, porcine carotid artery rings were rinsed briefly in a modified Krebs HEPES buffer solution (KHBS, 120 mM NaCl, 4.7 mM KCl, 1.18 mM KH2PO4, 20 mM HEPES, 2.5 mM CaCl2, 1.17 mM MgSO4, and 25 mM NaHCO3). The rings were then cut open longitudinally and then into approximately 5-mm × 5-mm pieces. Assay tubes (12 mm × 75 mm) were filled with 500 μL of KHBS, lucigenin (5 μM). The reagents were gently mixed in the tubes and the vessel segments were placed endothelium-side-down in the tubes. Measurements were taken every 15 s for a total of 12 min. The data in relative light units per second (RLU/s) for each sample were averaged between 7 and 10 min. Values of blank tubes containing the same reagents as the vessel ring samples were subtracted from their corresponding vessel samples. The area of each vessel segment was measured using a caliper and used to normalize the data for each sample, the final units thus being RLU/s/mm2.

Statistics

Data from the different groups were analyzed using an unpaired Student’s t test (Minitab software, Sigma Breakthrough Technologies, Inc., San Marcos, TX). In addition, analysis of variance test was used to analyze data of endothelium-dependent relaxation in response to bradykinin. P < 0.05 was considered statistically significant. Statistics are reported as the mean ± the standard error of the mean (SEM).
RESULTS

Homocysteine Impairs Endothelium-Dependent Relaxation

The effect of homocysteine on the porcine coronary artery rings (n = 9) is shown in Fig. 1. This demonstrates the maximal vessel tension after addition of thromboxane A2 analog U46619 (10^{-7} M) showed significant differences between control and homocysteine groups (P < 0.05). Control vessels (n = 9) contracted 103 ± 12 mN in response to 10^{-7} M U46619, and the vessels treated with 50 μM homocysteine contracted 108 ± 16 mN (Fig. 1A). In addition, homocysteine significantly changed endothelium-dependent vasorelaxation in response to bradykinin in precontracted vessels (Fig. 1B). At all concentrations of bradykinin, homocysteine-treated vessels relaxed differently from controls (P < 0.05). The relaxation at maximal bradykinin concentration (10^{-5} M) was 62% toward baseline compared to 89% for controls. Endothelium-independent relaxation was unchanged by homocysteine, as seen in Fig. 1C.

Estrogen Does Not Change Endothelium-Dependent Relaxation

The effect of estrogen on the porcine coronary artery rings (n = 9) is shown in Fig. 2. This demonstrates the maximal vessel tension after addition of U46619 (10^{-7} M) showed no significant differences between control and estrogen groups (P > 0.05). Control vessels (n = 9) contracted 103 ± 12 mN in response to 10^{-7} M U46619, and the vessels treated with 10^{-7} M of estrogen contracted 112 ± 17 mN (Fig. 2A). In addition, estrogen did not significantly change endothelium-dependent vasorelaxation in response to bradykinin in precontracted vessels (Fig. 2B). At all concentrations of bradykinin, estrogen-treated and control vessels relaxed similarly (P > 0.05). The relaxation at maximal bradykinin concentration (10^{-5} M) was 85% toward baseline compared with 89% for controls. Endothelium-independent relaxation was also unchanged by estrogen, as seen in Fig. 2C.

Combining Estrogen With Homocysteine Significantly Blocked the Effect of Homocysteine on Decreasing Endothelium-Dependent Vasorelaxation

The effect of estrogen combined with homocysteine on the porcine coronary artery rings (n = 9) is shown in Fig. 3. The maximal vessel tension after addition of U46619 (10^{-7} M) showed no significant differences between control and the combination estrogen-homocysteine groups (P > 0.05). Control vessels (n = 9) contracted 103 ± 12 mN in response to 10^{-7} M U46619, and the vessels treated with 10^{-7} M of estrogen contracted 112 ± 17 mN (Fig. 3A). In addition, the combination of estrogen and homocysteine did not significantly change endothelium-dependent vasorelaxation in response to bradykinin in precontracted vessels (Fig. 3B). At all concentrations of bradykinin, the vessels treated with homocysteine plus estrogen did not relax differently from controls (P > 0.05). The relaxation at maximal bradykinin concent-
tration \((10^{-5}\text{ M})\) was 79% toward baseline compared with 89% for controls. Endothelium-independent relaxation was unchanged by the combination of estrogen and homocysteine, as seen in Fig. 3C. Thus, estrogen blocks the effect of homocysteine on endothelial cells.

**FIG. 2.** The effect of estrogen on vasomotor activity of porcine coronary arteries. A, The maximal vessel tension after addition of U46619 \((10^{-7}\text{ M})\) showed no significant differences between control and estrogen groups \((P > 0.05, n = 9)\). B, Estrogen did not significantly change endothelium-dependent vasorelaxation in response to bradykinin in precontracted vessels. \((P > 0.05, n = 9)\). C, Endothelium-independent relaxation, induced by addition of sodium nitroprusside \((10^{-5}\text{ M})\) was unchanged by estrogen compared with controls \((P > 0.05, n = 9)\).

**FIG. 3.** The effect of estrogen on vasomotor activity of porcine coronary arteries. A, The maximal vessel tension after addition of U46619 \((10^{-7}\text{ M})\) showed no significant differences between control and estrogen groups \((P > 0.05, n = 9)\). B, Estrogen did not significantly change endothelium-dependent vasorelaxation in response to bradykinin in precontracted vessels. \((P > 0.05, n = 9)\). C, Endothelium-independent relaxation, induced by addition of sodium nitroprusside \((10^{-5}\text{ M})\) was unchanged by estrogen compared with controls \((P > 0.05, n = 9)\).
munohistochemical staining for the eNOS protein in the estrogen-treated tissues. Fig. 4C shows the same for homocysteine treated vessels. There appears to be no difference from control in the estrogen group, whereas the homocysteine group appears reduced compared with controls. The combination of estrogen and homocysteine seen in Fig. 4D shows an improvement in the eNOS protein level compared with homocysteine alone. In addition, endothelial integrity was damaged in homocysteine-treated vessels and this damage was also blocked by coinubcation with estrogen.

Effects of Homocysteine and Estrogen on Superoxide Anion Production

Fig. 5 illustrates the chemiluminescence of the endothelium, a marker for superoxide anion production. Control endothelium had a peak chemiluminescence of $10 \pm 0.3$ RLU/s/mm$^2$ ($n = 4$); estrogen alone endothelium was $5 \pm 3$ RLU/s/mm$^2$ ($n = 4$); homocysteine alone endothelium was $39 \pm 7$ RLU/s/mm$^2$ ($n = 4$); and the combination estrogen and homocysteine endothelium was $8 \pm 4$ RLU/s/mm$^2$ ($n = 4$). Thus, homocysteine significantly increases superoxide anion production as compared to controls ($P < 0.05$), whereas estrogen blocks this effect of homocysteine.

DISCUSSION

We have used myographic analysis coupled with superoxide free radical and immunohistochemical analyses in this study to help understand the change in endothelial function of porcine coronary arteries when exposed to estrogen, homocysteine, and a combination of the two compared with controls. As major observations, the homocysteine-treated vessels showed significantly impaired endothelium-dependent relaxation, but the endothelium-independent vasorelaxation and smooth muscle contractility were unchanged from controls. The vessels treated with estrogen showed no significant impairment of endothelium-dependent relaxation. More importantly, the combination of the two compounds showed no significant difference in endothelium-dependent vasorelaxation compared with controls, and this was in sharp contrast to the
homocysteine-alone group. In addition, the immunohistochemical staining reinforced these observations by showing a decline in eNOS levels in the homocysteine group; no major change in eNOS protein levels in estrogen versus controls; and no major change in the combination group. The superoxide free radical data again reaffirmed our observation by showing a marked increase in the homocysteine group; no major change from controls in the estrogen group; and a much-lessened effect in the combination of homocysteine and estrogen. These data demonstrate that estrogen blocks homocysteine-induced endothelial dysfunction in porcine coronary arteries.

Endothelium-dependent vasorelaxation is an important normal endothelial cell function. Development of atherosclerosis has been linked to impairment of this function [11]. It is widely known that NO is the most important endothelium-derived relaxation factor, and stimuli that activate eNOS increase its production. Bradykinin induces vasorelaxation by engaging specific endothelial cell receptors that stimulate production of NO and other vasodilator substances [12, 13]. In this study, endothelium-dependent vasorelaxation in response to bradykinin was significantly reduced in homocysteine-treated vessels. However, contractility from thromboxane A2 analog U46619 and endothelium-independent vasorelaxation from sodium nitroprusside did not differ from controls in the homocysteine group. These data are consistent with previous work published by our laboratory [1]. Studies by Lang et al. [14] also showed impairment of endothelium-dependent vasorelaxation by homocysteine in a rabbit aortic ring model. Ex vivo studies with isolated pancreatic arteries showed similar results [15]. Diet-induced hyperhomocystinemia in monkeys also exhibited impaired endothelium-dependent vasorelaxation [16]. The findings of no effect on the endothelium-independent function due to hyperhomocystinemia were also proven in a mice model by Watanable et al. [17].

The mechanism of estrogen inhibiting the detrimental effects of homocysteine on endothelium-dependent relaxation is likely due to multiple factors. Our data showed a decrease in eNOS levels by immunohistochemistry in the homocysteine treated group. The combination of estrogen and homocysteine improved these eNOS levels, and it can be inferred this would increase NO production and endothelium-dependent vasorelaxation. In addition, endothelial integrity was damaged in homocysteine-treated vessels and this damage was also blocked by co-incubation with estrogen. However, these data could not be quantified for statistical analysis due to insensitivity of intensity measurement and small sample size. In future studies, Western blot could be used to semiquantitatively measure eNOS protein levels.

Many authors have postulated the mechanism of homocysteine-induced cellular damage is caused by these superoxide free radicals [14, 18, 19]. NADPH oxidase generates superoxide anion, which in turn causes atheroma formation by lipoprotein oxidation and neutralization of nitric oxide. These react to form peroxynitrite [20]. Any substance that affects production of superoxide levels will affect NO levels directly. HMG-CoA reductase inhibitors have been shown to increase endothelium-dependent relaxation by this superoxide mechanism [21]. Lucigenin-enhanced chemiluminescence is one mechanism to quantify superoxide anion, a very short-lived molecule [22]. Our studies showed a significant decrease in superoxide anion levels in the combination group from the homocysteine group. The estrogen group alone showed a decrease in superoxide anion production as compared to controls, while the homocysteine group showed marked increase from controls. The conclusion is therefore drawn that the decrease in superoxide in the combination estrogen and homocysteine group compared to homocysteine alone causes decreased consumption of NO, therefore improving endothelium-dependent vasorelaxation.

Anti-oxidative effect of estrogen is an important observation in this study. However, we did not compare estrogen with any known antioxidants such as vitamins C and E for blocking homocysteine-induced superoxide anion production and endothelial dysfunction in this study. This comparison would be interesting in the future study.

Our data are consistent with a recent publication with different approaches. Tsen et al. [23] reported that human umbilical core vein endothelial cells treated with homocysteine (250 μM) for 24 h in vitro
showed an increase of ROS production by flow cytometry analysis, and estrogen (1 μM) significantly reduced this effect of homocysteine. However, the dose of homocysteine in their study is much higher than that in our study (50 μM), which is equivalent to the plasma level of patients with moderate hyperhomocystinemia [24].

Also, estrogen dose (1 μM) in their study is higher than that in our study (0.1 μM), which represents a plasma level of the patient who receives HRT [25, 26]. In addition, our models of coronary artery tissues include both functional and molecular analyses, which may be more relevant than human umbilical core vein endothelial cell cultures. Another recent publication in a human trial with HRT supports our conclusions [27].

After 6 months of HRT, homocysteine concentrations showed a statistically significant reduction in post-menopausal women. Women with the highest baseline levels of homocysteine experienced the greatest reduction. No significant variations in homocysteine concentrations were found in the control group. In addition, the positive effects of estrogen on endothelium have been published. The focus on clinical data showing a decrease in cardiac events in women on hormone replacement therapy has spawned a number of models showing the effect of estrogen on endothelial-dependent vasorelaxation. Mendelsohn et al. showed estrogen receptor-α-mediated an immediate nongenomic effect on eNOS. They also showed that genomic mechanisms controlled more long-term effects on eNOS [28–30]. Lack of estrogen impaired endothelium-dependent relaxation was demonstrated based on a human brachial artery model [31–33].

In summary, we believe that adding estrogen to vessels exposed to moderate hyperhomocystinemia decreases superoxide production and increases eNOS levels. This decreases the damage of homocysteine alone on the endothelium-dependent relaxation mechanism. This observation does not translate directly from porcine coronary arteries to human arteries, but the implication is seen. Perhaps estrogen-based pharmacotherapy might be used in the future to combat the detrimental effects of hyperhomocystinemia, although the side effects of estrogen may dissuade this type of therapy. These in vitro data will need to be pursued with chronic in vivo data to search for effects of estrogen on homocysteine-induced atherosclerosis. Ultimately, human studies might be indicated with potentially important results. This study should also encourage the search for other substances that might decrease the detrimental effects of hyperhomocysteinemia.

REFERENCES


