CALL FOR PAPERS | Sex and Gender Differences in Cardiovascular Physiology—Back to the Basics

Age and sex differences in vascular responsiveness in healthy and trauma patients: contribution of estrogen receptor-mediated Rho kinase and PKC pathways

Tao Li,1,* Xudong Xiao,1* Jie Zhang,1* Yu Zhu,1 Yi Hu,2 Jiatao Zang,1 Kaizhi Lu,3 Tiande Yang,4 Hengjiang Ge,2 Xiaoyong Peng,1 Dan Lan,1 and Liangming Liu1

1State Key Laboratory of Trauma, Burns and Combined Injury, Second Department of Research Institute of Surgery, Daping Hospital, Third Military Medical University, Chongqing, People’s Republic of China; 2Department of Anesthesiology, Research Institute of Surgery, Daping Hospital, Third Military Medical University, Chongqing, People’s Republic of China; 3Department of Anesthesiology, Xinqiao Hospital, Third Military Medical University, Chongqing, People’s Republic of China; 4Department of Anesthesiology, South Western Hospital, Third Military Medical University, Chongqing, People’s Republic of China

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Li T, Xiao X, Zhang J, Zhu Y, Hu Y, Zang J, Lu K, Yang T, Ge H, Peng X, Lan D, Liu L. Age and sex differences in vascular responsiveness in healthy and trauma patients: contribution of estrogen receptor-mediated Rho kinase and PKC pathways. Am J Physiol Heart Circ Physiol 306: H1105–H1115, 2014. First published February 15, 2014; doi:10.1152/ajpheart.00645.2013.—Several medical conditions exhibit age- and sex-based differences. Whether or not traumatic shock exhibits such differences with regard to vascular responsiveness is not clear. In a cohort of 177 healthy subjects and 842 trauma patients (21–82 years) as well as different ages (4, 8, 10, 14, 18, and 24 wk; 1 and 1.5 years) and sexes of Sprague-Dawley normal and traumatic shock rats, the age- and sex-based differences of vascular responsiveness were closely related to G protein-coupled receptor (GPR)30, estrogen receptor-mediated Rho kinase, and PKC pathway activation. Vascular responsiveness exhibits age- and sex-based differences in healthy subjects and trauma patients. Estrogen and its receptor (GPR30) mediated activation of Rho kinase and PKC using genomic and nongenomic mechanisms to elicit protective effects in vascular responsiveness. This finding is important for the personalized treatment for several age- and sex-related diseases involving estrogen.

IN RECENT YEARS, INTEREST in studying the effects of sex hormones and gender on cardiovascular function has been growing. Basic and clinical investigations have shown important differences in the structure and function of the cardiovascular system between men and women (32). Several studies have shown that premenopausal women are protected from most cardiovascular events as compared with men (26). However, after the menopause, women are at an increased risk of cardiovascular complications as compared with premenopausal women (26). The pathophysiological mechanisms for such differences are not clear, but studies have shown that sex steroids make some contribution (30, 36, 42).

Trauma is often seen in civilian and military situations. Hemorrhage and hemorrhagic shock are the major causes of early deaths in injured soldiers and in injuries due to accidents. Studies have shown that traumatic hemorrhagic shock accounts for ∼50% of deaths of battle personnel and for 66%–80% of trauma deaths (8, 13). Vascular responsiveness is a key factor in maintaining vascular tone and hemodynamic stability, helps to determine tissue perfusion, and affects other organ functions (23, 24). Vascular responsiveness decreases significantly after severe trauma and shock, which greatly interferes with the development and outcome of trauma and shock. It is the physiologic basis for refractory hypotension (6, 17, 18, 22, 41). If age and sex differences exist with respect to vascular responsiveness in victims of trauma and shock, and if and how estrogen has affects this process, is not clear.

Chaudry and colleagues (1, 11, 34, 46) found that females tolerate trauma and sepsis stimuli better than males and that estrogen has a protective effect. The estrogen level varies but also between individuals of different ages. Estrogen has affects this process, is not clear.

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Chaudry and colleagues (1, 11, 34, 46) found that females tolerate trauma and sepsis stimuli better than males and that estrogen has a protective effect. The estrogen level varies considerably in females of different ages. Our previous studies showed that Rho kinase (an important member of the small G protein family) and PKC had an important role in the regulation of vascular reactivity after trauma and shock. Thus we hypothesized that the changes in vascular responsiveness after trauma and shock were different not only in males and females but also between individuals of different ages. Estrogen and its
receptors may have important roles through Rho kinase and PKC in this process.

To elucidate these issues, age and sex differences in vascular responsiveness under healthy and traumatic shock conditions and the role of estrogen and its receptors as well as the mechanisms were investigated in healthy people, trauma patients, and hemorrhagic shock rats.

MATERIALS AND METHODS

Ethical Approval of the Study Protocol

The protocol for human and animal studies was approved by the Ethics Committee of the Research Institute of Surgery, Daping Hospital, Third Military Medical University (Chongqing, China) and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (8th Edition, 2011). All participants provided written, informed consent to participate in this human study, and our Ethics Committee approved this consent procedure. None of the authors of this manuscript are members of the Ethics Committee that approved this protocol. The clinical trial number for the human study is ChiCTR-OCC-11001876.

Human Studies

Healthy participants. Endonasal perfusion after postural change can be used to reflect vascular reactivity (38). Healthy humans of different ages and sexes were recruited for measurement of vascular responsiveness by determining endonasal perfusion after postural change. Two hundred fifty-five healthy participants, 21–82 years of age, were screened and 177 (81 men and 96 women) enrolled. The subjects were divided prospectively into three age groups: young (21–44 years, 36 men and 46 women); middle aged (45–59 years, 24 men and 24 women); and old (≥60 years, 21 men and 26 women) (Table 1). Participants in each group were divided further into male and female subgroups. Subjects did not have structural abnormalities of the nasal cavity or acute airway diseases and did not use medications (particularly cardiovascular drugs). Subjects with diabetes mellitus (DM) or hypertension were excluded from the study. Endonasal perfusion after postural change was measured using a rhinometer (Rhinolux; Rhios GmbH, Großkmehlen, Germany) (38). Briefly, subjects were acclimatized to room conditions for 1 h before commencement of measurements. To achieve constant baseline readings, subjects were asked to stand during a run-in phase of varying duration (5–10 min) while continuous measurements were taken. Stable baseline conditions were indicated automatically by an acoustic signal emanating from the rhinometer. Once stable baseline conditions were achieved while standing, a reference baseline level was set [optical density (OD) = 0]. Study participants were required to stand for a further 5 min. After this 5-min baseline period, subjects were placed in the supine position with continuous measurement of optical density for another 30 min. Changes in endonasal blood perfusion (OD detected by the optical rhinometer) after postural change and the time to reach maximal perfusion of endonasal blood (T-value) as well as the 30% and 100% recovery rates of endonasal blood perfusion were used to reflect vascular reactivity. Two milliliters of venous blood were obtained for measurement of the plasma level of estrogen using a chemical analyzer (LX20; Beckman Coulter, Brea, CA) after measurement of the perfusion of endonasal blood.

Trauma patients. Based on 842 trauma patients from Daping Hospital, Southwestern Hospital, and Xinqiao Hospital in Chongqing, China, between 1 May 2007 and 30 April 2012, we retrospectively investigated age and sex differences in vascular reactivity to commonly used vasoactive agents; that is, ephedrine (5–15 mg iv), dopamine (3–5 μg·kg⁻¹·min⁻¹ iv), and phenylephrine (10–50 mg iv). The inclusion criterion for this analysis was trauma patients aged 21–82 years who needed vasoactive agents to increase blood pressure except for basal fluid infusion before and during surgery. When systolic blood pressure was <80–90 mmHg or the mean arterial blood pressure increased by >30% of the basal blood pressure, vasoconstrictors were adopted. Exclusion criteria included individuals with hypertension, DM, or cardiac diseases and patients who received two types of vasoconstrictors. Among 1,345 trauma patients, 204 had hypertension, 188 had DM, and 111 had cardiac diseases and so were excluded from this analysis. Eight hundred forty-two patients were enrolled in the present study. Injury types were traffic accidents (698 patients), simple falls (56), stabwounds (49), and explosives (39). Among them, there were 773 penetrating injuries and 69 blunt injuries. The study cohort was divided into three age groups: 20–44 years; 45–59 years; and ≥60 years. Vascular reactivity was reflected by an increase in MAP after administration of vasoconstrictors (ephedrine, dopamine, or phenylephrine) (Table 2).

Animal Studies

Age and sex differences with respect to vascular responsiveness in normal and traumatic shock rats. NORMAL RATS. Male (64) and female (64) immature-to-mature and reproductive-to-aged Sprague-Dawley (SD) rats (4, 8, 10, 14, 18, and 24 wk and 1 and 1.5 years; n = 8 rats/sex/age group), whereby 4 wk represents immature, 8 wk-24 wk represents mature and reproductive age, respectively, and 1 year and 1.5 years represent old age, were used (2, 40). On the day of experimentation, rats were anesthetized with pentobarbital sodium (50 mg/kg ip). Right femoral arteries were catheterized with polyethylene catheters for blood sampling for measurement of estrogen levels (DX800 Biochemical Analyzer; Beckman Coulter). After blood sampling, rats were euthanized by an overdose of pentobarbital sodium and underwent laparatomies. Superior mesenteric arteries (SMAs) were isolated and made into rings of length 2 to 3 mm (an identical euthanization method was used in subsequent experiments) for determination of vascular reactivity [i.e., response of SMAs to graded concentrations of norepinephrine (NE)] (22, 41). Briefly, artery rings were mounted on wire and suspended between a force transducer and a post attached to a micrometer. They were then immersed in a 10-ml isolated organ chamber (AD Instruments, Castle Hill, NSW, Australia) containing Krebs-Henseleit (K-H) solution, which was bubbled continuously with 95% O₂ and 5% CO₂ while the temperature was maintained at 25°C. A 0.5-g preload was given, and the K-H solution was replaced every 20 min. The tension of the artery ring was determined by a Power Lab System via a force transducer (AD Instruments). After a 2-h equilibration, the response of SMAs to NE (10⁻⁹, 10⁻⁸, 10⁻⁷, 10⁻⁶, and 10⁻⁵ mol/l) was determined. The maximal contraction (Emax) and pD₂ [−log (50% effective concentration, EC₅₀)] of agonists and NE were obtained from the concentration-response curves and used to compare vascular reactivity.

HEMORRHAGIC SHOCK RATS. The same amounts, ages (4, 8, 10, 14, 18, and 24 wk and 1 and 1.5 years), and sexes of SD rats as in the normal rat experiments were used in this part of the experiment (n = 8 rats/sex/age group). Rats were anesthetized with pentobarbital

Table 1. Demographic parameters of healthy people

<table>
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<tr>
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<th>N</th>
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<td>66.6 ± 8.3</td>
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<tr>
<td>45–59</td>
<td>24</td>
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<td>118.3 ± 16.2</td>
<td>76.3 ± 11.7</td>
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<td>68.6 ± 4.8</td>
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<td>119.6 ± 12.3</td>
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<td>46</td>
<td>30.8 ± 7.8</td>
<td>55.1 ± 11.2</td>
<td>107.0 ± 13.2</td>
<td>72.0 ± 9.7</td>
</tr>
<tr>
<td>45–59</td>
<td>24</td>
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<td>56.0 ± 6.3</td>
<td>118.0 ± 12.3</td>
<td>74.0 ± 9.4</td>
</tr>
<tr>
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<td>57.1 ± 7.2</td>
<td>114.0 ± 15.2</td>
<td>75.9 ± 10.6</td>
</tr>
</tbody>
</table>

Values are means ± SD.
sodium (50 mg/kg ip). Right femoral arteries were catheterized with polyethylene catheters for monitoring MAP. After catheterization, rats underwent laparotomies and were phlebotomized from the right femoral arterial catheter to a MAP of 40 mmHg and maintained at this MAP for 2 h. Rats were then euthanized by an overdose of pentobarbital sodium and underwent laparotomies. SMAs were obtained for the measurement of vascular reactivity as described in the section describing experiments on normal rats.

**Protective effects of exogenous supplementation of estrogen on vascular reactivity and survival rate in normal and shock rats of different ages and sexes.** On VASCULAR REACTIVITY IN NORMAL RATS. Male and female rats (8 and 14 wk and 1.5 years) were used as representatives (whereby 8 wk represented just mature, 14 wk represented reproductive, and 1.5 years represented aged rats; n = 8 rats/sex/age group) to receive exogenous supplementation of estrogen (17β estradiol, 0.1 mg/kg iv) for 1 wk (once daily). The control group received the same volume of saline. One week after, SMAs were obtained for measurement of vascular reactivity as described in Age and sex differences with respect to vascular responsiveness in normal and traumatic shock rats.

On VASCULAR REACTIVITY IN HEMORRHAGIC SHOCK RATS. Male and female rats (8 and 14 wk and 1.5 years) were also used as representatives to receive management of hemorrhagic shock (n = 8 rats/sex/age group). After MAP was maintained at 40 mmHg for 2 h, rats received exogenous treatment with estrogen (17β estradiol, 0.1 mg/kg in 2 times volumes of lactated Ringer’s solution; the infusion rate was 25 μl/h) 2 h after estrogen was given. SMAs were obtained, and SMA rings created for measurement of vascular reactivity as described above. The control group received only an infusion of two-times volume of lactated Ringer’s solution. We selected rats aged 8 and 14 wk and 1.5 years as representatives to observe the protective effects of estrogen on vascular reactivity under normal and shock conditions for two main reasons. First, the results in the experiment in Age and sex differences with respect to vascular responsiveness in normal and traumatic shock rats showed that sexually immature rats (aged 4 wk) exhibited no sex-based differences in vascular responsiveness under normal and shock conditions. Sex-based differences in vascular responsiveness were mainly in rats aged 8–24 wk. Hence, we selected rats aged 8 wk (representing only sexual immaturity) and 14 wk (representing the reproductive period) as representatives in this part of the experiment (2, 40). Second, to further clarify whether hormone-replacement treatment can improve cardiovascular function in the elderly (4, 15, 27, 37), 1.5-year rats, even though they also showed no obvious sex-based differences in vascular reactivity as 4-wk-old rats, were chosen as representatives in this part of the experiment.

On SURVIVAL TIME IN SHOCK RATS. One hundred eight male and female rats aged 14 wk were selected as representatives to determine the protective effect of estrogen on vascular reactivity and survival after hemorrhagic shock. Rats were divided randomly into three groups: shock; shock + lactated Ringer’s solution (LR) resuscitation; and shock + estrogen treatment. After completion of preparation of hemorrhagic shock (maintaining the MAP at 40 mmHg for 2 h), rats were resuscitated with LR or LR + estrogen (17β estradiol, 0.1 mg/kg). The volume of LR was also two times the volume of blood loss. The infusion rate was 25 μl/h. Rats in the shock control group were not resuscitated. Sixty rats (n = 10/group/sex) were used to determine the 24-h survival rate. Forty-eight rats (n = 8/group/sex) were used to determine the pressor response of NE and the contractile response of SMAs to NE.

For measurement of the pressor response of NE and contractile response of SMAs to NE, the increased value of MAP before and after NE (3 μg/kg, bolus injection) administration and changes in SMA diameter after NE administration were measured at baseline and at the end of shock, as well as at 10 min, 30 min, 1 h, and 2 h after estrogen was given. The pressor effect of NE was expressed as the increased MAP before and after NE was given. The contractile response of SMAs to NE was expressed as a reduced percentage of the SMA diameter after NE administration. The SMA diameter was measured using an IntraVital video system (S6D; Leica, Wetzlar, Germany). For observation of animal survival, all catheters were removed and incisions sutured. To relieve postoperative pain, Jingsongling (xylidithaizole, 0.15 mg/kg im) was given. Rats were in articulo mortis during the period of survival observation or who survived >24 h were euthanized by overdosing of pentobarbital sodium.

**Regulatory effect of estrogen on rho kinase and PKCe.** AGE AND SEX DIFFERENCES IN PROTEIN EXPRESSION OF RHO KINASE AND PKCe IN NORMAL RATS. Eighteen rats (8 and 14 wk and 1.5 years, representing mature, reproductive, and aged stages, respectively; n = 3/each age and sex) were used. SMAs were isolated and total proteins extracted according to instructions given in the extraction kit. The expression of Rho kinase and PKCe protein was determined using Western blotting.

**Effects of estrogen on the activity and expression of Rho kinase and PKCe** in normal and hemorrhagic shock rats. Twenty-four female rats aged 14 wk (n = 3/each group) were used in this part of the experiment. SMAs were isolated from normal and hemorrhagic shock rats. SMAs from normal rats were incubated with estrogen (17β estradiol, 10^-6 mol/l) for 15 min and 1, 4, and 12 h. SMAs from hemorrhagic shock rats were incubated with estrogen (17β estradiol, 10^-6 mol/l) for 15 min and 4 h. Cytoplasmic and membrane proteins were extracted from SMAs using an extraction kit. The activity of Rho kinase was reflected by the level of phosphorylation of the myosin phosphatase target subunit (MYP1). PKC activity was reflected by the ratio of membrane-to-cytoplasmic PKC. Protein expressions of Rho kinase and PKCe were determined using Western blotting.

**Effects of Y-27632 (specific antagonist of Rho kinase) and PKCe pseudosubstrate inhibitory peptide on estrogen-induced protection of vascular reactivity after hemorrhagic shock in rats.** Fourteen-week-old female rats were used. Fifty-six SMA rings obtained from normal and hemorrhagic shock rats were divided randomly into seven groups: normal control; 2 h shock; 2 h shock + estrogen (17β estradiol, 10^-6 mol/l); 2 h shock + Y-27632 [Rho kinase inhibitor (10^-5 mol/l)]; 2 h shock + PKCe pseudosubstrate inhibitory peptide (10^-5 mol/l); 2 h shock + PKCe pseudosubstrate inhibitory peptide + estrogen; and 2 h shock + Y-27632 + estrogen (n = 8/group). SMAs from hemorrhagic shock rats were incubated with estrogen, PKCe pseudosubstrate inhibitory peptide, Y-27632, PKCe pseudosubstrate inhibitory peptide + estrogen, or Y-27632 + estrogen for 20 min. Vascular reactivity of SMA was observed as described above. The doses of Y-27632 and PKCe pseudosubstrate inhibitory peptide selected in the present study were based on our previous study (20).

Age and sex differences in estrogen receptor (ER) expression and the ER subtype that participates in the regulation of vascular reactivity. AGE AND SEX DIFFERENCES IN ER EXPRESSION IN NORMAL RATS. Male and female rats [8 and 14 wk and 1.5 years (n = 3/each age and sex)] were used. SMAs were isolated and total protein extracted. Expressions of ER-α, ER-β, and GPR30 were determined by Western blotting.

**Subtype of ER that participates in the regulation of vascular reactivity, Rho kinase, and PKCe.** Female rats aged 14 wk (n = 8/each group) were used. SMA rings were isolated for measurement of vascular reactivity as well as for the activities of Rho kinase and PKCe. The antisense oligodeoxynucleotides (AODNs; final concentration, 100 μmol/l) of ER-α, ER-β, and GPR30 were transfected into SMAs with transfection reagent (5:1 vol/vol; Qiagen, Stanford, VA) 24 h before hypoxic conditions were established. SMAs were then exposed to hypoxic conditions (oxygen concentration <0.2%) for 2 h. The contractile response of SMAs to NE was observed.
and activities of Rho kinase and PKCε were determined. The AODN sequences of ER-α, ER-β, and GPR30 were 5′-TCA TGT TTC CCT TCT CGC TG-3′, 5′-GTA CCC ACA CCG TTC TCT CCT-3′, and 5′-GCT CCT CTG CGC CAC AT-3′, respectively. The AODN of the ER needs 24 h to elicit effects and, during this time, SMAs from shocked animals would lose the shocked state. Hence, hypoxia-treated SMAs to mimic the shocked state were used to complete this part of the experiment.

Statistical Analyses

Data are means ± SD of n observations. The 30% and 100% recovery rates of endonasal blood perfusion are presented as recovery percentages and account for the total subjects detected in that group. Differences in parameters between ages, sexes, or experimental groups were analyzed by one-way, two-way, and three-way ANOVA, followed by the post hoc Tukey test. Analyses of correlation between changes in blood estrogen and changes in vascular reactivity in different ages and sexes of the healthy population and rats were done using the Pearson coefficient. Animal survival was evaluated by Kaplan-Meier survival analyses. P < 0.05 (two-tailed) was considered significant.

RESULTS

Human Studies

Healthy participants. With aging, average perfusion in the nasal mucosa was increased significantly, the T-value (time to reach the maximal endonasal blood perfusion) was prolonged significantly, and the recovery proportion of 30% and 100% endonasal blood perfusion after 30 min was decreased significantly; females had less changes in endonasal blood perfusion after postural change and shorter time to reach maximal perfusion as well as higher recovery rates of endonasal blood perfusion (Fig. 1, A–D). However, in subjects aged ≥60 years, the sex-based difference in vascular reactivity was not obvious. Middle-aged and young women had higher blood estrogen levels than older women (≥60 years). The blood estrogen level in men was too low to be detected (Fig. 1H). Changes in the blood estrogen level in women were positively associated with changes in vascular reactivity with aging. The coefficient between blood estrogen level and vascular reactivity in healthy women was 0.9734 (P < 0.01).

Trauma patients. The amount of blood loss and MAP before administration of vasoactive agents in all 842 patients in the three age groups and two sex groups were not significantly different (Table 2). Among all 842 patients, 520 received ephedrine, 228 received dopamine, and 94 received phenylephrine. Similar to that of the healthy subjects, vascular responsiveness (pressor effect of ephedrine, dopamine, and phenylephrine) in middle-aged (45–59 years) and young (21–44 years) patients was greater than that in elderly trauma patients (≥60 years). The females were greater than the males, whereas in the ≥60-year group there were no obvious sex-based differences in the pressor effects of ephedrine, dopamine, and phenylephrine (Fig. 1, E–G).

Animal Studies

Age and sex differences in vascular responsiveness in normal and hemorrhagic shock rats. Normal rats. Vascular reactivity in 8–24 wk female rats was increased and higher as compared with age-matched male rats and 4-wk-old rats. After 24 wk (1 year and 1.5 years), vascular reactivity decreased gradually, but no sex difference was observed (4-wk-old rats also showed no sex-based differences in vascular reactivity) (Fig. 2A). pD2 decreased gradually with aging, but there was no sex-based difference among the age groups (Fig. 2B). Changes in the blood estrogen level in normal female rats (Fig. 2C) were positively associated with changes in vascular reac-

Fig. 1. Age- and sex-based differences in vascular reactivity in healthy controls and trauma patients. A–D: changes in vascular reactivity at different ages and sexes of healthy controls, i.e., 21–44 years age group (36 men and 46 women); 45–59 years age group (24 men and 24 women); ≥60 years age group (21 men and 26 women). A: changes in perfusion in the nasal mucosa (optical density [OD]) after postural change. B: time at which the perfusion in the nasal mucosa reached a maximum (T) value after postural change. C: proportion of the population in which perfusion in the nasal mucosa reached 30% recovery. D: proportion of the population in which perfusion in the nasal mucosa reached 100% recovery. E–G: response of trauma patients to vasoactive agents. E: increased mean arterial blood pressure (MAP) after ephedrine administration (Adm; n = 520). F: increased MAP after dopamine administration (n = 228). G: increased MAP after phenylephrine administration (n = 94). H: plasma estrogen level in women. *P < 0.05, **P < 0.01, vs. the same age female group; #P < 0.05, ##P < 0.01, vs. the same sex <44-year-old group.
### Table 2. Demographic parameters of trauma patients

<table>
<thead>
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<th>Parameter</th>
<th>N</th>
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<th>Weight, kg</th>
<th>Blood Loss, ml</th>
<th>Mean Arterial Blood Pressure, mmHg</th>
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<td>83</td>
<td>32.3 ± 10.5</td>
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<td>36</td>
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<td>Phenylephrine, 10–50 mg iv</td>
<td>94</td>
<td>Male, years</td>
<td></td>
<td></td>
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<tr>
<td>≤44</td>
<td>12</td>
<td>29.9 ± 7.9</td>
<td>54.0 ± 10.84</td>
<td>1232.7 ± 1159.9</td>
<td>66.0 ± 12.5</td>
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<tr>
<td>45–59</td>
<td>27</td>
<td>52.4 ± 2.1</td>
<td>51.3 ± 12.19</td>
<td>1050.0 ± 1172.9</td>
<td>67.2 ± 10.2</td>
</tr>
<tr>
<td>≥60</td>
<td>15</td>
<td>71.3 ± 6.6</td>
<td>55.6 ± 10.66</td>
<td>1112.5 ± 502.6</td>
<td>67.9 ± 9.9</td>
</tr>
<tr>
<td>Female, years</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤44</td>
<td>13</td>
<td>29.2 ± 10.3</td>
<td>59.6 ± 15.33</td>
<td>1080.4 ± 1112.8</td>
<td>70.3 ± 12.2</td>
</tr>
<tr>
<td>45–59</td>
<td>18</td>
<td>50.9 ± 5.8</td>
<td>61.5 ± 9.68</td>
<td>1069.1 ± 1110.5</td>
<td>68.3 ± 16.2</td>
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<tr>
<td>≥60</td>
<td>9</td>
<td>72.6 ± 9.2</td>
<td>59.3 ± 12.00</td>
<td>1106.3 ± 831.6</td>
<td>71.9 ± 10.9</td>
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Values are means ± SD. Mean arterial blood pressure is before administration of vasoactive agents. No difference in these parameters among the different age and sex groups exists.

Activity with aging. The coefficient between the blood estrogen level and vascular reactivity in normal rats was 0.67 (P < 0.01). Blood estrogen in normal male rats could not be detected.

**Hemorrhagic Shock Rats.** When compared with that of normal rats of the same age and sex, vascular reactivity was decreased after hemorrhagic shock (Fig. 2, D and E). Male rats lost more vascular reactivity than female rats at 8–24 wk of age after shock (Fig. 2, D–F). There was no sex-based difference in loss of vascular reactivity in rats aged 4 wk or 1 or 1.5 years.

**Protective effects of estrogen on vascular reactivity in normal and hemorrhagic shock rats.** On vascular reactivity in normal and hemorrhagic shock rats. Exogenous supplementation of estrogen (17-β estradiol, 0.1 mg/kg iv, once daily for 1 wk in normal rats or 0.1 mg/kg of 17-β estradiol infused 2 h after shock in hemorrhagic shock rats) increased vascular reactivity significantly in 8 and 14-wk-old male and female normal or hemorrhagic shock rats. For rats aged 1.5 years, supplementation or treatment with estrogen increased vascular reactivity only slightly in normal female rats, whereas there was no protective effect in normal and shocked male rats, or in shocked female rats (Fig. 3, A and B). The pD2 of SMA to NE did not change after estrogen incubation in all age groups, including male and female rats (data not shown).

**On animal survival in hemorrhagic shock rats.** Infusion of exogenous estrogen (17-β estradiol, 0.1 mg/kg) significantly increased the MAP and increased animal survival (including the survival number and survival time of hemorrhagic shock.
vascular responsiveness that was closely related to activation
results suggested that estrogen had a protective effect on
rats after hemorrhagic shock. NE challenge in hemorrhagic shock rats (Fig. 3, D) as compared with the control group (Fig. 3, C and F). Estrogen infusion significantly increased the pressor effect of NE as well as the contractile response of SMAs to NE (changes in SMA diameter are shown in Fig. 3, D and E). These results suggested that estrogen not only protects vascular reactivity in healthy subjects but also increases the ability to fight against the insults elicited by trauma or shock.

**Regulatory effect of estrogen on Rho kinase and PKCε and the relationship with vascular reactivity.** Protein expression of Rho kinase and PKCε in rats aged 8 and 14 wk and 1.5 years exhibited differences based on age and sex. With regard to expression of Rho kinase, it was higher in all ages of normal female rats (8 and 14 wk and 1.5 years) than in male rats. When compared with 8-wk-old rats, 14-wk-old female rats had greater expression than 8-wk-old and 1.5-year-old rats. Rats aged 1.5 years had the lowest expression, especially in male rats (Fig. 4, A and B).

As rats aged, PKCε expression decreased gradually, and male rats showed lower expression than female rats (Fig. 5, A and B). Incubation with estrogen (10^{-6} mol/l) increased the protein expression of Rho kinase and PKCε in normal and shock rat SMAs and also increased the activities of Rho kinase (rate of p-MYPT/MYPY increased) and PKCε (PKC level in cell membrane increased) in the SMAs of normal and shocked rats (Fig. 4, D–I, and 5, D–J). Interestingly, irrespective of whether normal SMAs or shocked SMAs were used, short-term incubation with estrogen (15 min or 1 h) could increase the activities of Rho kinase and PKCε, whereas only longer-term incubation with estrogen (4 h or 12 h) could increase the expression of Rho kinase and PKCε. The Rho kinase inhibitor Y-27632 (10^{-5} mol/l) and PKCε pseudosubstrate inhibitory peptide (10^{-7} mol/l) antagonized the effect of estrogen on vascular reactivity in shocked rats (Fig. 4C and 5C). These results suggested that estrogen had a protective effect on vascular responsiveness that was closely related to activation of Rho kinase and PKCε and that estrogen had genomic and nongenomic effects.

**Age and sex differences in ER expression and the ER subtype that participates in regulation of vascular reactivity**

There were no differences in the expression of ER-α in normal rats based on age and sex; ER-β expression was decreased in 1.5-year-old male and female rats compared with 8- and 14-wk-old male and female rats, and there were no changes at the other ages tested. GPR30 expression was lower in 1.5-year-old male and female rats and 14-wk-old male rats than 8-wk-old male and female rats and 14-wk-old female rats (Fig. 6, A and B). Incubation of SMAs from 14-wk-old female rats with GPR30 AODN (but not the AODNs of ER-α and ER-β) abolished the protective effect of estrogen on vascular reactivity (Fig. 6C). Further investigation found that GPR30 AODN significantly decreased the upregulation effect of estrogen on the activity of Rho kinase and PKCε in hypoxia-treated SMAs from 14-wk-old female rats (Fig. 6, D–G).

**DISCUSSION**

Several diseases exhibit age- and sex-based differences with respect to developmental and clinical characteristics (31, 35). Chaudry and colleagues (1, 11, 34, 46) found that the tolerance to trauma also exhibits differences between males and females. Vascular responsiveness is a key factor in the maintenance of cardiovascular function and hemodynamic stability. It is not known, however, whether vascular responsiveness elicits age and sex differences in healthy and traumatic-shock patients, and whether estrogen and ERs make important contributions to the regulation of vascular responsiveness.

Our human studies showed that vascular responsiveness exhibited differences based on age and sex. In healthy subjects, vascular reactivity decreased gradually as aged. Middle-aged
and young healthy women were higher than men of the same age. Sex-based differences in vascular reactivity were not obvious in aged population. Similar to the healthy participants, vascular responsiveness in middle-aged and young trauma patients was greater than in elderly trauma patient. Females had stronger responsiveness than males, whereas aged population had no obvious sex-based differences in vascular reactivity. Animal study confirm this results and further found reproductive age female rats to be better equipped to fight against shock-induced decreases in vascular reactivity than age-matched male rats and older rats (Fig. 2). Further studies showed that middle-aged and young healthy women and reproductive-age female rats had higher stable estrogen levels that were positively associated with changes in vascular reactivity. Exogenous supplementation of estrogen (17-β estradiol) not only improved vascular reactivity in normal reproductive-aged rats but also improved vascular reactivity and survival in hemorrhagic shock rats. Exogenous supplementation of estrogen did not improve vascular reactivity in older rats, which was not in accordance with the literature (Fig. 3) (31). These findings suggest that estrogen contributes to age- and sex-based differences in vascular reactivity and provide important evidence that estrogen supplementation can improve vascular reactivity in healthy, reproductive-age humans and potentiate the treatment of severe trauma and shock. Also, these findings provide further support to the notion that hormone-replacement therapy cannot improve cardiovascular function in the elderly (4, 15, 27, 37). In addition to the important regulatory effect of estrogen in age- and sex-based differences in vascular reactivity, hypertension, DM, and hyperlipidemia-induced arteriosclerosis may also participate in the decrease in vascular reactivity in the elderly.

Our previous studies showed that vascular reactivity is regulated by Rho kinase and PKCε (9, 18, 19, 21, 44, 45). Whether estrogen regulates vascular reactivity associated with Rho kinase and PKCε is not known. The present study suggested that estrogen significantly increased the expression and activity of Rho kinase and PKCε in SMAs from 14-wk-old normal and hemorrhagic shocked female rats. Antagonists of Rho kinase and PKCε antagonized the protective effect of estrogen on vascular reactivity after shock (Figs. 4 and 5). These results suggest that the regulation of vascular reactivity

Fig. 4. Relationship between the protective effect of estrogen on vascular reactivity in normal and shock conditions to Rho kinase. A and B: protein expression of Rho kinase in SMAs from normal (N) rats aged 8 wk (w), 14 wk, and 1.5 years (y), respectively (n = 3). C: effect of estrogen on vascular reactivity in hemorrhagic shock rats and relationship with Rho kinase (n = 8). D–F: effect of estrogen incubation on the activity [p-myosin light chain phosphatase subtype (MYPT/MYPT) and protein expression of Rho kinase in SMAs from normal rats (n = 3). G–I: effect of estrogen incubation on the activity (p-MYPT/MYPT) and protein expression of Rho kinase in SMAs from hemorrhagic shock rats (n = 3). Y, Y-27632; F, female; M, male. *P < 0.05, **P < 0.01, vs. normal group; #P < 0.05, ##P < 0.01, vs. shock group; @@@P < 0.01, vs. estrogen group; +P < 0.05, vs. female of the same age; ^^P < 0.05, vs. 8-wk-old group of the same sex.
by estrogen is closely related to activation of Rho kinase and PKCε.

Three subtypes of the ER have been identified: ER-α/H9251, ER-β/H9252, and GPR30 (3, 7, 29, 39, 43). Some studies have shown that the ERs have different patterns of expression and different effects at different ages (12, 14). The present study showed that expression of ER-α/H9251 did not exhibit differences based on age and sex. Expression of ER-β/H9252 and GPR30 was decreased in 1.5-year-old male and female rats but did not decrease in 8- and 14-wk-old rats. GPR30 with GPR30 AODN abolished the protective effect of estrogen on the vascular reactivity of SMAs. These results suggested that GPR30 is the main ER in the regulation of estrogen with respect to vascular reactivity.

What can account for these differences in ER levels? Several factors regulate the level of ER expression. Estrogen can positively and negatively regulate ER levels. Other hormones, such as progesterone and vitamin D, have been reported to negatively regulate the levels of ERs (16, 33). Here, we showed that a decrease in the level of estrogen in elderly rats could be the main reason for a decrease in expression of the ER, which is why exogenous supplementation with estrogen did not have a protective effect on vascular reactivity in normal and traumatic shocked elderly rats.

Estrogen has genomic and nongenomic effects (25, 31). The present study showed that short-term incubation with estrogen could increase the phosphorylation of Rho kinase and the activity of PKCε in SMAs from normal or shocked rats and that longer-term incubation with estrogen could increase the protein expression of Rho kinase and PKCε in SMAs from normal or shocked rats. Our results showed that early rescue of vascular responsiveness after trauma could be related to the nongenomic effect of estrogen and that a prolonged effect could be related to the genomic and nongenomic effects of estrogen. Some studies have shown that the mechanism by which estrogen induces rapid nongenomic effects is related mainly to the PI3k-Akt-eNOS and MAPK-ERK pathways (5, 10, 28). The present study suggests that new genomic and nongenomic signaling pathways (Rho kinase and PKC) have protective effects on vascular function.

Our previous studies showed that hypoxic treatment in vitro could induce the same change of vascular reactivity in vascular smooth muscle cells (VSMCs) and isolated blood vessels such as the SMA as that seen in vivo during hemorrhagic shock in rats (20, 22, 23, 24). Those data suggested that hypoxia-treated VSMCs and isolated SMAs in vitro could model the hypoxic condition of the shocked state. Longer-term incubation (24 h)
of isolated blood vessels with AODNs in a model of hemorrhagic shock would lose the features and state of shock. Hence, hypoxia-treated SMAs were used to mimic the shocked state in the present study to investigate the mechanisms of estrogen on vascular reactivity. Nevertheless, hypoxia can only partially mimic the hypoxic state of shock; a powerful in vitro model that can mimic the hypoxic and ischemic state of shock is needed.

In the present study, we used endonasal perfusion after postural change to reflect vascular reactivity in the human study, which is an indirect method. Endonasal perfusion is affected not only by vascular reactivity but is also regulated by the nervous system. Hence, using it to reflect “true” vascular reactivity as determined in rats has some limitations. To overcome this limitation, we used different ages and sexes of rats to confirm the results of our human study. In addition, our study and previous studies have shown that vascular reactivity exhibits organ-based differences after trauma or shock, but that the tendency of these changes is identical. The pattern of change in SMAs is a classic feature and has been used in many studies (17–22). Other limitations of the present study need to be overcome in future work. First, the disorders we studied

Fig. 6. Age- and sex-based differences in estrogen receptor (ER) expression and the ER subtype that participates in regulation of vascular reactivity. A and B: protein expression of ER-α, ER-β, and G protein-coupled receptor (GPR30) in SMAs from normal rats of different ages and sexes (n = 3). C: effects of different ER antisense oligodeoxynucleotides (AODNs) on the protective effect of estrogen on vascular reactivity in shocked rats (n = 8). D–G: effect of estrogen and GPR30 AODN on the activity of Rho kinase (p-MYPT/MYPT) and PKCε (membrane PKCε/cytoplasmic PKCε) in SMAs from shocked rats (n = 3). Hy, hypoxia; M-PKCε, membrane PKCε; S-PKCε, cytoplasmic PKCε. **P < 0.01, vs. normal group; ###P < 0.01, vs. hypoxia group; @P < 0.05, @@P < 0.01, vs. estrogen group; +P < 0.05, + +P < 0.01, vs. female of the same age; ^P < 0.05, vs. 8-wk-old group of the same sex.
were trauma and hemorrhagic shock; age- and sex-based differences in cardiovascular function in other diseases need investigation. Second, the precise mechanism by which estrogen regulates Rho kinase and PKC needs further investigation. Third, only rats aged 8 and 14 wk and 1.5 years were chosen as representatives to observe the protective role of exogenous supplementation of estrogen on vascular reactivity in normal and shocked rats. A wider range of ages should be used in future studies.

Conclusion
Vascular reactivity exhibits age- and sex-based differences in healthy humans, trauma patients, normal rats, and traumatic shocked rats. Reproductive-age female rats and middle-aged and young women had higher vascular responsiveness than age-matched male rats and elderly men, a stronger ability to fight the insults elicited by trauma and shock, in which estrogen had an important role. Realization of this protective effect of estrogen was mainly via activation of Rho kinase and the PKC pathway by genomic and nongenomic mechanisms, in which GPR30 was the main ER involved. This finding is important for the personalized treatment for many age- and sex-related diseases involving estrogen. A new mechanism for the effect of estrogen on vascular function via GPR30-Rho kinase and PKC pathways is proposed.

GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES