Mechanisms of endothelium-dependent vasodilation in male and female, young and aged offspring born growth restricted

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Morton JS, Rueda-Clausen CF, Davidge ST. Mechanisms of endothelium-dependent vasodilation in male and female, young and aged offspring born growth restricted. Am J Physiol Regul Integr Comp Physiol 298: R930–R938, 2010. First published January 6, 2010; doi:10.1152/ajpregu.00641.2009.—Numerous epidemiological studies have shown that cardiovascular dysfunction in adult life may be programmed by compromised growth in utero. Aging is a risk factor for vascular endothelial-dependent dysfunction. After birth, the impact of intrauterine growth restriction (IUGR) on normal aging mechanisms of vascular dysfunction is not known. We hypothesized that IUGR would cause changes in vascular function that would affect the mechanisms of endothelium-dependent vasodilation later in life in an age- or sex-dependent manner. To create an IUGR model, pregnant Sprague-Dawley rats were placed in a hypoxic (12% O2) or control (room air, 21% O2) environment from days 15 to 21 of the pregnancy, and both male and female offspring were investigated at 4 or 12 mo of age. Endothelial function was assessed in small mesenteric arteries using methacholine (MCh)-induced vasodilation in a wire myograph system. The involvement of nitric oxide (NO), prostaglandins, and endothelium-derived hyperpolarizing factor (EDHF) was assessed using the inhibitors Nω-nitro-l-arginine methyl ester hydrochloride, meclofenamate, or a combination of apamin and TRAM-34 (SKCa and IKCa blockers), respectively. EDHF-induced vasodilation was further investigated by using inhibitors of P450 epoxidases [N-methylsulfonyl-6-(2-propargyloxyphenyl) hexanamide] and gap junctions (18-glycyrrhetinic acid). NO-mediated vasodilation was significantly reduced in aged controls and both young and aged IUGR females. EDHF-mediated vasodilation was maintained in all groups; however, an additional involvement of gap junctions was found in females exposed to hypoxia in utero, which may represent a compensatory mechanism. A change in the mechanisms of vasodilation occurring at an earlier age in IUGR offspring may predispose them to adult cardiovascular diseases.

IUGR; hypoxia; nitric oxide; EDHF; myoendothelial gap junctions

INTRAUTERINE GROWTH RESTRICTION (IUGR) is a condition of pregnancy resulting in offspring who do not reach their growth potential for a given gestational age. A large proportion of pregnancies result in IUGR offspring, 3–32% worldwide (41), and in the last decade awareness of the potential clinical significance of this condition to chronic diseases later in life has increased. A number of studies have demonstrated an association between low birth weight and an increased mortality later in life (reviewed in Ref. 2). In fact, there is a growing body of evidence suggesting that a suboptimal in utero environment can cause permanent changes in the fetal development of the cardiovascular system that programs or predisposes it to develop cardiovascular and metabolic conditions such as endothelial dysfunction (15, 17, 23, 24), hypertension (38), coronary artery disease (22), obesity (11), and diabetes (1) later in life.

Cardiovascular diseases are the leading cause of death in the world today and, in addition, are closely associated with aging; the incidence of cardiovascular disease rises dramatically after 60 yr of age in both males and females. However, in recent years the incidence of deaths due to cardiovascular diseases has increased in a younger age group; those <59 yr old (25, 26).

Until now, primary care strategies for the prevention of cardiovascular diseases have centered on the modification of adult lifestyle: smoking cessation, increased activity, and a healthy diet (29). With the establishment of the theory of developmental origins of health and disease, we must now consider both whether IUGR could reduce the age at which cardiovascular diseases develop and also give us reason to extend our focus into the prenatal environment. However, our understanding of the mechanisms that mediate fetal programming, and could therefore be targeted in the development of potential prophylactic and therapeutic interventions, is very limited. By studying this phenomenon in detail, we may be able to move to a more proactive therapeutic approach.

IUGR has many different causes but is most commonly due to a placental insufficiency leading to an inadequate nutrient and oxygen supply to the fetus. Animal models developed to investigate this area have utilized a decrease in the supply of nutrients, oxygen, or both to the fetus; however, each model has its limitations. The fetus has some recourse to adjust for a limited nutrient supply (21); however, there are few mechanisms to compensate for a limited oxygen supply, which is a common occurrence in several obstetric pathological conditions that may lead to IUGR, such as preeclampsia, placenta previa, maternal anemia, and smoking. Thus, we have used the rat model of hypoxia-induced IUGR to address the long-term effects of limited oxygen during the development of the fetus on the cardiovascular system.

Using this reduced oxygen model of growth restriction in rats, we have previously observed reduced endothelial function in mesenteric arteries of adult offspring (18, 43). The three main pathways of endothelium-dependent vasodilation involve nitric oxide (NO), prostaglandins, and endothelium-dependent hyperpolarizing factor (EDHF). However, the exact nature of EDHF has not yet been determined and can be considered more as a group of factors. Some of the proposed mechanisms of EDHF-induced vasodilation include 1) epoxygenase-cosatirnecic acids (EETs) (12); 2) activation of endothelial channels including small conductance calcium-activated potassium

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channels (SKCa) and intermediate conductance calcium-activated potassium channels (IKCa) (14); 3) activation of smooth muscle channels including Na+/K+-ATPase, inward-rectifying potassium channels (Kir), voltage-gated potassium channels (Kv), and large conductance calcium-activated potassium channels (BKCa) (3, 10, 19); 4) transfer of hyperpolarization via myoendothelial gap junctions (MEGJ) (7), hydrogen peroxide (27), C-type natriuretic peptide (6), and anandamide (33) to name but a few. Despite variability in the predicted identity of EDHF, it is commonly accepted that inhibition of endothelial SKCa and IKCa channels inhibits most EDHF-mediated vasodilation. We hypothesized that hypoxia-induced IUGR would cause changes in vascular function that would affect the mechanisms of endothelium-dependent vasodilation. Since vascular endothelial function is also known to be affected by age (5, 20) and sex (32, 36), we also investigated whether changes associated with IUGR were age or sex dependent.

METHODS

Animal model of IUGR. All protocols were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee in accordance with the Canadian Council on Animal Care guidelines. Three-month-old female Sprague-Dawley rats (Charles River, Wilmington, MA) were maintained on ad libitum standard rat chow and tap water in a 12:12-h light-dark cycle. Females were acclimatized in-house before breeding. Day 0 of pregnancy was determined by the presence of sperm in a vaginal smear following an overnight introduction of a male. Dams were weighed daily throughout pregnancy. On day 15 of pregnancy, rats were randomly allocated to control or hypoxia groups. Those in the hypoxia group were individually housed in a standard cage inside a Plexiglas chamber, and the environmental oxygen partial pressure (PO2) was reduced to 12% using a continuous infusion of an air/nitrogen mixture. Oxygen levels were monitored throughout the treatment using a portable oxygen analyzer calibrated daily (Hud-son RCI, Temecula, CA). Placement of pregnant dams in the hypoxic chamber significantly decreased their weight gain from day 16 onward as previously reported (42). Control animals were housed in a room air (21% oxygen) environment.

Females were removed from the chamber on day 21 and allowed to give birth in a normal oxygen environment. At birth, litters were reduced to eight pups, four males and four females, to avoid the impact of competition for milk on the subsequent growth of the offspring. Our pilot studies have demonstrated that cross-fostering hypoxic pups to control dams and vice versa had no impact on vascular function results. Offspring were weaned into same-sex pairs in standard cages and maintained under standard conditions with a normal room air environment until 4 or 12 mo of age. At the point of death, offspring were selected from different litters for experimental procedures (n = 14 dams, 7 control, 7 IUGR), thus ensuring experimental groups did not contain littersmates. This model has been well characterized in our laboratory and, in a separate set of animals (n = 8 litters, 4 control, 4 IUGR), weight just prior to birth was shown to be reduced in both male [4.57 ± 0.19 g (n = 23)] controls vs. 4.00 ± 0.08 g (n = 22) IUGR, P < 0.05) and female offspring [4.44 ± 0.19 g (n = 27) controls vs. 3.87 ± 0.11 g (n = 27) IUGR, P < 0.05]. The IUGR offspring were found to be in the 15th percentile of the control population. In addition, placenta weight was reduced [0.60 ± 0.01 g (n = 50) control vs. 0.51 ± 0.01 g (n = 49) IUGR, P < 0.0001], while litter size remained unchanged [17.0 ± 0.7 IUGR vs. 18 ± 0.7 IUGR, P = 0.36]. Therefore, the experimental group was referred to as IUGR as an indicator of exposure to a compromised intrauterine environment, caused in this case by chronic fetal hypoxia.

Vascular function. At 4 or 12 mo of age, animals were euthanized by exsanguination via excision of the superior vena cava under inhaled isoflurane anesthesia. Tissue dissections were performed in ice-cold physiological saline solution (in mmol/l: 10 HEPES, 5.5 glucose, 1.56 CaCl2, 4.7 KCl, 1.17 MgSO4, 1.18 KH2PO4, pH 7.5). Small mesenteric (<250 μm, mean 143 ± 2 μm) arteries were isolated from young adult (2.6 to 4.7 mo old) and aged adult (11.3 to 13.0 mo old), control and IUGR, male and female Sprague-Dawley rats. Arteries were cleaned of all surrounding adipose and connective tissues and mounted on two 25-μm wires attached to a wire myograph (DMT, Copenhagen, Denmark) to allow isometric tension recordings. Vessels were normalized through a series of stepwise increases in diameter to determine their optimal resting tension, set to 0.8 × IC100 (the internal circumference equivalent to a transmural pressure of 100 mmHg).

Following a 30-min equilibration period, vessels were twice exposed to a single dose of phenylephrine (10 μmol/l PE) followed by a single dose of methylycholine (3 μmol/l MCh) to check functional endothelial and smooth muscle integrity. A cumulative concentration-response curve (CCRC) to noradrenaline (NA) was performed to determine the EC50 of the maximum response (Emax) for the vasoconstrictor. NA was chosen since it demonstrated stable maintenance of tone; in particular in the presence of the MEGJ inhibitor 18α-glycyrretinic acid (18α-GA), which affected PE-induced tone. To investigate vascular responses to MCh (0.003 to 3 μmol/l), a CCRC was performed following preconstriction with the EC50 concentration of NA. CCRCs to MCh were performed in the absence or presence of inhibitors. To investigate some of the possible pathways involved in endothelium-dependent vasodilation, we inhibited NO synthase (NOS) with Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME; 100 μmol/l), production of prostaglandins with the cyclooxygenase inhibitor meclofenamate (1 μmol/l), or EDHF-induced vasodilation with a combination of apamin (100 nmol/l) and TRAM-34 (10 μmol/l), which block SKCa and IKCa channels, respectively. In addition, blockade of EET, with an inhibitor of P450 epoxygenase N-methylsulfonyl-6-(2-propargyloxyphenyl) hexanamide (MS-PPOH, 50 μmol/l) or myoendothelial gap junction inhibition with 18α-GA (3 μmol/l) were used to further investigate EDHF-mediated vasodilation.

Statistical analysis. All data were presented as means ± SE of the pEC50 (negative log of EC50) or the Emax (maximum response). All data were normally distributed (Kolmogorov-Smirnov test for Gaussian distribution). The significance of the difference in mean values of continuous variables between groups was determined by a one- or two-way ANOVA, with the Bonferroni posttest for multiple groups or a Student’s t-test. Differences in the proportions of categorical variables were determined by a χ2 test. A P value < 0.05 was considered statistically significant. Male and female offspring were analyzed separately. Given the dispersion of the data, and accepting a type 1 error of 0.05, we calculate that this study has an average power of 80% to detect a difference of 1.0 in the pEC50.

RESULTS

Vasoconstrictor and vasodilator capacity. At the time of death, the body weights of females exposed to hypoxia in utero were not significantly different from controls at 4 mo (310 ± 7 g IUGR vs. 317 ± 5 g controls) or 12 mo (442 ± 18 g IUGR vs. 449 ± 11 g controls). In males, however, offspring born growth restricted remained significantly lighter than controls at both 4 mo (526 ± 14 g IUGR vs. 588 ± 12 g controls) and 12 mo (736 ± 22 g IUGR vs. 794 ± 15 g controls). There were no significant differences in the size of vessels from each group with the exception of the aged IUGR male group in which arteries were significantly larger than those from the young male control group (157 ± 6 μm vs. 135 ± 5 μm).
µm, \( P = 0.029 \)). Maximal vasoconstrictor responses to NA, but not sensitivity, were significantly increased in aged control females compared with young female control and IUGR (Fig. 1). This increase was not present in aged females exposed to hypoxia in utero or in males. In mesenteric arteries from all groups, the endothelium-dependent vasodilator MCh caused 87.6 ± 6.8% to 96.7 ± 2.4% relaxation with a pEC\(_{50}\) of 6.6 ± 0.1 to 7.9 ± 0.7. Responses to the NO donor sodium nitroprusside were unaltered between groups or in the presence of inhibitors (data not shown). Although overall vasodilation to MCh was unaltered by hypoxia in utero, a combination of age and hypoxia in utero, the underlying mechanisms of vasodilation were found to be affected in these vessels. A source of underestimation of endothelial dysfunction may be that vessels were discarded if there was no steady maintenance of NA tone from which to perform MCh CCRCs. It was interesting to note that there was an increased number of discarded vessels with age and hypoxia in utero [9.9% in young control, 4.2% in young IUGR, 15.2% in aged control, and 19.0% in aged IUGR (\( \chi^2, P < 0.0001 \))].

**Mechanisms of vasodilation in females.** In young (4 mo) control females, inhibition of NOS (100 µmol/l, L-NAME) or EDHF (100 nmol/l apamin and 10 µmol/l TRAM-34) reduced vasodilator responses to MCh (Fig. 2). L-NAME significantly decreased the pEC\(_{50}\) from 6.9 ± 0.1 to 6.3 ± 0.2 (\( P = 0.04 \)), while apamin and TRAM-34 decreased maximal vasodilation from 89.5 ± 2.7% to 55.0 ± 3.5% (\( P < 0.0001 \)). Neither inhibition of prostaglandins (1 µmol/l meclofenamate), EET (50 µmol/l MS-PPOH), or MEGJ (3 µmol/l 18α-GA) inhibited vasodilation to MCh (Fig. 2, Table 1).

Contrary to their controls, in young females exposed to hypoxia in utero MCh-induced vasodilation was not inhibited by L-NAME. However, it was significantly inhibited by apamin and TRAM-34 reducing maximal vasodilation from 96.7 ± 2.4% to 70.1 ± 5.5% (\( P = 0.0005 \)) and, in addition, was significantly inhibited by 18α-GA reducing maximal vasodilation from 96.7 ± 2.4% to 58.4 ± 10.5% (\( P = 0.003 \)) (Fig. 2). Inhibition of prostaglandins or EET had no effect in this group (Table 1).

In the aged (12 mo) female control group, vasodilation to MCh was only inhibited by apamin and TRAM-34 reducing maximal responses from 88.4 ± 5.0% to 47.8 ± 7.6%, \( P = 0.001 \) (Fig. 3, Table 1).

Hypoxia in utero had a similar effect in aged females to that in young females. In the aged IUGR group, sensitivity to MCh was not affected by L-NAME, but maximal vasodilation was inhibited by apamin and TRAM-34 reducing responses from 89.3 ± 4.0% to 55.4 ± 9.7% (\( P = 0.007 \)) and, in addition, was inhibited by 18α-GA reducing maximal vasodilation from 89.3 ± 4.0% to 59.4 ± 3.1% (\( P = 0.002 \)) (Fig. 3). Meclofenamate and MS-PPOH had no effect on vasodilation in this group (Table 1).

**Mechanisms of vasodilation in males.** In young control males, vasodilation was significantly inhibited by L-NAME, reducing the pEC\(_{50}\) from 6.7 ± 0.1 to 6.3 ± 0.2, \( P = 0.033 \) (Fig. 4). In addition, maximal vasodilation was reduced by EDHF inhibitors from 94.6 ± 1.2% to 57.0 ± 12.3% (\( P = 0.007 \)) and was reduced by 18α-GA from 94.6 ± 1.2% to 66.3 ± 8.3% (\( P = 0.004 \)). Inhibition of prostaglandins or EET had no effect in this group (Table 1).

In young males exposed to hypoxia in utero, vasodilation was similarly inhibited by L-NAME, reducing the pEC\(_{50}\) from 7.1 ± 0.1 to 6.5 ± 0.1, \( P = 0.010 \) (Fig. 4, Table 1). Apamin and TRAM-34 reduced maximal dilation from 96.2 ± 2.2% to 42.4 ± 4.5% (\( P < 0.0001 \)), and 18α-GA reduced maximal dilation from 96.2 ± 2.2% to 75.9 ± 7.0% (\( P = 0.013 \)).

In contrast, in aged control males L-NAME did not inhibit vasodilation (Fig. 5, Table 1). However, maximal vasodilation was inhibited by apamin and TRAM-34 from 90.2 ± 3.6% to 53.3 ± 7.8% (\( P = 0.0009 \)) and by 18α-GA from 90.2 ± 3.6% to 73.7 ± 6.4%, \( P = 0.036 \).

In aged males exposed to hypoxia in utero, vasodilation was only inhibited by apamin and TRAM-34 reducing maximal...
Table 1. Vasodilation to methacholine in mesenteric arteries from male and female rats

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Data are summarized as pEC50 (negative log of the concentration required to give 50% maximal constriction) in means ± SE. Vasodilation was performed in the absence (control) or presence of inhibitors of cyclooxygenase [1 μM meclofenamate (Meclo)] or P450 epoxygenase [50 μM/l N-methylsulfonyl-6-(2-propargyloxyphenyl) hexanamide (MS-PPOH)], IUGR, intrauterine growth restricted. There were no significant differences as assessed using a one-way ANOVA followed by a Bonferroni posttest to compare each inhibitor to its group control.
females exposed to hypoxia in utero at a young, 4-mo time point. Previous studies have demonstrated the effect of a compromised pregnancy in young male animals: 1) NO-mediated vasodilation was reduced in 7-mo-old rat offspring from a hypoxia model of IUGR (43); 2) endothelial NOS activity and expression were reduced in 3.5- and 5-mo-old rat offspring from a nutrient restriction model of IUGR (13, 34); 3) NO synthesis was also reduced in 3-mo-old rat offspring from a reduced placental perfusion model (30, 31); and 4) in chicken embryos from a hypoxia model of IUGR (35). However, this is the first study to demonstrate that a loss of NO-induced vasodilation occurs at an earlier time point in females exposed to a compromised in utero environment compared with their controls.

In addition, few studies have investigated whether endothelium-dependent pathways other than NO are affected by growth restriction in utero. Since overall vasodilator responses were maintained in both males and females exposed to a compromised uterine environment, this suggests that there was either a redundancy in the mechanisms of vasodilation or compensatory mechanisms active in the presence of a stressor-induced reduction of NO. As previously mentioned, the three major endothelium-dependent vasodilator pathways are NO, prostaglandins, and EDHF. In mesenteric arteries from all groups, incubation with a cyclooxygenase inhibitor had no effect on MCh-induced vasodilation, suggesting that prostaglandins played little or no role in normal vasodilation. The concentration of meclofenamate used has been previously validated in our own and other labs (4, 8). Therefore, prostaglandins were also not responsible for vasodilation in the face of a loss of NO-mediated pathways. In contrast, EDHF-mediated vasodilation was both present and maintained in all groups, male and female, young and aged, control and IUGR. Therefore, there may be a relative redundancy between the NO and EDHF pathways such that EDHF-mediated vasodilation can maintain vascular dilator function in the absence or reduction of NO bioavailability. However, since EDHF is not a single factor, the precise mechanisms behind this require clarification.

As previously discussed, EDHF-induced vasodilation may be mediated by a number of factors or pathways. To study this in a little more detail, we targeted two of the most supported theories: 1) transfer of hyperpolarization from the endothelium to the smooth muscle cells via gap junctions and 2) activation of smooth muscle potassium channels by EET. Interestingly, while vasodilation in mesenteric arteries from both young and aged females exposed to hypoxia in utero demonstrated a reduction in NO-mediated vasodilation, vasodilation was inhibited by both potassium channel blockers and a gap junction inhibitor. However, vasodilation in both young and aged controls demonstrated no involvement of gap junctions. This suggests that in females suboptimal conditions in utero had the effect of recruiting MEGJ. The inhibition of P450 exoperoxogenases or gap junctions had no effect in mesenteric arteries from
female controls. Therefore, hyperpolarization of the endothelial membrane via movement of potassium ions through SKCa and IKCa channels was not initiated by EET nor transmitted to the smooth muscle via MEGJ. As with meclofenamate, the concentration of MS-PPOH used has been previously validated by our own and other labs (9, 16, 40). It has been suggested that accumulation of potassium ions in the intracellular space may activate either Na+/H+-ATPase, KIR, Kv, or BKCa channels on the smooth muscle cells as previously described (3, 10, 19). Since inhibitors of these pathways were not included in the current study, these possibilities would require further investigation.

In contrast, in males there appeared to be no general effect of hypoxia in utero on the overall mechanisms of vasodilation. NO, EDHF, and gap junctions, but again neither prostaglandins nor EET, were involved in the vasodilation of both young control and IUGR groups. However, while NO involvement was decreased and EDHF was maintained in both aged control and IUGR animals, the involvement of MEGJs was only decreased in aged animals exposed to hypoxia in utero. Therefore, while the overall contribution of EDHF to vasodilation was unchanged, there appeared to be a noticeable sex-difference in mechanisms of translation of endothelial hyperpolarization to a smooth muscle response. In males, EDHF-mediated responses were transmitted via MEGJ and potentially via other, unstudied, potassium channels. However, the combination of age and hypoxia in utero (reduction in both NO- and MEGJ-mediated mechanisms) compromised the diversity of vasodilator pathways. In females, however, the connection of vasodilator responses and operating MEGJ appears to be active only in the absence of NO-mediated pathways following hypoxia in utero. To determine whether this change represents an overall increase in numbers of MEGJs or an alteration in the structure that affects function would be important to address in future studies. While MEGJs could be visualized by using fluorescence or electron microscopy, e.g., by staining for connexins, it would be critical to perform more involved studies to assess the functionality of MEGJs in each of the experimental groups. The greater involvement of MEGJ may represent a compensatory mechanism in females to maintain vasodilation in the presence of an earlier loss of NO-mediated pathways and may explain why some previous studies have described a greater impact of a loss of NO function on male vasodilator function and suggest that females are in some manner cardioprotected (37, 39, reviewed in Ref. 28).

The present study has focused on endothelial factors by using endothelium-dependent vasodilators and inhibitors. The responses of the smooth muscle to an NO donor and maximal responses to the adrenergic agonist PE were found to be unchanged. However, the endothelium and the smooth muscle act as a closely integrated unit to produce vascular reactivity, and it is possible that a compromised in utero environment could have effects on all aspects of this functional unit, including smooth muscle reactivity to other vasoactive compounds.

Fig. 4. Vasodilation to MCh in mesenteric arteries from young (4 mo) control (n = 6–7) and IUGR (n = 6–7) males. Vasodilation was performed in the absence (●, black bars) or presence (○, white bars) of inhibitors of NO (100 μmol/l L-NAME), EDHF (100 nM apamin and 10 μmol/l TRAM-34), or MEGJ (3 μmol/l 18α-glycyrrhetinic acid). Insets show the effect of the inhibitors on pEC50 (NO) or Emax (EDHF and MEGJ). Vasodilation in the presence or absence of an inhibitor was compared by Student’s t-test, *P < 0.05, **P < 0.01, ***P < 0.001.
Furthermore, while we have considered the pathways of endothelium-dependent vasodilation, there are also many important endothelial vasoconstrictors, such as endothelin, which may play an important role in fetal programming. Also, structural factors, such as the availability of MMPs or vascular remodeling may also have roles to play. Nevertheless, the current study has made progress toward understanding how the endothelium is affected by the in utero environment and the consequences this could have for adult cardiovascular function.

**Perspectives and Significance**

Compromised growth in utero was found to affect the mechanisms of vasodilation, reducing the NO component (in females) and maintaining an EDHF component (in both sexes). Age alone also decreased the NO component without affecting EDHF-mediated vasodilation in all groups. In this study, the loss of NO-mediated vasodilation occurred at an earlier time point in females, but not males, exposed to hypoxia in utero. Therefore, while overall vasodilation was maintained, a loss of particular vasodilator pathways such as NO may cause the vasculature to be more susceptible to further dysfunction due to a lack of redundant mechanisms. It would be interesting to investigate whether a second insult, such as a high-fat diet or type II diabetes, would exacerbate the vascular system to a point of overt vascular dysfunction. Interestingly, females, but not males, appeared capable of compensating for a decrease in NO function via recruitment of alternative pathways such as MEGJ, furthering the hypothesis that females are cardioprotected. Given that many studies have focused on improving the NO pathway in pathological states, but that this pathway appears to be the most commonly affected, it may be more beneficial to target remaining functioning pathways, such as the EDHF pathways, to improve vascular function. The determination of the mechanisms of compromised vascular function in different sexes may help to direct future treatments to more susceptible groups.

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**DISCLOSURES**

No conflicts of interest are declared by the authors.
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