Investigation of Vascular Responses in Endothelial Nitric Oxide Synthase/Cyclooxygenase-1 Double-Knockout Mice

Key Role for Endothelium-Derived Hyperpolarizing Factor in the Regulation of Blood Pressure in Vivo

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Background—Endothelium-dependent dilatation is mediated by 3 principal vasodilators: nitric oxide (NO), prostacyclin (PGI2), and endothelium-derived hyperpolarizing factor (EDHF). To determine the relative contribution of these factors in endothelium-dependent relaxation, we have generated mice in which the enzymes required for endothelial NO and PGI2 production, endothelial NO synthase (eNOS) and cyclooxygenase-1 (COX-1), respectively, have been disrupted (eNOS−/− and COX-1−/− mice).

Methods and Results—In female mice, the absence of eNOS and COX-1 had no effect on mean arterial blood pressure (BP), whereas BP was significantly elevated in eNOS−/−/COX-1−/− males compared with wild-type controls. Additionally, endothelium-dependent relaxation remained intact in the resistance vessels of female mice and was associated with vascular smooth muscle hyperpolarization; however, these responses were profoundly suppressed in arteries of male eNOS−/−/COX-1−/− animals. Similarly, the endothelium-dependent vasodilator bradykinin produced dose-dependent hypotension in female eNOS−/−/COX-1−/− animals in vivo but had no effect on BP in male mice.

Conclusions—These studies indicate that EDHF is the predominant endothelium-derived relaxing factor in female mice, whereas NO and PGI2 are the predominant mediators in male mice. Moreover, the gender-specific prevalence of EDHF appears to underlie the protection of female eNOS−/−/COX-1−/− mice against hypertension. (Circulation. 2005;111: 796-803.)

Key Words: endothelium ■ hypertension ■ nitric oxide
ic effects in vivo. Moreover, NO and PGI₂ can also elicit dilatation via hyperpolarization of vascular smooth muscle,¹²,¹³ which results in difficulties in separating the actions of these endothelium-derived vasodilators. Thus, the physiological significance of EDHF remains unsubstantiated, and its potential as a novel therapeutic target has yet to be explored.

Herein, we describe the vascular phenotype of mice with targeted disruption of both the predominant endothelial isoforms of NOS (eNOS) and COX (COX-1) and demonstrate that this “EDHF mouse” is the ideal model with which to investigate the identity and physiological role of EDHF, both in vitro and in vivo. Moreover, using this model, we describe a novel, gender-specific, cardioprotective role for EDHF that may account for the relative protection of premenopausal females from cardiovascular disorders such as hypertension and atherosclerosis.

Methods

Generation of eNOS⁻/⁻/COX-1⁻/⁻ Mice

Animals with targeted disruption of the eNOS (eNOS⁻/⁻) and COX-1 (COX-1⁻/⁻) genes, both on a C57BL6 background, were mated to yield animals heterozygous for both loci (eNOS⁻/⁺/COX-1⁻/⁺). These animals were then crossed with eNOS⁻/⁻ mice to yield eNOS⁻/⁻/COX-1⁻/⁻, which in turn were brother-sister mated to yield mice doubly deficient in both genes (eNOS⁻/⁻/COX-1⁻/⁻, or dKO). The offspring resulted in a 1:1 population of eNOS⁻/⁻/COX-1⁻/⁻ and dKO, which were genotyped by polymerase chain reaction (Figure 1). Litters from dKO×dKO matings were stillborn.

Genotyping

DNA was extracted with a DNeasy tissue kit (Qiagen). The presence of eNOS, neomycin (NEO), or COX-1 was determined by polymerase chain reaction with the following primers (Figure 1): eNOS⁻/⁺ forward: 5'-GGTTGTTGCTGCAGCACTG-3'; eNOS⁻/⁻ reverse: 5'-GCAGCAACGCTGGTGAAC-3'; NEO forward 5'-GCGATCTGGTGTGCTGAC-3'; NEO reverse 5'-GAGGCGATGCGCTGGAATC-3'; COX-1⁻/⁺ forward: 5'-GCAGCCCTCTGTTCTCACACATAC-3'; COX-1⁻/⁻ reverse: 5'-AATTGTGATTTGCTAGTGCC-3'.

Resistance Artery Experiments

Male and female mice, 8 to 12 weeks old (weight 20 to 27 g), were killed by neck dislocation; the mesentery was removed and placed in physiological salt solution (PSS) composed of (in mmol/L) NaCl 119, KCl 4.7, CaCl₂ 2.5, MgSO₄ · 7H₂O 1.2, NaHCO₃ 25, KH₂PO₄ 1.2, and glucose 5.5. Second-order arteries were mounted in a tension myograph (Danish Myotechnology) and bathed in PSS (37°C gassed with 5% CO₂ in O₂). After equilibration, six vessels were stimulated with thromboxane A₂ mimetic U46619 (1 μmol/L) until responses were reproducible. Vessels were precontracted with a submaximal concentration of U46619 (50% to 70% of response to 1 μmol/L), and the endothelium was stimulated by a single application of ACh (1 μmol/L) or BK (1 μmol/L). Full concentration-response curves were not constructed because typically these small arteries do not hold a stable level of tone for prolonged periods.¹⁶

The contribution of NO and prostanooids was assessed with the NOS and COX inhibitors N⁶-nitro-L-arginine methyl ester (L-NAME; 300 μmol/L, 30 minutes) and indomethacin (5 μmol/L, 30 minutes), respectively. Involvement of EDHF was determined with agents thought to block EDHF release and activity: KCi (30 mmol/L),¹⁸ apamin (100 nmol/L) and charybdotoxin (ChTx; 100 nmol/L),¹⁹ or Ba²⁺ (30 μmol/L) and ouabain (1 μmol/L). In some arteries, the endothelium was removed by passing a human hair twice through the lumen.¹⁹ In some experiments, tension and membrane potential were measured simultaneously as described previously.²¹ Small mesenteric arteries from female dKO mice were stimulated with a single application of ACh (10 μmol/L) either in the absence of or after precontraction with U46619 (10 to 20 nmol/L). Membrane potential was measured with aluminum silicate microelectrodes with a resistance of 50 to 100 MΩ, when filled with 3 mol/L KCl, and recorded on an Intra-676 amplifier (WPI Inc).

Conduit Artery Experiments

Segments of thoracic aortas were mounted in organ baths under a basal tension of 0.3g. Relaxation response curves to ACh were constructed in phenylephrine (PE; 10 to 30 μmol/L, EC₅₀)-precontracted vessels in the absence or presence of L-NAME and indomethacin. At the end of each experiment, sodium nitroprusside (SNP; 1 μmol/L) was applied to test the integrity of the vascular smooth muscle.

Measurement of EDHF Responses In Vivo

Female and male dKO mice were anesthetized with isoflurane (2%). The left common carotid artery was cannulated for measurement of arterial BP and the right femoral artery for drug administration. BK was used for examination of EDHF responses in vivo, because unlike ACh, it has little direct cardiac activity. BK (0.1 to 10 μg/kg) and SNP (3 μg/kg) were given as single bolus doses of 50 μL. Responses were calculated as the maximum change in BP minus the response to bolus injection of 50 μL of saline.

Measurement of BP in Conscious Mice

Systolic BP (SBP) was measured by tail-cuff plethysmography (XBP 1000, Kent Scientific) in conscious animals in a heated room (26°C to 27°C). Mean arterial BP (MAP) in conscious female dKO and wild-type (WT) animals was also assessed with a tethered mouse model.²² After a 12-hour recovery period after surgery, MABP was recorded over the following 12 hours.

Plasma 6-Keto-Prostaglandin F₁α Measurement

Blood was collected from the tail vein and mixed 1:10 with 3.15% (vol/vol) citrate. Plasma PGI₂ was assessed by measuring PGI₂ metabolite 6-keto-prostaglandin F₁α by enzyme immunoassay (Amersham Biosciences).

Materials and Solutions

Drugs were purchased from Sigma, except U46619 (Biomol) and apamin and charybdotoxin (Alomone Labs). Stock solutions were made in water, except U46619 (ethanol), ouabain (DMSO), and indomethacin (5% NaHCO₃).
Statistical Analysis

Relaxation responses were calculated as percentage reversal of preconstriction. Data shown are mean±SEM. Data were analyzed by 1- or 2-way ANOVA followed by Bonferroni multiple comparison test.

Results

Resistance Artery Studies

Endothelium-Dependent Relaxation in Arteries of WT Animals

There was no significant difference in diameter or basal tension of arteries between the groups of mice (Table). Furthermore, the contractile response to U46619 (1 μmol/L) and the relaxant response to the endothelium-independent vasodilator SNP (1 μmol/L) were similar between groups. Together, these data suggest no gross morphological differences between arteries of the different genotypes.

To compare endothelial function between genotypes, we assessed responses to ACh. ACh caused relaxation of 84.3±5.1% (n=4; Figure 2A) in mesenteric resistance arteries of female WT mice. Pharmacological blockade of NOS or COX individually did not alter this response (n=4, P>0.05; data not shown); however, combined NOS/COX inhibition attenuated ACh-induced relaxation by 29.3±4.0% (n=4; Figure 2A). ACh-induced relaxation in arteries from male WT mice was not significantly different from female vessels.

Comparison of Structural and Functional Properties of Mesenteric Resistance Arteries From Mice With Different Genotypes

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<th>Male</th>
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<td>WT</td>
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<tr>
<td>Diameter, μm</td>
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<td>168±8.00</td>
<td>183±6.40</td>
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<td>Basal tension, mN</td>
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<td>2.6±0.16</td>
<td>2.7±0.12</td>
<td>2.1±0.25</td>
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<td>Contraction to 1 μmol/L U46619, mN</td>
<td>11.1±1.70</td>
<td>11.4±0.80</td>
<td>9.8±0.48</td>
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<td>Relaxation to 1 μmol/L SNP, %</td>
<td>90.6±2.30</td>
<td>92.5±2.41</td>
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<td>181±7.60</td>
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n>12. Data are mean±SEM.

Figure 2. EDHF is predominant in female but not male resistance arteries. A. Relaxation to ACh in absence and presence of endothelium, inhibitors of NO and PGJ2, or inhibitors of EDHF (apamin/ChTx, or K+, or Ba2+/ouabain). *P<0.05 vs control (n=4 to 8). B. Relaxation of arteries of male WT or COX−1−/− animals is abolished by inhibition of NO synthesis. In arteries of eNOS−/− animals, responses to ACh are abolished by inhibition of PGJ2 release. ACh produces weak relaxation of arteries from male dKO mice (n=4 to 7). C. ACh-induced relaxation of dKO mesenteric resistance arteries is associated with change in membrane potential. D. Endothelium-dependent relaxation of female aortas is mediated exclusively by NO. Application of ACh produces concentration-dependent relaxation of aortic rings from WT and COX−−/− that is completely blocked by NO inhibition. Aortas that lack eNOS (eNOS−−/− or dKO) do not relax to ACh (n=4 to 7).
(71.4±5.1%; n=14, P>0.05 versus female arteries) but was abolished by NOS inhibition (Figure 2B).

Effect of eNOS Gene Disruption on Vascular Responses

ACh caused relaxation of 42.8±4.8% (n=5; Figure 2A) in mesenteric resistance arteries of female eNOS−/− mice. This response was unaffected by L-NAME or indomethacin treatment either alone or in combination (Figure 2A). Block of EDHF release and activity, using raised [K+] or Ba2+/ouabain, respectively, abolished relaxations to ACh in these arteries. In contrast, ACh produced only weak relaxation of mesenteric arteries from male eNOS−/− animals (26.1±4.7%; n=7; Figure 2B). This response was not affected by treatment with L-NAME but was markedly attenuated by indomethacin.

Effect of COX-1 Gene Disruption on Vascular Responses

ACh caused relaxation of 58.1±14.1% (n=4; Figure 2A) in mesenteric resistance arteries of female COX-1−/− animals. These responses were resistant to inhibition of NOS and COX but markedly attenuated by inhibitors of EDHF (ie, raised [K+] or Ba2+/ouabain). In contrast, ACh-induced relaxation of male COX-1−/− mesenteric arteries (45.5±11.9%; n=5; Figure 2B) was abolished by L-NAME (Figure 2B).

Vascular Responses in dKO Mice

ACh caused relaxation of 59.6±6.8% (n=13; Figure 2A) in mesenteric resistance arteries of female dKO mice. In accord with observations in female eNOS−/− and COX-1−/− arteries, ACh evoked responses were unaffected by combined NOS/COX blockade and were mediated exclusively by EDHF, because raised [K+], ChTx/apamin, or Ba2+/ouabain profoundly suppressed these responses (Figure 2A). BK (1 μmol/L) also relaxed these arteries, a response that was unaffected by combined L-NAME and indomethacin treatment (54.8±10.2% and 50.8±13.0%, respectively; n=4 for both; P<0.05 versus control). In contrast, ACh caused comparatively weak relaxations in mesenteric arteries of male dKO mice (Figure 2B) that were L-NAME and/or indomethacin resistant (data not shown). A relaxant response to BK was not observed in these arteries (data not shown).

Electrophysiology

Basal membrane potential of arteries of female dKO mice was −55.1±1.6 mV (n=6). In the absence of tone, ACh produced a hyperpolarization of −10±2.7 mV (n=3). Simultaneous recording of membrane potential and isometric tension showed that contraction to U46619 (≈EC50 10 to 20nmol/L) was associated with a depolarization of 14±3.8 mV. Application of ACh produced a repolarization of 9±3 mV (n=3; Figure 2C).

Conduit Artery Studies

Relaxation to ACh in aortas of male or female WT and COX-1−/− animals were entirely mediated by NO (n=4 to 7; see Figure 2D for female data). Aortas from male or female eNOS−/− and dKO animals did not relax in response to ACh (n=4 to 7; see Figure 2D for female data). There were no gender differences in the responses to ACh in aortas (male data not shown).

Assessment of EDHF Responses In Vivo and Correlation to Systemic BP

BK produced a dose-dependent decrease in BP in anesthetized female dKO mice (n=4; Figures 3A and 3C) but had little or no effect in male dKO animals (n=3; Figures 3B and C); however, SNP (3 μg/kg) produced similar hypotension in female (−29.2±0.7 mm Hg, n=4) and male (−24.0±4.84 mm Hg, n=3; P>0.05) dKO mice.

There was no significant difference in SBP, measured by tail-cuff plethysmography, between different genotypes of female mice compared with WT (Figure 4A). In marked contrast, SBP was significantly elevated in male eNOS−/− and dKO animals, respectively. There was no difference in SBP between male COX-1−/− and WT animals. In addition, continuous BP measurement in vivo in conscious animals demonstrated that MABP in female dKO and WT mice was not significantly different (Figure 4B).

Plasma 6-Keto-Prostaglandin F1α Measurement

6-Keto-PGF1α was substantially elevated in male eNOS−/− compared with WT mice (Figure 5); however, these enhanced levels were absent in male dKO mice. In contrast, 6-keto-PGF1α levels were equivalent to WT in female eNOS−/− animals. However, 6-keto-PGF1α levels were significantly lower in COX-1−/− and dKO animals, which confirms that COX-1 is the source of PGI2 in these animals and that there is no upregulation of COX-2 to compensate in both males and females. Western blot analysis of mesenteric arteries from animals of each genotype and gender confirmed a lack of COX-2 expression (data not shown).

Discussion

Herein, we demonstrate a crucial role for EDHF in maintaining normal levels of BP and highlight a potential cardioprotective effect of this factor in females. These novel physiological roles for EDHF are supported by several observations. The targeted disruption of both eNOS and COX-1 genes results in elevated BP of male, but not female, mice. Moreover, significant vasodilator endothelial function, determined both in vitro and in vivo, remains in female, but not male, dKO animals. Hence, the generation of this “EDHF mouse” has created an ideal model to characterize the identity and biological actions of EDHF in vivo and in vitro (without the need for pharmacological inhibitors of NOS and COX) and revealed novel, gender-specific cardioprotective functions of this mediator. Further characterization of the vascular physiology and pathology of this animal is likely to identify new therapeutic targets to treat cardiovascular disease.

EDHF Is the Predominant Endothelium-Derived Relaxing Factor in Female Mice

Using specific gene-knockout technology, we were able to directly assess the involvement of eNOS and COX-1 in endothelium-dependent relaxation of resistance arteries. The results of this study show a clear distinction between the mediators involved in female and male arteries. In female WT mesenteric small arteries, combined NOS and COX inhibition suppressed ACh-induced relaxation by only 30%, which indicates a predominant role for EDHF in this response.
Disruption of either eNOS or COX-1, or both genes, resulted in a residual EDHF-dependent relaxation of between 50% and 90% of that in WT animals. In addition, BK also caused NO- and PGI2-independent relaxation of female dKO arteries, which implicates EDHF as the common mediator of endothelium-dependent dilatation in these mice.

Confirmation of EDHF as the mediator of these non-NO, nonprostanoid responses was achieved with classic inhibitors of EDHF release and activity. A definitive feature of EDHF-mediated responses is the sensitivity to combined inhibition of endothelial SKCa (with apamin) and IKCa (with charybotoxin), which is thought to inhibit EDHF responses by blocking the passage of EDHF from the generating endothelial cell to the target underlying smooth muscle. Pretreatment with these K+ channel blockers, or mechanical removal of the endothelium, virtually abolished relaxation to ACh in female dKO mesenteric arteries. Similarly, elevation of extracellular [K+]18 eliminated non-NO, nonprostanoid relaxations in female dKO arteries, which demonstrates the crucial role of endothelial SKCa (with apamin) and IKCa (with charybotoxin), which is thought to inhibit EDHF responses by blocking the passage of EDHF from the generating endothelial cell to the target underlying smooth muscle.19 Pretreatment with these K+ channel blockers, or mechanical removal of the endothelium, virtually abolished relaxation to ACh in female dKO mesenteric arteries. Similarly, elevation of extracellular [K+]18 eliminated non-NO, nonprostanoid relaxations in female dKO arteries, which demonstrates the crucial role of

Figure 3. BK reduces BP in female but not male dKO mice in vivo. Original trace depicting typical responses to bolus injections of BK in (A) female and (B) male dKO mice. C, Mean dose-dependent decrease in BP to BK in female (n=4) and male dKO mice (n=3). **Significantly different (P<0.01) from female.

Figure 4. Female mice are not hypertensive. A, Measurement of systolic BP by tail-cuff plethysmography in conscious female and male mice (n=6 to 9). *Significantly different (P<0.01) from corresponding WT and female. B, Measurement in vivo of MABP in conscious female dKO and WT mice (n=5).
hyperpolarization in these responses. Indeed, direct measurement of membrane potential demonstrated that ACh hyperpolarizes smooth muscle of arteries from female dKO animals in the absence of a constrictor and that relaxations of arteries contracted (and depolarized) with U46619 occur simultaneously with a repolarization of membrane potential. Moreover, concomitant blockade of KIR and Na+/K+-ATPase by Ba²⁺ and ouabain, respectively (believed to underlie EDHF-mediated hyperpolarization of vascular smooth muscle⁶⁻²³), blocked relaxant responses to ACh in female arteries of all genotypes. Together, these findings demonstrate that relaxant responses to endothelium-dependent stimuli, irrespective of genotype, are dependent on EDHF and involve smooth muscle hyperpolarization.

**Male Mice Exhibit Dramatically Less EDHF Activity Than Female Mice**

Unlike resistance arteries of female mice, NO and PGI₂ are the predominant endothelium-derived dilators in resistance arteries of male mice. In male WT and COX-1⁻/⁻ animals, NO accounts almost entirely for endothelium-dependent relaxation to ACh. The finding that endothelium-dependent relaxation of eNOS⁻/⁻ arteries is markedly suppressed (≈75%) highlights the pivotal role of NO in male arteries. Coupled with the fact that responses to ACh were suppressed in COX-1⁻/⁻ male arteries (≈45%) compared with WT arteries, these data suggest that in male arteries, only weak compensatory mechanisms exist. In clear contrast, the absolute capacity of resistance arteries of females to relax to endothelium-dependent stimuli, irrespective of genotype, is largely maintained.

It is thought that one of the physiological roles of EDHF is to function as a salvage pathway in conditions of insufficient NO release and therefore that EDHF activity is only evident in the presence of NOS inhibition; however, in male eNOS⁻/⁻ mesenteric arteries, in which NO-mediated responses are absent, no EDHF activity is observed. Rather, COX activity partially compensates for the loss of NO, as demonstrated by the sensitivity of ACh-induced responses to indomethacin. This upregulation of COX activity is likely to be a general phenomenon in arteries of male eNOS⁻/⁻ mice, because relaxation of coronary²⁵ or superior mesenteric arteries¹⁷ to ACh and flow-mediated dilation of skeletal muscle arterioles²⁶ are also sensitive to COX inhibition. Indeed, in the present study, assay of plasma 6-keto-PGF₁α, a stable PGI₂ metabolite, demonstrated an elevation in the levels of this product in male eNOS⁻/⁻ mice. Western blot analysis of arteries taken from these animals showed no COX-2 expression, which suggests that the enhanced PGI₂ synthesis likely relates to elevated COX-1 rather than COX-2 activity. Because COX-1 activity appears to be upregulated in male eNOS⁻/⁻ mice, we speculated that in the absence of both eNOS and COX-1, EDHF activity would be induced or upregulated to normalize vasomotion. Surprisingly, ACh produced negligible relaxation of male dKO arteries.

This difference between endothelium-derived mediators in male and female mice suggests that sex hormones are likely to be important for EDHF activity. Indeed, there is evidence hinting at a link between estrogen and EDHF activity. EDHF-mediated responses to ACh,²⁷ shear stress,²⁸ ADP,²⁹ or Ca²⁺ ionophore³⁰ are all reduced by ovariectomy; however, E₂ is known to target several signal transduction pathways in the vasculature, including the muscarinic receptor M2 and M3 pathways,³¹ which suggests that alternative mechanisms may be responsible at least for the changes in EDHF activity in response to ACh. It is unlikely that this latter mechanism is at play in the present study, because BK also produced relaxation of dKO arteries that persisted in the presence of NOS and COX inhibition, which demonstrates that EDHF-mediated relaxation is a general phenomenon of these arteries and is not due to an increase in sensitivity to ACh.

Mechanisms by which estrogens might increase EDHF responses are unclear, but possibilities include induction or upregulation of an EDHF synthase or enhancement of EDHF signal-transduction pathways. Communication between endothelial and vascular smooth muscle layers via myoendothelial gap junctions has been implicated as an integral part of EDHF-mediated signaling.¹⁰,³² Interestingly, there is evidence that E₂ enhances the expression of connexin-43, one of the connexin protein components that form the gap junctions of resistance arteries, in ovariectomized rats.³⁰ Such changes may be one process by which estrogen stimulates changes in EDHF activity. In addition, we have recently demonstrated that CNP is an EDHF in mesenteric resistance arteries;³³ estrogen enhances transcription of CNP in mouse uterus,³³ and relaxations to CNP in female porcine coronary artery are greater than in males,³⁴ which suggests that increased CNP release/activity may account for enhanced EDHF activity in female mice. Further studies investigating the effects of ovariectomy on vascular responses in these DKO mice are required to more fully examine the mechanisms of the gender-specific EDHF response. An alternative explanation for the apparent gender differences may be related to an effect of male sex hormones rather than estrogen. Recent evidence demonstrated that testosterone suppresses EDHF responses in rat middle cerebral arteries.³⁵ Whether it is the presence of estrogen upregulating EDHF activity or the absence of a suppressive influence of testosterone on EDHF in the female DKO mice is uncertain and warrants further investigation.
In contrast to mesenteric resistance arteries, there is no gender difference in relaxations of aortic rings to ACh. In agreement with previous studies, we found that NO release accounts for ACh-induced relaxation of thoracic aortas (i.e., responses in WT or COX-1/-/- are abolished by NOS inhibition, and responses are absent in eNOS/-/- and dKO mice). These observations are consistent with a fundamental role of EDHF in resistance arteries, whereas NO is the sole mediator of endothelium-dependent relaxation in conduit arteries.

**Female dKO Mice Are Not Hypertensive**

A substantial body of evidence demonstrates a lower incidence of hypertension and heart disease among premenopausal women than among age-matched men. This difference has been attributed to the effects of estrogen, although this theory has come under considerable scrutiny of late and is highly controversial. Nevertheless, evidence in a number of animal models supports the concept that the BP-lowering effects of estrogen are due in part to its ability to stimulate eNOS-derived NO release (for review, see Mendelsohn and Karas); whether NO is solely responsible for this specific protection is uncertain. Transgenic mice that lack the estrogen receptor [ER]-β, which is more prevalent in females than males, are hypertensive; however, evidence supports a predominant role for ER-α in estrogen-mediated NO signal transduction. Together, these data suggest that the effects of estrogen on BP may not be entirely dependent on NO release. Indeed, the BP-lowering effects of E2 are unaltered by targeted disruption of eNOS. In the present study, we have circumvented the need to use pharmacological inhibitors by creating eNOS/COX-1 dKO mice and thereby have, for the first time, permitted characterization of the physiological role of EDHF in vivo. Administration of BK in female dKO animals produced a dose-dependent decrease in MABP. In contrast, BK had little or no effect on MABP in male dKO animals. Such an observation represents the first definitive demonstration of an EDHF-evoked change in BP in vivo and indicates that EDHF is an important modulator of vascular tone physiologically. Moreover, the present studies show a clear EDHF-mediated regulation of BP apparent in females but not males.

**Implications for Study of EDHF and Novel Therapeutic Targets**

By generating animals that lack both eNOS and COX-1, i.e., the EDHF mouse, we have produced animals that display substantial in vitro and in vivo EDHF activity. These animals are an invaluable new tool for the study of the nature and actions of EDHF. The results of the present study demonstrate a clear link between gender and EDHF-mediated responses in resistance arteries. Stimulation of endothelial cells of female arteries, regardless of their genotype, results in significant EDHF activity. Moreover, this ability to release EDHF appears to be integral to BP regulation. Together, these findings suggest that EDHF may contribute to the lower incidence of cardiovascular disease in premenopausal women.

**Acknowledgments**

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**References**


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