Responses of carotid artery in mice deficient in expression of the gene for endothelial NO synthase

FRANK M. FARACI, CURT D. Sigmund, EDWARD G. SHESELY, NOBUYO MAEDA, AND DONALD D. HEISTAD (With the Technical Assistance of Kristen Rummelhart)

Departments of Internal Medicine, Pharmacology, and Physiology, Cardiovascular Center, University of Iowa College of Medicine, Iowa City, Iowa 52242; and Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

Faraci, Frank M., Curt D. Sigmund, Edward G. Shesely, Nobuyo Maeda, and Donald D. Heistad. Responses of carotid artery in mice deficient in expression of the gene for endothelial NO synthase. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H564–H570, 1998.—We examined the hypotheses that responses to acetylcholine are impaired and responses to NO are enhanced in carotid artery from mice made deficient in endothelial nitric oxide synthase (eNOS) by gene targeting (eNOS-deficient mice). We also tested the hypothesis that deletion of one copy of the eNOS gene is sufficient to alter vascular responses. Vessels were studied in vitro from heterozygous (+/−) and homozygous (−/−) eNOS-deficient mice as well as wild-type [eNOS(+/+)] littermates.

After precontraction with prostaglandin F2α, acetylcholine produced marked relaxation of carotid arteries in eNOS(+/+) mice, with impaired vasorelaxation in eNOS(−/−) mice. For example, 1 µM acetylcholine relaxed carotid arteries by 55 ± 5% (mean ± SE) in eNOS(+/+) mice (n = 13) compared with 83 ± 3% in eNOS(+/+) mice (n = 14, P < 0.001 vs. +/+). In contrast, acetylcholine caused no relaxation in carotid arteries from eNOS(−/−) mice (P < 0.001 vs. +/+ and −/−).

Relaxation of the carotid artery in response to nitroprusside [a nitric oxide (NO) donor] was enhanced (P < 0.001) in eNOS-deficient mice. For example, in response to 10 nM nitroprusside, the carotid artery relaxed by 18 ± 2% in eNOS(+/+) mice (n = 14), 33 ± 2% in eNOS(+/+) mice (n = 13), and 47 ± 4% in eNOS(−/−) mice (n = 5). Thus relaxation of the carotid artery is impaired with acetylcholine and enhanced with the NO donor nitroprusside in eNOS-deficient mice. Enhanced responses to NO may represent a compensatory response expressed in the absence of eNOS. The findings that vascular responses to acetylcholine and NO are altered in eNOS(+/−) mice compared with those observed in eNOS(+/+) mice suggest a “gene-dosing” effect.

gene targeting; nitric oxide; acetylcholine; nitroprusside; soluble guanylate cyclase; N5-nitro-L-arginine; endothelial nitric oxide synthase

PRODUCTION AND RELEASE of nitric oxide (NO) by NO synthase (NOS) in endothelium are thought to play a major role in regulation of vascular tone (8, 14). Three isoforms of NOS, which are products of separate genes, have been identified and are designated as neuronal, inducible, and endothelial (eNOS) NOS (9, 14). We and others have used pharmacological inhibitors of NOS to examine the role of different isoforms of NOS in regulation of vascular tone (see Ref. 8 for review). A common approach is to use analogs of L-arginine, including N5-nitro-L-arginine (L-NNA), to inhibit activity of NOS. Although inhibitors of NOS are very useful in examination of the role of NO in vascular responses, a major limitation is that most agents nonselectively inhibit all isoforms of NOS (20, 25). There are no known selective inhibitors of eNOS.

Mice with targeted disruption of the gene encoding eNOS (11, 22) provide an opportunity to examine the role of eNOS in complex physiological systems. Through studies performed in eNOS-deficient mice, the role of the endothelial isoform of NOS can be defined without relying on pharmacological inhibitors that lack specificity for NOS isoforms.

Recent studies suggest that, in the presence of inhibition or impairment of eNOS (19, 23), compensatory mechanisms may regulate vascular tone. In mice that are deficient for the eNOS gene, dilatation of cerebral arteries in response to acetylcholine has been reported to be normal in magnitude but mediated by non-eNOS-dependent mechanisms (16). In contrast to cerebral arteries, relaxation of the aorta in response to acetylcholine is absent in eNOS-deficient mice (11).

The first goal of the present study was to examine the hypothesis that responses of the carotid artery to acetylcholine are absent in mice lacking the gene for eNOS. Because the previous study used only aorta, this represents the first study of a large muscular artery from eNOS-deficient mice. The study was performed in vitro, which allows examination of mechanisms without the potential influence of NOS in surrounding tissue. As part of these experiments, we also examined mechanisms that mediate responses to acetylcholine in carotid arteries from normal [wild-type, eNOS(+/+)] mice. Because mechanisms that mediate endothelium-dependent responses in mouse carotid arteries have not been studied previously, it was important to exclude the possibility that non-NO-dependent mechanisms may mediate relaxation to acetylcholine.

Relaxation of blood vessels in response to NO or NO donors is typically enhanced after acute inhibition of NOS (1, 6, 7, 17, 23). Thus the second goal of the present study was to examine the hypothesis that relaxation of the carotid artery in response to nitroprusside (an NO donor) is enhanced in mice lacking the gene for eNOS.

To our knowledge, previous studies have not examined vascular responses in any heterozygous gene-targeted animals. Thus our third goal was to examine the hypothesis that deletion of one copy of the eNOS gene was sufficient to alter vascular responses. Altered vascular responses in eNOS(+/−) mice compared with eNOS(+/+) mice would suggest that a “gene-dosing” effect was present for eNOS gene expression.
METHODS

Animals. eNOS-deficient mice were produced as described previously (22). We interbred eNOS(+/−) mice to generate eNOS(+/+), eNOS(+−), and eNOS(−−) mice within the same litter. This approach allowed us to use eNOS(+−) littersmates as controls. For all studies in which we compared responses of eNOS(+/+), eNOS(+−), and eNOS(−−) mice, we used littermates as the wild-type control. For these studies, each mouse was genotyped. For some studies of mechanisms of vasorelaxation in wild-type mice, C57BL/6J mice were obtained from Jackson Laboratories.

Genotyping of the animals was performed by Southern blotting DNA from tail biopsies. High-molecular-weight genomic DNA was isolated using a random primer-labeled 1.4-kb eNOS cDNA probe that was described previously (22). Briefly, 10-µg samples were digested with BamHI, separated on 0.8% agarose gels, and then transferred to nylon-supported nitrocellulose. The blots were then hybridized using a random primer-labeled 1.4-kb eNOS cDNA probe that was described previously (22). A 5.3-kb fragment was diagnostic of the endogenous eNOS locus, and a 6.4-kb fragment was diagnostic of the targeted allele.

Vascular ring preparation. Mice were anesthetized with pentobarbital sodium (75–100 mg/kg ip), and both carotid arteries were quickly removed and placed in Krebs buffer with the following ionic composition (mmol/l): 118.3 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, and 11 glucose. Loose connective tissue in the adventitia was removed, and each carotid artery was cut into two rings (3–4 mm in length). Vascular rings were suspended in an organ bath containing 25 ml Krebs solution maintained at 37°C.

Protocols. Vessels were contracted submaximally (30–55% of maximum) using prostaglandin F2α (PGF2α). In initial studies, we found that PGF2α produces stable and reproducible contraction of carotid arteries. After reaching a stable contraction plateau, dose-response curves were obtained for acetylcholine or sodium nitroprusside. Dose-response curves were obtained for each agonist. Acetylcholine was used to assess endothelial function, and nitroprusside was used as an NO donor to examine direct effects on smooth muscle. We have used these techniques for studies of mouse vessels in vitro previously (3).

We examined mechanisms that mediate relaxation to acetylcholine and nitroprusside in carotid arteries from eNOS(+−) mice. For these studies, responses to acetylcholine and nitroprusside were obtained in the absence and presence of L-NNA (100 µM), an inhibitor of NOS, or 1H-(2,4)oxadiazolo(4,3-α)quinoxalin-1-one (ODQ, 10 µM), an inhibitor of soluble guanylate cyclase (18, 24). Between each concentration-response curve, the vessels were washed at least three times with fresh Krebs buffer and allowed to reequilibrate. Vasoconstrictor responses to PGF2α tended to be enhanced in arteries taken from eNOS(+−) mice and treated with L-NNA or ODQ. Thus higher concentrations of PGF2α were sometimes used in untreated vessels to produce similar contraction to that seen in vessels treated with either inhibitor. Similarly, vasoconstrictor responses to PGF2α tended to be enhanced in arteries taken from eNOS(−−) and eNOS(+−) mice compared with arteries from eNOS(+−) mice, and higher concentrations of PGF2α were sometimes used in vessels from eNOS(+−) mice to produce contraction that was similar to that seen in vessels from eNOS(+−) and eNOS(−−) mice.

At the end of each experiment, we examined responses of carotid arteries to the thromboxane analog 9,11-dideoxy-11α, 9α-epoxy-methanoprostanoglandin F2α (U-46619) to determine maximal contractile responses. In preliminary experiments, we found that U-46619 produces greater maximal contraction of mouse carotid arteries than high concentrations of KCl. Maximum contraction was similar in carotid arteries from eNOS(+−), eNOS(+/−), and eNOS(−−) mice (0.66 ± 0.06, 0.65 ± 0.05, and 0.71 ± 0.10 g, respectively).

Statistical analysis. All data are expressed as means ± SE. Statistical analysis of relaxation responses to acetylcholine and nitroprusside were expressed as the percent relaxation from the amount of preconstriction produced by PGF2α. Comparisons were made using analysis of variance followed by Bonferroni's multiple comparison test. Statistical significance was accepted at P < 0.05.

RESULTS

Vascular responses in eNOS(+−) mice. In vessels from normal mice, acetylcholine produced concentration-dependent relaxation (Figs. 1 and 2). In normal mice, relaxation of the carotid artery in response to
Acetylcholine was markedly attenuated by L-NNA (100 µM; Figs. 1 and 2) or ODQ (Figs. 3 and 4). For example, maximum vasorelaxation in response to acetylcholine was 85 ± 2 and 10 ± 3% in the absence and presence of ODQ, respectively (Fig. 4). These findings suggest that relaxation in response to acetylcholine is mediated almost exclusively by NO and activation of soluble guanylate cyclase in the mouse carotid artery.

Relaxation of the carotid artery in response to nitroprusside was enhanced in the presence of L-NNA (Fig. 2). In contrast to L-NNA, ODQ produced marked inhibition of vasorelaxation in response to nitroprusside (Fig. 4). For example, relaxation in response to 3 µM nitroprusside was 80 ± 4 and 5 ± 3% in the absence and presence of ODQ, respectively (Fig. 4). These findings suggest that relaxation of the mouse carotid artery in response to nitroprusside is mediated by activation of soluble guanylate cyclase.

Vascular responses to acetylcholine in eNOS(+/−) mice. In eNOS(+/−) mice, the lower concentrations of acetylcholine produced relaxation of the carotid artery that was similar to that observed in eNOS(+/+) mice (Figs. 5 and 6). However, higher concentrations of acetylcholine produced less relaxation in eNOS(+/−) mice than in eNOS(+/+) mice. For example, 1 µM acetylcholine produced 55 ± 5 and 83 ± 3% relaxation in eNOS(+/+) and eNOS(+/−) mice, respectively [P < 0.001 vs. eNOS(+/+); Fig. 6]. Furthermore, higher concentrations of acetylcholine often produced contraction of the carotid artery in eNOS(+/−) mice (Fig. 5). In these studies of carotid arteries, contraction to higher concentrations of acetylcholine was observed in only 1 out of 14 eNOS(+/+) mice but was seen in 9 out of 13 eNOS(+/−) mice. Altered responses to acetylcholine suggest that endothelial function is altered in eNOS(+/−) mice.

Vascular responses to sodium nitroprusside. Sodium nitroprusside caused marked relaxation of the carotid
artery from eNOS(+/+) mice, eNOS(+/-), and eNOS(-/-) mice (Fig. 8). Relaxation of the carotid artery in response to nitroprusside was augmented in eNOS-deficient mice, particularly in eNOS(-/-) mice (Fig. 8). For example, in response to 10 nM nitroprusside, the carotid artery relaxed by 18 ± 6% in eNOS(+/+) mice, 33 ± 2% in eNOS(+/+) mice, and 47 ± 4% in eNOS(-/-) mice. In contrast to responses to acetylcholine in eNOS(+/-) mice, transient contractions of the carotid artery were not observed during application of nitroprusside in any group of mice. Maximum relaxation to 10 µM nitroprusside was similar in eNOS(+/+) mice, eNOS(+/-), and eNOS(-/-) mice (90 ± 6, 89 ± 3, and 93 ± 4%, respectively). Thus relaxation of the carotid artery to low concentrations of the NO donor nitroprusside is increased in eNOS(+/-) and eNOS(-/-) mice.

**DISCUSSION**

There are several major new findings in the present study. First, the pharmacological data obtained using l-NNA and ODQ suggest that relaxation of the mouse carotid artery to acetylcholine is mediated normally by NO and activation of soluble guanylate cyclase. Absence of relaxation to acetylcholine in eNOS(-/-) mice provides direct evidence that relaxation of the carotid artery in response to acetylcholine is mediated by eNOS. Second, altered responses to acetylcholine suggest that altered endothelial function is present in eNOS(+/-) mice. Third, data obtained with ODQ suggest that relaxation of the mouse carotid artery to nitroprusside is mediated by activation of soluble guanylate cyclase. Interestingly, vasorelaxation in response to nitroprusside is enhanced in both eNOS(+/+) and eNOS(-/-) mice. The finding that responses to nitroprusside are increased in eNOS(+/-) mice also suggests that deletion of one copy of the eNOS gene is sufficient to alter vascular responses. Thus responses to both an endothelium-dependent and -independent agonist are altered in eNOS(+/-) mice.

To our knowledge, the only study that examined responses of isolated vessels from eNOS-deficient mice [eNOS(-/-)] is that of Huang et al. (11), who studied the aorta in vitro. Our findings confirm and extend results obtained previously using aorta and indicate that acetylcholine does not produce relaxation in the carotid artery (a large muscular artery) from eNOS(-/-) mice. In addition, we obtained two major new findings
in vessels from eNOS-deficient mice. The first finding is that deletion of one copy of the eNOS gene is sufficient to alter vascular responses to the endothelium-dependent agonist acetylcholine. The second finding is that relaxation of the carotid artery to NO is enhanced in eNOS-deficient mice and that deletion of one copy of the eNOS gene is sufficient to alter vascular responses to NO.

Relaxation of carotid artery to acetylcholine in eNOS-deficient mice. Although the use of pharmacological inhibitors of NOS is useful in examination of the role of NO in blood vessels, a major limitation is that these analogs of L-arginine nonselectively inhibit all isoforms of NOS (14, 20, 25). Importantly, there are no selective inhibitors of eNOS. Studies of vessels in eNOS-deficient mice allow the definition of the contribution of the endothelial isoform of NOS in producing relaxation in response to vasoactive stimuli, without relying exclusively on inhibitors that lack specificity for NOS isoforms.

In the present study, we found that relaxation of the carotid artery in response to acetylcholine is absent in eNOS(−/−) mice. This response is in marked contrast to that observed in eNOS(+/+) mice. In eNOS(−/−) and eNOS(+/−) mice, acetylcholine often produced contraction of the carotid artery. This latter effect could be the result of direct contractile effects of acetylcholine on vascular muscle or possible release of an endothelium-derived contracting factor. These findings provide direct evidence that relaxation of the carotid artery to acetylcholine is mediated by eNOS.

Few studies have examined endothelium-dependent relaxation of mouse blood vessels in vitro. In the aorta from mice, we (3) and others (11, 13, 21) have shown that relaxation in response to acetylcholine is attenuated substantially by inhibitors of NOS, suggesting that the response is mediated by NO (endothelium-derived relaxing factor). All previous studies have examined responses in aorta, and this is the first study to examine responses of carotid or other muscular arteries from mice. In normal mice, acetylcholine produced relaxation of the carotid artery, which was markedly attenuated by the NOS inhibitor L-NNA. Thus genetic evidence obtained with eNOS(−/−) mice is consistent with pharmacological data obtained using L-NNA in eNOS(+/+) mice. In addition, vasorelaxation in response to acetylcholine was almost completely inhibited by ODQ, indicating that relaxation of normal carotid artery in response to endogenously produced NO (endothelium-derived relaxing factor) is mediated by activation of soluble guanylate cyclase.

In eNOS(+/+) mice, low concentrations of acetylcholine produced relaxation of the carotid artery that was similar to that observed in eNOS(+/+) mice. Higher concentrations of acetylcholine produced less relaxation in eNOS(+/+) mice than in eNOS(+/−) mice. The data suggest that deletion of one copy of the eNOS gene is sufficient to alter endothelial function in the carotid artery. Differences in vascular responses to acetylcholine in the eNOS(+/−) and eNOS(+/+) mice suggest a gene-dosing effect. Previous studies have provided evidence for gene dosing in relation to regulation of blood pressure (12, 15). To our knowledge, the present study provides the first...
evidence to suggest that gene dosing may also be present for vascular responses.

Relaxation of carotid artery to nitroprusside in eNOS-deficient mice. NO can potentially produce relaxation of vascular muscle by guanylate cyclase-dependent or by guanylate cyclase-independent mechanisms. In the present study, vasorelaxation to nitroprusside was attenuated markedly by ODQ, a recently developed inhibitor of soluble guanylate cyclase that appears to be very specific (18, 24). For example, ODQ inhibits vasorelaxation in response to acetylcholine and nitroprusside but not adenosine or an analog of guanosine 3',5'-cyclic monophosphate (24). The efficacy of ODQ suggests that relaxation of mouse carotid artery in response to nitroprusside is mediated essentially entirely by activation of soluble guanylate cyclase.

Relaxation of the carotid artery in response to low concentrations of nitroprusside was augmented in eNOS(+/−) and eNOS(−/−) mice. Thus relaxation to the NO donor nitroprusside is augmented in eNOS-deficient mice, perhaps as a compensatory response to reductions in the amount of eNOS in blood vessels. The finding that vasorelaxation in response to nitroprusside is enhanced in eNOS-deficient mice is similar to results published by us (6, 7, 17) and others (1, 23) in which relaxation of cerebral vessels in response to NO or NO donors is enhanced after acute pharmacological inhibition of NOS with agents such as L-NNa. In the present study in eNOS(+/+) mice, we also observed enhancement of responses of the carotid artery to nitroprusside after treatment with L-NNa. Thus genetic evidence obtained with eNOS-deficient mice is consistent with pharmacological evidence obtained using L-NNa in eNOS(+/+) mice. Similarities in findings suggest that enhanced vasorelaxation in response to NO donors obtained in previous studies (1, 6, 7, 17, 23) was not due to a nonspecific effect of pharmacological inhibitors of NOS. The finding that relaxation of the carotid artery to nitroprusside was enhanced in eNOS(+/−) mice compared with eNOS(+/+) mice is additional evidence that deletion of one copy of the eNOS gene is sufficient to alter vascular responses.

Compensatory mechanisms in eNOS-deficient mice. Both our genetic and pharmacological data indicate that relaxation of the carotid artery in response to acetylcholine is mediated by eNOS. The finding that acetylcholine does not produce relaxation in eNOS(−/−) mice indicates that formation of a non-NO endothelium-derived hyperpolarizing factor does not contribute to endothelium-dependent relaxation. After selective deletion of a gene, it is not uncommon for such gene-targeted animals to display no or minimal differences in phenotype (26). Such findings may suggest the presence of redundant or compensatory mechanisms after selective gene deletion. In pial arterioles of eNOS(−/−) mice, responses to acetylcholine are thought to be mediated by neuronal NOS (16).

Although expression of compensatory mechanisms can occur in gene-deficient mice, we found no evidence for such compensation in the carotid artery with regard to responses to acetylcholine. Because NO is thought to inhibit production or release of endothelium-derived hyperpolarizing factor in the carotid artery (2), eNOS-deficient mice seemed to be an ideal model in which to test for the presence of non-NO endothelium-dependent relaxation (such as vasorelaxation mediated by endothelium-derived hyperpolarizing factor). We found, however, no evidence for a non-NO endothelium-derived relaxing factor in mouse carotid artery. Our data, which suggest that responses of the carotid artery to acetylcholine are mediated exclusively by eNOS and soluble guanylate cyclase, are consistent with a recent study using rabbit carotid artery (4). Nevertheless, our findings do not exclude the possibility that NO itself may function as an endothelium-derived hyperpolarizing factor in the carotid artery, as suggested previously (4).

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Address for reprint requests: F. M. Faraci, Department of Medicine, University of Iowa Hospitals and Clinics, Iowa City, IA 52242-1081.

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