Quantification of mRNA for Endothelial NO Synthase in Mouse Blood Vessels by Real-Time Polymerase Chain Reaction

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Abstract—The mouse is useful in studies of vascular biology because of its well-defined genetics and because the mouse genome can be manipulated. However, because only small amounts of mRNA can be extracted from blood vessels, the quantification of gene expression in individual mice is difficult. Endothelial NO synthase (eNOS) plays a major role in the regulation of vascular tone and growth. In addition, there appear to be sex differences in the production of NO under basal conditions in mouse aortas. The goals of this study were to develop a real-time polymerase chain reaction (PCR) method to quantify eNOS mRNA in blood vessels from mice and to examine eNOS mRNA levels in vessels from male and female mice. Blood vessels were isolated from C57BL/6 mice. Total RNA from individual mice was isolated and reverse-transcribed. The number of molecules of eNOS mRNA (after reverse transcription) was determined against cDNA standards, with 18S rRNA used as a control for RNA input and reverse-transcription efficiency. When expressed as copy numbers per nanogram of total RNA or as the ratio of eNOS to 18S rRNA, eNOS mRNA was lower in the aortas of female mice than in those of male mice at 7 to 9 months of age. In contrast, no difference in eNOS mRNA was found in the aortas of 2-month-old mice. In addition, eNOS mRNA levels were similar in the carotid, cerebral, and coronary arteries. These findings provide the first quantitative measurements of eNOS mRNA by using real-time PCR in the vessels of mice and suggest age- and sex-related differences in the basal levels of eNOS mRNA in mice. In addition, the eNOS region that was used for real-time PCR was amplified and sequenced for monkeys and other species. With modifications, this region may be used to design real-time PCR for eNOS in other species. (Arterioscler Thromb Vasc Biol. 2002;22:lll–lll.)

Key Words: endothelial NO synthase • real-time polymerase chain reaction • mice • aortas • sex

Endothelial NO synthase (eNOS) plays a major role in the regulation of vascular tone and growth in large arteries and in the microcirculation. In addition to mechanisms that regulate enzyme activity acutely, eNOS is regulated at mRNA and protein levels. Steady-state levels of eNOS mRNA are increased by shear stress, estrogen, and exercise, but these levels are decreased during heart failure and possibly other disease states. A sensitive and reproducible method to quantify eNOS expression is of great importance in studies of vascular physiology and pathophysiology.

Quantification of relative eNOS mRNA levels has been accomplished previously by using Northern blotting, RNase protection assay, and competitive reverse transcription (RT)–polymerase chain reaction (PCR). Real-time PCR (after RT) is a new, sensitive, and accurate method for the quantification of mRNA levels and has had limited application in vascular studies. It requires a small amount of starting RNA and uses the initial (linear) rate of PCR (Cp) as the measure of the original amount of cDNA. With at least 5 orders of linear dynamic range, there is no need to analyze dilutions of each sample, and this, coupled with no need for post-PCR processing, makes the method particularly suitable for quantification of mRNA obtained from small samples, such as blood vessels from individual mice. Because the mouse genome can be manipulated by using transgenic and gene-targeting approaches, mice are used widely in studies of vascular biology.

In the present study, we report a real-time PCR method (after RT) to quantify eNOS mRNA in blood vessels of individual mice. Because the mouse aorta has been reported to produce more NO under basal conditions in males than in females, we quantified eNOS mRNA in blood vessels from males and females by using the real-time PCR method. To examine the usefulness of the method in much smaller blood vessels, we also studied cerebral and coronary arteries pooled from 2 or 3 mice.

Methods

Animals

C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Me) and maintained in the Animal Care Facility of the University of Iowa, in accordance with the Guide for the Care and
Use of Laboratory Animals (National Institutes of Health). Two groups of mice were studied: young (2-month-old) mice with body weights of 22±1 g (mean±SE, n=7) for males and 20±1 g (n=7) for females, and old (7- to 9-month-old) mice with body weights of 34±1 g (n=10) for males and 25±1 g (n=10) for females.

RNA Preparation

After pentobarbital anesthesia, thoracic aortas and carotid arteries were harvested, with careful removal of fat tissue surrounding the adventitia and blood inside the lumen. The vessels were minced with a razor blade and immediately added to a 1.5-mL tube (RNase-free containing 1.0 mL of TriReagent (Molecular Research Center). After extraction with 300 μL chloroform and centrifugation at 15,000 rpm for 45 minutes, the aqueous phase was transferred into a fresh 1.5-mL tube, and 20 μg glycogen (Roche Molecular) and 0.8 mL isopropanol were added to precipitate RNA. After centrifugation at 15,000g for 20 minutes at 4°C, the precipitate was rinsed in 70% ethanol, centrifuged, and then resuspended in 10 μL of RNase-free water (Sigma Chemical Co.). RNA (1 μL) was added to 49 μL of water for the measurement of RNA concentration with the use of a Beckman DU-640B spectrophotometer. The remaining 9 μL of RNA was immediately placed in a −80°C freezer for storage. The yield of RNA from an individual mouse was typically 1.2 to 1.5 μg for the aorta and 0.6 to 0.8 μg for the carotid artery.

To examine the feasibility of measuring eNOS mRNA from much smaller arteries from mice, cerebral arteries (basilar artery and large arteries of the circle of Willis) and coronary arteries (left anterior descending and right coronary arteries) were carefully isolated by using fine-point tweezers under a dissecting microscope and directly added into a 1.5-mL tube containing TriReagent. For these experiments, arteries were pooled from 2 or 3 mice to increase RNA yield. The RNA yield from cerebral arteries per mouse (7 to 9 months old) was 0.46±.09 μg (mean±SE, n=6) for males and 0.39±0.05 μg (n=9) for females. The RNA yield from coronary arteries per mouse (7 to 9 months old) was 0.32±0.02 μg (n=6) for males and 0.31±0.03 μg (n=8) for females.

RT Reaction

RNA (300 to 400 ng; typically, 400 ng) was used for RT in a final volume of 20 μL. The reaction mixture consisted of 3.125 mM random hexamer, 0.5 mM MgCl₂, deoxynucleotide triphosphates, 50 mM Tris (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, and 10 U/μL Moloney murine leukemia virus reverse transcriptase (Life Technologies). The reaction was incubated at 37°C for 90 minutes and subsequently stored at −20°C until use.

Design and Preparation of Primers and Probe

Primers and probe for mouse eNOS mRNA were designed to satisfy the following conditions: First, the 5’ and 3’ primers, in addition to being specific for eNOS, overlap 2 neighboring exons so that they hybridize only with cDNA but not with possibly genomic sequences of eNOS. This approach ensures that only mRNA, after RT, is measured in the sample. Second, primers, probe, and the length of PCR product satisfy the requirements as specified in Primer Express (version 1.5) software (PE Biosystems). Third, primers and probe, with minor modifications, may be adapted to be used for quantification of eNOS mRNA in other species (see below).

The sense primer (nucleotides 1419 to 1437, spanning exons 10 and 11 corresponding to the human eNOS genomic sequence(6), 5’-CCCTCCGCTACACGCAG-3’, and the antisense primer (1523 to 1500, spanning exons 11 and 12), 5’-CAGAGATCTTACGATGCTAGTA-3’, were synthesized by Integrated DNA Technologies. The dual-labeled probe (1467 to 1497, within exon 11), 5’-6-carboxy fluorescein-CAGGCTACACGAGAGACCTTTAAGGA-6-carboxy tetramethylrhodamine-3’, was synthesized by PE Biosystems.

As a control for RNA input and RT efficiency, 18S rRNA in each RNA sample was quantified with real-time PCR by using a kit purchased from PE Biosystems (TaqMan ribosomal control reagents, catalogue No. 4308329).

Preparation of DNA Standards

The products of conventional PCR for mouse eNOS (105 bp) and 18S rRNA (187 bp), with the use of the primers designed for the real-time PCR, were cloned into pCR3.1 vector (Invitrogen). A clone each of pCR3.1/eNOS and pCR3.1/18S, after confirmation of correct sequences, was propagated. Plasmid DNAs were purified, linearized with BamH I and XhoI, respectively, and quantified after purification with use of a Beckman DU-640B spectrophotometer. Concentration in molecules of single-stranded DNA (ssDNA) per milliliter was obtained by using the following formula: concentration of plasmid DNA (mg/mL) · 6.022 × 10⁸ · number of base pairs of plasmid DNA/650, where 650 is the average molecular weight of 1 bp. The conversion from the measured concentration of double-stranded DNA to the calculated concentration of ssDNA was the correct use as a standard, because the cDNA derived from RT (first-strand synthesis) is single-stranded. The standards used in real-time PCR were 2, 20, . . . , and 2 × 10⁶ molecules (ssDNA) for mouse eNOS and 300, 3000, . . . , and 3 × 10⁶ molecules (ssDNA) for 18S rRNA.

Real-Time PCR

Real-time PCR was performed according to the recommendations of PE Biosystems. Briefly, 1.1 μL of DNA standard or RT product (22 ng equivalent of total RNA) was added (using a P2 pipette and acrosol-free tips) to an aliquot of 26.4 μL of the stock buffer, bringing the mixed solution to a final concentration of 1 × TaqMan mixture (5.5 mM/L MgCl₂, 200 μM/L dATP/dCTP/dGTP, 400 μM/L dUTP, 400 nM/L primers, 100 nM/L probe, 0.01 U/μL AmpErase, and 0.025 U/μL AmpliTaq Gold DNA polymerase). Purchased from PE Biosystems, 2× TaqMan PCR Master Mix was used to make the stock buffer. From the 27.5-μL mixture, 25.0 μL (20 ng total RNA in 1× TaqMan mix) was transferred to a 96-well plate. PCR was performed at 50°C for 2 minutes and at 95°C for 10 minutes and then run for 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute on the ABI Prism 7700 Detection System (PE Biosystems). Cₚ, which is the threshold cycle number at which the initial amplification becomes detectable by fluorescence (defined as ΔRn=0.1 in our experiments), was determined. A standard curve was established in Cₚ versus copy number of ssDNA (equivalent to cDNA after RT), and the copy number of cDNA was determined for each RT sample as an approximation of mRNA copies. All analyses were standard procedures of the 7700 detection system. For 18S rRNA, a 2-pg equivalent of total RNA after RT was used because of its great abundance. Quantification of eNOS mRNA was expressed as copy-numbers per nanogram of total RNA and also as the ratio of eNOS to 18S rRNA. The value for each sample was an average of 3 independent PCR measurements. Intraexperimental variation (SD/mean) of a sample was within 10%, and interexperimental variation (SD/mean) was within 20%.

Sequencing of the eNOS Region Used in Real-Time PCR in Other Species

To extend the use of real-time PCR beyond the mouse, the region of eNOS was amplified from other species by use of the 5’ primer, whose sequence is identical among mice, humans, bovines, and guinea pigs (BLAST at GenBank). A “universal” 5’ primer was designed on the basis of the sequence of mouse, human, and bovine eNOS, which is located downstream from the 3’ primer, 5’-YTTCAWCGCTTSSGCCATCA-3’. The RT reaction was performed as described above on RNA samples obtained from monkey middle cerebral arteries, porcine aortic endothelial cells, and aortas from rabbits, dogs, and mice (as a positive control). A single amplification product of 143 bp, as predicted, was obtained from monkeys, pigs, rabbits, dogs, and mice. The monkey, pig, rabbit, and canine PCR products were purified from a low-melting-point gel after electrophoresis and sequenced bidirectionally by using the 5’ primer and the 3’ universal primer, respectively.

Statistical Analysis

Differences between mRNA levels were evaluated by using an unpaired Student t test. Values were mean±SE. A value of P<0.05 was considered significant.
Results

Using the primers and probe developed for real-time PCR, we were able to quantify eNOS mRNA in aortas and carotid arteries of individual mice and in cerebral and coronary arteries pooled from 2 or 3 mice. Because both primers span 2 exons, only hybridization with cDNA occurred; i.e., no amplification was observed with the use of RT control (reverse transcriptase omitted during RT) or even genomic DNA preparations (data not shown). A single product of the expected length (105 bp) was amplified from real-time PCR, which was confirmed by agarose gel electrophoresis (data not shown).

An example of a real-time PCR experiment is shown (Figure 1). The reaction was linear over 6 orders of magnitude of starting cDNA standards, with a detection sensitivity of 10 molecules. Correlation coefficients of such standard curves were consistently >0.99.

In aortas, eNOS mRNA levels were similar in 2-month-old mice of both sexes (Figure 2). Levels of eNOS mRNA were similar in young and older male mice. In contrast, eNOS mRNA levels were lower in the aortas of older female mice than in those of male mice (Figure 2). No differences were found in the carotid arteries between male and female mice in either age group (Figure 3). In addition, no differences between males and females were found in cerebral or coronary arteries (pooled from 2 or 3 mice) in older mice (Figure 4). Results based on the ratio of eNOS to 18S rRNA were consistent with those expressed as eNOS mRNA copies per nanogram of RNA equivalent (Figures 2, 3, and 4).

The eNOS region used for real-time PCR was amplified from several species by the use of RT products from endothelial cells or arteries (Figure 5); the PCR products were used directly as templates for bidirectional sequencing with the use of the 5′ and the universal 3′ primers, respectively. Unequivocal sequences were obtained (Figure 6), suggesting that no mutation had occurred during the PCR reaction. The sequences for pig,17 rabbit,18 and canine9,19 eNOS were later found to have been reported in GenBank. The sequences of the eNOS region were compared (Figure 6), and primers/probe may be designed from the sequences. For example, with a change of T for A in the sense primer, this method will readily be adapted for quantification of rat eNOS expression.

Figure 1. A, Amplification of eNOS detected by changes in fluorescence (ΔRn) in a real-time PCR setting. Ninety RT samples (red) were assayed against 6 DNA standards spanning 6 logs (black). The amplification cycle at which ΔRn equals 0.1 was assigned as the threshold cycle (Ct). B, Standard curve of threshold cycle against known quantities of DNA standards. Ninety RT samples (unknown) were determined for the number of copies of eNOS by use of the standard curve.

Discussion

We developed a real-time PCR method to quantify eNOS mRNA in aortas and carotid arteries from individual mice and cerebral and coronary arteries from 2 or 3 mice. The levels of eNOS mRNA were examined in males and females. No sex differences in eNOS mRNA levels were found in the aortas or carotid arteries of younger mice. In older mice, levels of eNOS mRNA in aortas but not in carotid, cerebral, or coronary arteries were lower in female mice than in male mice. We also amplified and sequenced the eNOS region used for real-time PCR in several species, so that this real-time PCR method could be extended to species beyond the mouse with modifications of primers and/or probe in the region.
Quantification of eNOS mRNA in Mice

eNOS mRNA had not been quantified to an absolute amount with the use of a real-time PCR method until the present study. In an excellent study with complex methodologies used, quantification of eNOS mRNA by competitive RT-PCR indicated that there were 600 eNOS mRNA copies per nanogram total RNA in aortas and 2 to 3 times more eNOS mRNA in right coronary arteries and left anterior descending arteries in dogs. These relative levels of eNOS mRNA in aortas and coronary arteries in dogs are consistent with those from our findings in mice, whereas the absolute number was ~2 times lower than our measurements.

Only a few studies have examined mRNA levels for eNOS in vessels from mice. By use of the competitive RT-PCR technique, eNOS mRNA was quantified in a single study of carotid arteries from individual mice. There were ~36,000 copies of eNOS per nanogram of total RNA in the carotid artery. This value is larger than that observed with the use of real-time PCR in the present study. Our method used 18S rRNA as an internal control for RNA input and RT efficiency.

Figure 2. A, Number of molecules of eNOS mRNA (left) and ratio of eNOS to 18S rRNA (right) in thoracic aorta of 2-month-old male (n=7) and female (n=7) C57BL/6 mice. B, Number of molecules of eNOS mRNA (left) and ratio of eNOS to 18S rRNA (right) in thoracic aorta of 7- to 9-month-old male (n=10) and female (n=10) C57BL/6 mice. Values are mean±SE. *P<0.01 vs male by an unpaired Student t test.

Figure 3. A, Number of molecules of eNOS mRNA (left) and ratio of eNOS to 18S rRNA (right) in carotid artery of 2-month-old male (n=7) and female (n=7) C57BL/6 mice. B, Number of molecules of eNOS mRNA (left) and ratio of eNOS to 18S rRNA (right) in carotid artery of 7- to 9-month-old male (n=10) and female (n=10) C57BL/6 mice. Values are mean±SE.
However, because RT efficiency is always <100%, quantification for eNOS mRNA may represent an underestimation. RT efficiency was determined to range from 24% to 60% with the RT condition that we used (personal communication with the manufacturer of Moloney murine leukemia virus reverse transcriptase). In a previous study,22 RT efficiency of eNOS mRNA was controlled by an added competitor RNA. Inasmuch as random hexamers that were used in RT could bind unequally to the native and competitor eNOS RNA because of differences in length and configuration, this control may have limitations. Variations in the estimation of eNOS mRNA with the use of competitive RT-PCR may be further introduced by the inherent problems of an end-point PCR assay, post-PCR processing, and densitometry. Thus, a difference between the 2 methods in eNOS mRNA levels results from the different methodologies used. Both methods may be valid in the quantification of eNOS mRNA, as long as the method is executed consistently within a study.

Variation in eNOS mRNA by Sex
A previous study provided evidence that the aorta produces more NO under basal conditions in males than in females.13 The sex difference detected in the present study in eNOS mRNA in the aorta is a new finding and is consistent with the finding of a previous study. The sex difference in the present study was age-related and was observed only in older mice. Interestingly, older apoE-deficient or LDL receptor–deficient female mice develop more severe atherosclerosis in the aorta than do male apoE-deficient or LDL receptor–deficient mice.23–25 Considering the antiatherogenic effects of eNOS (NO), we speculate that the sex difference in eNOS expression may contribute to the sex difference in susceptibility to atherosclerosis in mice.

Quantification of relative eNOS mRNA levels has been accomplished previously by using Northern blotting,5,7,8 RNase protection assay,6 and competitive RT-PCR.9 The former 2 methods require relatively large amounts of starting

Figure 5. Conventional RT-PCR for eNOS of different species with modified primers. To expand the use of real-time PCR beyond mice, the region of eNOS was amplified from different species by using the conserved 5’ primer and a universal 3’ primer, as described in Methods. An amplification product (143 bp) was obtained from monkey, pig, rabbit, dog (not shown), and mouse (as a positive control). MCA indicates middle cerebral arteries; PAEC, porcine aortic endothelial cell; and Ao, thoracic aorta.
RNA material (10 to 30 μg total RNA per sample per assay, typically) and, thus, can be used only in cultured cells or in blood vessels isolated from either large species or pooled vessels from small species. The latter method requires several laborious steps, including titration to ensure that the end point of PCR falls within a quantitative range, and post-PCR processing, which may result in variations of end results (eg, the shape and evenness of bands and framing of bands for densitometry). As more studies of vascular biology use the mouse,1,2,26 this real-time PCR method to quantify eNOS mRNA should find broad applications. Its simplicity, sensitivity, broad sample range, and high throughput are far more advantageous than other methods to quantify gene expression.

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