cAMP Signal Transduction Cascade, a Novel Pathway for the Regulation of Endothelial Nitric Oxide Production in Coronary Blood Vessels

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Abstract—The aim of this study was to determine whether cAMP signal transduction plays a role in the regulation of endothelial nitric oxide (NO) production. Canine coronary blood vessels were isolated, and nitrite, the hydration product of NO, from these vessels was quantified by using the Griess reaction. Forskolin \( (10^{-2} \text{ mol/L}) \), 8-bromo-cAMP \( (10^{-2} \text{ mol/L}) \), or isoproterenol \( (10^{-4} \text{ mol/L}) \) significantly increased nitrite release to 168\( \pm \)6 pmol/mg, 162\( \pm \)13 pmol/mg, or 149\( \pm \)6 pmol/mg, respectively, from isolated coronary microvessels (all \( P<0.05 \); control, 86\( \pm \)3 pmol/mg). Adrenomedullin and calcitonin gene–related peptide (CGRP), both potent vasodilator peptides, also increased coronary microvascular nitrite production. \( \text{N}^\text{G}-\text{nitro-L-arginine methyl ester}, \) a competitive inhibitor of NO synthase, or Rp-cAMP, a protein kinase A inhibitor, markedly blocked the nitrite release induced by these agents. Forskolin and adrenomedullin also potentiated coronary NO production induced by bradykinin. In large coronary arteries, removal of the endothelium eliminated nitrite production to both forskolin and acetylcholine. Our data demonstrate that stimulation of cAMP signal transduction can substantially increase coronary NO production, indicating that there is a cAMP-mediated, endothelial NO–forming system in coronary blood vessels. Because the cAMP signal cascade can be activated by CGRP or adrenomedullin and enhance kinin-mediated nitrite production, the cAMP-NO pathway may play an important role in the regulation of cardiovascular function. (Arterioscler Thromb Vasc Biol. 2001;21:797-803.)

Key Words: cAMP | nitric oxide | endothelium | protein kinase B | coronary blood vessels

\( \beta \)-Adrenoceptor agonists and adenosine have been regarded as “archetypal” endothelium-independent vasodilators that cause vasodilation by increasing intracellular cAMP, with subsequent activation of protein kinase A (PKA) and myosin light-chain kinase within smooth muscle cells. However, recent studies have challenged this concept. Growing evidence indicates that a number of classic endothelium-independent vasodilator drugs also cause endothelium-dependent vasodilation, and this process seems to be related to endothelial NO production. These include (1) the inhibition of isoprenaline-induced vasorelaxation in rat aorta by hemoglobin and methylene blue; (2) the inhibition of prostacyclin- and forskolin-induced vasorelaxation in pig coronary artery in vitro by hemoglobin and methylene blue; (3) the inhibition of salbutamol- and epinephrine-induced vasorelaxation in vivo and in vitro in the rat by the NO synthase (NOS) inhibitor \( \text{N}^\text{G}-\text{nitro-L-arginine methyl ester} \) (L-NAME); (4) the inhibition of isoproterenol-induced resistance coronary vessel dilation in the conscious dog by L-NAME; and (5) the inhibition of isoproterenol-induced vasodilation in human forearm by \( \text{N}^\text{G-} \)-monomethyl-L-arginine. These results indicate that in blood vessels, there is an NO component to the vasorelaxant response to all of these agonists. This may be of particular physiological importance because many endogenous factors that affect cAMP production, such as ATP or adenosine, norepinephrine or epinephrine, and adrenomedullin or calcitonin gene–related peptide (CGRP), may therefore participate in the regulation of endothelial NO production. Indeed, studies by Graier et al and Li et al have found that adenosine significantly enhances basal or agonist-induced NO release from cultured porcine artery endothelial cells. Kanai et al also found that norepinephrine and epinephrine evoke detectable NO release from individual rat ventricular myocytes. However, the mechanism of NO formation from blood vessels induced by these cAMP-elevating agents remains unknown. Therefore, our study was designed to determine (1) whether stimulation of the cAMP signal-transduction pathway can increase endothelial NO production from coronary blood vessels; (2) whether there is a natural ligand of the cAMP-NO pathway in coronary microvessels from normal dog heart; (3) whether stimulation of cAMP signal transduction can affect kinin-mediated NO formation; and (4) whether this mechanism is endothelium dependent.

See page 729
Methods

Animal Preparation

All of the studies were approved by the institutional Animal Care and Use Committee of New York Medical College and conform to current National Institutes of Health and American Physiological Society guidelines for the use and care of laboratory animals. Twenty-nine adult mongrel dogs (body weight 21 to 29 kg) were anesthetized with pentobarbital (IV, 50 mg/kg). The heart was excised immediately and kept in ice-cold PBS containing 0.1% bovine serum albumin at pH 7.4.

Isolation of Coronary Microvessels and Large Coronary Arteries

Isolation of coronary microvessels from the left ventricular free wall of the dog heart was performed with the method used in previous studies.27,28 Coronary microvessels were obtained after separation from large arteries and veins, connective tissue, fat, and myocytes by a series of steps involving sequential dissection, homogenization, sieving, and glass bead purification. This preparation of microvessels (diameter range 20 to 70 μm) was virtually free of myocytes and consisted only of arterioles, venules, and capillaries. Approximately 2000 mg of microvessels was collected per heart (215 to 7 g). The left circumflex, left anterior descending, or right coronary artery from 7 dogs was removed and cut into rings (~20 mg in weight). To determine the role of the endothelium in NO production from cardiac blood vessels, from some coronary artery rings the endothelium was denuded by scraping with a wooden stick.

Incubation of Coronary Microvessels and Large Coronary Arteries

Microvessels, ~20 mg (wet weight) of tissue, were oxygenated with 95% O2 and 5% CO2 in PBS for 30 minutes, placed in 5-mL plastic tubes that contained chemical stimuli or inhibitors, and incubated for 20 minutes at 37°C. At the end of the incubation time, the tubes were removed from the tissue bath, and sulfanilamide (450 μL of a 1% solution) and N-(1-naphthyl)ethylenediamine (50 μL of a 0.2% solution) were added to each tube for diazotization of sulfanilic acid by NO. After 5 to 10 minutes' incubation at room temperature for full color (pink) development, the supernatant was removed from each tube. Formation of NO was measured as nitrite. Nitrite release was measured with a spectrophotometer (Uvikon 930 spectrophotometer, Kontron Instruments Inc) as the increase in absorbance at 540 nm and compared with known concentrations of nitrite. Absorbance was measured with a spectrophotometer (Uvikon 930 spectrophotometer, Kontron Instruments Inc) as the increase in absorbance at 540 nm and compared with known concentrations of nitrite. Absorbance was measured with a spectrophotometer (Uvikon 930 spectrophotometer, Kontron Instruments Inc) as the increase in absorbance at 540 nm and compared with known concentrations of nitrite. Absorbance was measured with a spectrophotometer (Uvikon 930 spectrophotometer, Kontron Instruments Inc) as the increase in absorbance at 540 nm and compared with known concentrations of nitrite.

Method to Dissect cAMP-NO Transduction

After stimulation of cAMP in microvessels with the use of agonists (ie, forskolin, an adenylyl cyclase activator; isoproterenol, a β-adrenoceptor agonist; and adrenomedullin and CGRP, both activators of adenylyl cyclase), we used a number of inhibitors to determine the signal-transduction pathway leading to NO formation. The specific inhibitor of phosphatidylinositol 3-kinase (PI3 kinase). We used the concentration used to inhibit protein kinase A. LY294002 is a specific inhibitor of protein kinase A, and 10 mol/L, Rp-cAMP (10 mol/L), dideoxyadenosine (10 mol/L), LY294002 (3×10 mol/L), wortmannin (10 mol/L), or pranopanol (10 mol/L) was also incubated with tissue before addition of the highest concentration of forskolin, 8-bromo-cAMP, or isoproterenol.

Experimental Protocols

Effects of Forskolin, 8 Bromo-cAMP, and Isoproterenol on cAMP Signal Transduction–Mediated NO Production in Coronary Microvessels

Increasing concentrations of forskolin (10 to 10 mol/L), 8-bromo-cAMP (10 to 10 mol/L), and isoproterenol (10 to 10 mol/L) were incubated with tissue for 20 minutes. Nitrite release was measured. L-NAME (10 mol/L), Rp-cAMP (10 mol/L), dideoxyadenosine (10 mol/L), LY294002 (3×10 mol/L), wortmannin (10 mol/L), or pranopanol (10 mol/L) was also incubated with tissue before addition of the highest concentration of forskolin, 8-bromo-cAMP, or isoproterenol.

Effects of Adrenomedullin and CGRP on cAMP Signal Transduction–Mediated NO Production in Coronary Microvessels

Increasing concentrations of adrenomedullin and CGRP (10 to 10 mol/L) were incubated with tissue for 20 minutes. Nitrite release was measured. L-NAME (10 mol/L), Rp-cAMP (10 mol/L), dideoxyadenosine (10 mol/L), LY294002 (3×10 mol/L), wortmannin (10 mol/L), or pranopanol (10 mol/L) was also incubated with tissue before addition of the highest concentration of adrenomedullin or CGRP.

Effects of Forskolin and Adrenomedullin on NO Production Induced by Bradykinin

The effects of forskolin and adrenomedullin on nitrite production induced by bradykinin were studied. Increasing concentrations of bradykinin (10 to 10 mol/L), alone and in the presence of a subthreshold concentration (a low concentration that has no effect on NO production) of forskolin (10 mol/L) or adrenomedullin (10 mol/L), were incubated with tissue for 20 minutes. Nitrite was measured. L-NAME (10 mol/L), HOE 140 (Icatibant, a specific B2-kinin receptor antagonist; 10 mol/L [Hoechst]), Rp-cAMP (10 mol/L), or dideoxyadenosine (10 mol/L) was also incubated with tissue before addition of the highest concentration of bradykinin combined with forskolin or adrenomedullin.

Role of Endothelium in Forskolin- or Acetylcholine-Mediated NO Production in Large Coronary Arteries

Increasing concentrations of forskolin (10 to 10 mol/L) or acetylcholine (10 to 10 mol/L) were incubated with large coronary arteries with or without endothelium for 20 minutes. Nitrite release was measured. L-NAME (10 mol/L), Rp-cAMP (10 mol/L), or atropine (10 mol/L) was also incubated with tissue before addition of the highest concentration of forskolin or acetylcholine.

Drugs and Chemicals

The PBS used in these studies consisted of (in mmol/L) NaCl 139, KCl 2.7, NaH2PO4 8.1, KH2PO4 1.5, CaCl2 0.68, and MgCl2 0.49; bovine serum albumin concentration was 0.1%. Drugs (adrenomedullin, CGRP, acetylcholine, and bradykinin) and chemicals (8-bromo-cAMP, isoproterenol, L-NAME, dideoxyadenosine, LY294002, wortmannin, propranolol, nitrite, and bovine serum albumin) were purchased from Sigma Chemical Co. Forskolin was purchased from Calbiochem-Novabiochem Corp. Rp-cAMP was purchased from Research Biochemicals International.

Statistical Analysis and Calculations

To construct a standard curve for nitrite, a stock solution of NaNO2 (10 mol/L) was prepared and diluted for each experiment. Sulfanilamide (450 μL of a 1% solution) and N-(1-naphthyl)ethylenediamine (50 μL of a 0.2% solution) were mixed with NaNO2 and allowed to stand at room temperature for 5 to 10 minutes for full color (pink) development. Absorbance of nitrite was measured at 540 nm and converted to a straight line by use of linear regression analysis (y=ax+b, R=0.99). Nitrite production was calculated with the linear regression formula. Data were expressed as mean±SEM in pmol/mg wet weight per 20 minutes. Differences in nitrite production versus control were determined with a 2-way ANOVA. The
and CGRP both significantly increased nitrite production from coronary microvessels. *P<0.05 vs control. Values are mean±SEM.

**Results**

Data in the figures are the changes in nitrite production in pmol/mg wet weight per 20-minute incubation, whereas data in the text are percentages of the change in nitrite and absolute values.

**Effects of Forskolin, 8-Bromo-cAMP, and Isoproterenol on cAMP Signal Transduction–Mediated NO Production in Coronary Microvessels**

Forskolin (10^-10 to 10^-8 mol/L), 8-bromo-cAMP (10^-8 to 10^-2 mol/L), and isoproterenol (10^-10 to 10^-4 mol/L) concentration-dependently increased nitrite production by 15±5% to 98±8%, 20±4% to 103±11%, and 17±3% to 78±11%, respectively (from a control value of 85±3 pmol/mg; all P<0.05). The actual changes in nitrite are shown in Figure 1. After incubation with l-NAME, Rp-cAMP, dideoxyadenosine, LY294002, and wortmannin, nitrite release induced by the highest concentration of forskolin, 8-bromo-cAMP, or isoproterenol was reduced by 90% to 100%, 32% to 88%, and 95% to 100%, respectively (all P<0.01). Propranolol entirely eliminated the effects of isoproterenol. The effects of these antagonists (except propranolol) on the actual changes in nitrite induced by forskolin, 8-bromo-cAMP, and isoproterenol are shown in Figure 2.

**Effects of Adrenomedullin and CGRP on cAMP Signal Transduction–Mediated NO Production in Coronary Microvessels**

The effects of adrenomedullin and CGRP on NO production are shown in Figure 3. Adrenomedullin (10^-12 to 10^-8 mol/L) and CGRP (10^-12 to 10^-7 mol/L) increased nitrite production by 18±7% to 86±17% and by 19±7% to 98±16%, respectively (from a control value of 84±3 pmol/mg; all P<0.05). After incubation with l-NAME, Rp-cAMP, dideoxyadenosine, LY294002, or wortmannin, nitrite release induced by the highest concentration of adrenomedullin or CGRP was concentration-dependently increased nitrite production by 17% and by 19% to 98% by 18% to 97%, respectively. Comparison of the effects of the highest concentrations of bradykinin showed that forskolin and adrenomedullin potentiated the change in nitrite production by 34% and 39% (P<0.05 vs bradykinin alone), respectively. These effects were synergistic. The effect of forskolin on nitrite production induced by bradykinin is shown in Figure 5. In the presence of l-NAME, HOE **Effects of Forskolin and Adrenomedullin on NO Production Induced by Bradykinin**

 Bradykinin (10^-10 to 10^-7 mol/L) concentration-dependently increased nitrite release by 14±4% to 95±21% (from a control value of 81±3 pmol/mg). After incubation with a low concentration of forskolin (10^-10 mol/L) or adrenomedullin (10^-12 mol/L), nitrite release induced by increasing concentrations of bradykinin was elevated by 51±8% to 171±10% and by 66±3% to 168±12%, respectively. Comparison of the effects of the highest concentrations of bradykinin showed that forskolin and adrenomedullin potentiated the change in nitrite production by 34% and 39% (P<0.05 vs bradykinin alone), respectively. These effects were synergistic. The effect of forskolin on nitrite production induced by bradykinin is shown in Figure 5. In the presence of l-NAME, HOE
140, Rp-cAMP or dideoxyadenosine, the effects of forskolin and adrenomedullin on nitrite release induced by the highest concentration of bradykinin were blocked by 83% to 99%, respectively (all \(P<0.01\)).

**Role of Endothelium in Forskolin- or Acetylcholine-Mediated NO Production in Large Coronary Arteries**

Forskolin (10^{-5} to 10^{-4} mol/L) and acetylcholine (10^{-5} to 10^{-3} mol/L) concentration-dependently increased nitrite production from large coronary arterial rings by 72±5% to 10±5% and by 4±2% to 87±13%, respectively (from a control value of 81±8 pmol/mg; all \(P<0.05\)). After incubation with L-NAME or Rp-cAMP, nitrite release induced by the highest concentration of forskolin was reduced by 87% or 85%, respectively (all \(P<0.01\)). After incubation with L-NAME or atropine or denudation of the endothelium, nitrite release induced by the highest concentration of acetylcholine was blocked by 95% and 96%, respectively (all \(P<0.01\)). After denudation of the endothelium, neither forskolin nor acetylcholine had an effect on nitrite production (all \(P>0.05\)).

**Discussion**

The most important finding in the present study is that stimulation of cAMP signal transduction can significantly increase NO formation from isolated canine coronary blood vessels. This effect is mediated by PKA or PI3 kinase. Two endogenous vasodilators, adrenomedullin and CGRP, also substantially increased coronary NO production via stimulation of cAMP signal transduction, indicating that there is an innate cAMP-NO pathway in coronary blood vessels. Another significant finding in this study is that stimulation of cAMP signal transduction by forskolin or adrenomedullin dramatically potentiated coronary vascular NO production induced by bradykinin, indicating synergism between kinin-mediated endothelial NO production and intracellular cAMP signal transduction. Because forskolin-induced NO production was eliminated by prior endothelium denudation in large coronary arteries, our results suggest the concept that coronary cAMP signal transduction may participate in the regulation of cardiovascular endothelial NO production.

NO, a potent vasodilator, has been identified as a major endothelium-derived relaxing factor synthesized by NOS in the endothelium.\(^1\)\(^-\)\(^3\) A number of physiological factors, such as shear stress, or vasoactive substances, such as acetylcholine and bradykinin, regulate NO production from endothelial cells.\(^2\)\(^-\)\(^3\) β-Adrenoceptor agonists or adenosine are widely believed to be another group of potent vasodilators that cause endothelium-independent vasodilatation by a different second messenger, ie, cAMP, in smooth muscle.\(^1\)\(^-\)\(^6\) However, recent studies suggest that vascular relaxant responses to many adenylate cyclase activators are, at least in part, also endothelium dependent. In our study, stimulating β-adrenoceptors by isoproterenol, activating adenylyl cyclase by forskolin, or activating PKA by 8-bromo-cAMP all evoked significant NO release from isolated coronary microvessels. The NOS inhib-
mediated relaxation. In our present study, inhibition of eNOS either directly or indirectly and evoke NO cGMP-rise in intracellular cAMP may activate endothelial NOS but not in smooth muscle cells. It has been suggested that a increase in intracellular calcium. However, it has been shown that forskolin and isoproterenol promotes NO production through activation of adenyl cyclase. Propranolol also inhibited the effect of isoproterenol on NO production, suggesting that isoproterenol induces NO release by stimulating β-adrenoceptors. Increasing the activity of adenyl cyclase could subsequently increase intracellular cAMP, which may occur in both smooth muscle cells and the endothelium. However, a study by Gray and Marshall found that in rat aortic rings, isoprenaline and forskolin increased cAMP accumulation in the endothelium but not in smooth muscle cells. It has been suggested that a rise in intracellular cAMP may activate endothelial NOS (eNOS) either directly or indirectly and evoke NO cGMP-mediated relaxation. In our present study, inhibition of cAMP-dependent protein kinase A with Rp-cAMP abrogated the NO release induced by isoproterenol, forskolin, and 8-bromo-cAMP, clearly showing an indirect effect of cAMP on NOS. It is generally accepted that in cell types other than vascular endothelial cells, stimulation by receptor-operated agonists evokes adenyl cyclase activation by a G protein to produce cAMP. This acts in conjunction with cAMP-dependent protein kinase A to induce calcium influx through voltage-gated calcium channels, resulting in an increase in intracellular calcium. However, it has been shown that endothelial cells lack voltage-gated calcium channels. Even so, activation of PKA-mediated potentiation of NOS activity has been suggested by many studies. Iranami et al found that the PKA inhibitor H-89 markedly inhibited endothelial NO-mediated relaxation induced by isoproterenol in rat aortic rings. Graier et al also reported that inhibition of cAMP-dependent protein kinase A abolished the stimulatory effects of cAMP-elevating, agonist-induced NO bio-synthesis in cultured porcine aortic endothelial cells. All of these data suggest a crucial role of protein kinase A in the stimulation of NO production mediated by cAMP.

A very recent study by Chen et al found that AMP-activated protein kinase commononprecipitates with cardiac eNOS and phosphorylates Ser-1177 to activate eNOS. In our experiments, a 20-minute incubation of all of the agents with isolated coronary microvessels significantly increased tissue NO release. The most probable explanation for this phenomenon is the increase in activity of the enzyme rather than an increase in expression of mRNA or protein for NOS. Our study also shows that the increased activity of NOS is unlikely to be mediated via phosphorylation by protein kinase A only, because 2 PI3 kinase inhibitors, LY294002 and wortmannin, also essentially abolished nitrite release induced by all of these agonists. These results indicate that PI3 kinase could be either a parallel or a downstream effector of protein kinase A on NOS. Importantly, 2 recent studies by Fulton et al and Dimmeler et al found that eNOS is an efficient substrate for PKB (serine/threonine protein kinase Akt). This enzyme can phosphorylate eNOS directly and increase its activity. This process is mediated by PI3 kinase. Taken together, their findings and our current results, it is interesting to speculate that the cAMP signal-transduction cascade increases coronary vascular NO release, perhaps via activation of PKA and subsequent phosphorylation of eNOS by PKB through a PI3 kinase-mediated mechanism.

A clinically significant finding in this study was that adrenomedullin and CGRP both markedly increased nitrate release from isolated canine coronary microvessels. It is thought that adrenomedullin induces vasorelaxation by activating adenylate cyclase and the subsequent increase in cAMP in vascular smooth muscle cells. Increasing evidence suggests that adrenomedullin also induces NO release from the endothelium. However, the intracellular signal-transduction pathway in the endothelium has never been addressed. CGRP, a vasodilator neuropeptide, is widely distributed in the autonomic nerve terminals supplying the cardiovascular system and is present in plasma. Recent studies have found that CGRP can also evoke endothelium-dependent and NO-mediated vasodilation. Adrenomedullin shares significant structural homology with CGRP. Both adrenomedullin and CGRP can increase intracellular cAMP in various tissues, including the endothelium. In the present study, both adrenomedullin and CGRP markedly increased NO production from isolated canine coronary microvessels, suggesting that coronary microvessels are capable of NO release in response to adrenomedullin and CGRP. t-NAME, Rp-cAMP, dideoxyadenosine, LY294002, and wortmannin significantly blocked this effect on NO formation, indicating that adrenomedullin and CGRP share a common mechanism with forskolin in NO formation, most likely by a cAMP-PKA and a PI3 kinase-regulated pathway. Both adrenomedullin and CGRP are endogenous biological factors. This may be of significant physiological or pathophysiological importance, because plasma adrenomedullin levels are elevated in a variety of disease states, including hypertension, congestive heart failure, and septic shock.

Another important finding in our study is that forskolin and adrenomedullin both significantly potentiated coronary NO formation induced by bradykinin, suggesting a dual regulatory effect on NO production in the endothelium. Even a low physiological concentration of adrenomedullin (10−12 mol/L, which alone has no significant effect on NO production) enhanced NO production induced by physiological concentrations of bradykinin (10−8 to 10−6 mol/L). NO production induced by bradykinin, after being combined with low
concentrations of forskolin and adrenomedullin, was almost abolished not only by the NOS inhibitor and B₂-kinin receptor antagonist but also by a PKA inhibitor and an adenylyl cyclase inactivator. Heart failure and many other cardiovascular diseases are associated with defective endothelial NO production. This has been recognized as an important pathophysiological mechanism. If stimulation of intracellular cAMP signal transduction promotes additional endothelial NO production, then cAMP-elevating agents may become a very useful tool for the treatment of many types of cardiovascular disease.

There are 2 limitations to our present study. First, we could not measure nitrite production from single endothelium-denuded coronary microvessels. Therefore, the conclusion that an endothelium-dependent mechanism in coronary microvessels controls NO production induced by forskolin, based on evidence in large coronary arteries, is still speculative. Second, the specificity of some of the antagonists used in this study is uncertain. For example, we cannot eliminate a possible effect of Rp-cAMP and LY294002 on PKB directly, although according to the literature, Rp-cAMP and LY294002 are very specific inhibitors for protein kininase A and PI3 kinase, respectively.

In summary, our data indicate that there is a cAMP-NO pathway in canine coronary blood vessels. Adrenomedullin or CGRP may be a natural ligand for activation of this signal-transduction system. Combining the stimulation of B₂-kinin receptors and a cAMP signal system can have a synergistic effect on coronary NO production. Because a similar concentration of forskolin that was used in a recent study could stimulate porcine coronary microvascular NO production and had a significant effect on NO-dependent coronary vasodilation and blood flow elevation, our data suggest that the cAMP-NO pathway may play a crucial role in the regulation of endothelium-dependent cardiac vascular function in physiological and pathophysiological states.

Acknowledgment

These studies were supported by PO-1-HL 43023, HL 50142, and HL 61290 from the National Heart, Lung, and Blood Institute, Bethesda, Md.

References

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doi: 10.1161/01.ATV.21.5.797
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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