Cardioprotective Effect of Angiotensin-Converting Enzyme Inhibition Against Hypoxia/Reoxygenation Injury in Cultured Rat Cardiac Myocytes

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Background—Although ACE inhibitors can protect myocardium against ischemia/reperfusion injury, the mechanisms of this effect have not yet been characterized at the cellular level. The present study was designed to examine whether an ACE inhibitor, cilazaprilat, directly protects cardiac myocytes against hypoxia/reoxygenation (H/R) injury.

Methods and Results—Neonatal rat cardiac myocytes in primary culture were exposed to hypoxia for 5.5 hours and subsequently reoxygenated for 1 hour. Myocyte injury was determined by the release of creatine kinase (CK). Both cilazaprilat and bradykinin significantly inhibited CK release after H/R in a dose-dependent fashion and preserved myocyte ATP content during H/R, whereas CV-11974, an angiotensin II receptor antagonist, and angiotensin II did not. The protective effect of cilazaprilat was significantly inhibited by Hoe 140 (a bradykinin B2 receptor antagonist), N\textsuperscript{G}-monomethyl-L-arginine monoacetate (L-NMMA) (an NO synthase inhibitor), and methylene blue (a soluble guanylate cyclase inhibitor) but not by staurosporine (a protein kinase C inhibitor), aminoguanidine (an inhibitor of inducible NO synthase), or indomethacin (a cyclooxygenase inhibitor). Cilazaprilat significantly enhanced bradykinin production in the culture media of myocytes after 5.5 hours of hypoxia but not in that of nonmyocytes. In addition, cilazaprilat markedly enhanced the cGMP content in myocytes during hypoxia, and this augmentation in cGMP could be blunted by L-NMMA and methylene blue but not by aminoguanidine.

Conclusions—The present study demonstrates that cilazaprilat can directly protect myocytes against H/R injury, primarily as a result of an accumulation of bradykinin and the attendant production of NO induced by constitutive NO synthase in hypoxic myocytes in an autocrine/paracrine fashion. NO modulates guanylate cyclase and cGMP synthesis in myocytes, which may contribute to the preservation of energy metabolism and cardioprotection against H/R injury. (Circulation. 1999;99:817-822.)

Key Words: angiotensin ■ hypoxia ■ bradykinin ■ nitric oxide ■ myocytes

Recent clinical and experimental studies have established the therapeutic benefits of ACE inhibitors, not only in treating hypertension and congestive heart failure, but also in reducing reinfarction, limiting infarct size, and preventing reperfusion arrhythmias.\textsuperscript{1–3} These cardioprotective effects of ACE inhibitors are thought to depend on their ability to attenuate the degradation of endogenous bradykinin as well as to decrease the synthesis of angiotensin II from angiotensin I. In addition to altering circulating concentrations of angiotensin II and bradykinin, thereby effecting hemodynamic changes, ACE inhibitors may participate in cardioprotection through the alteration of localized angiotensin II or bradykinin concentrations in cardiac tissue.\textsuperscript{4} Angiotensin II may induce cardiac myocyte necrosis and fibroblast proliferation, thereby exacerbating ischemia-reperfusion injury.\textsuperscript{5} In contrast, recent reports have indicated that kinin can induce an increase in coronary circulation and improve cardiac function in ischemia-reperfusion injury. Although inhibition of ACE is also known to cause coronary vasodilation and an improvement in contractile and metabolic function in ischemia-reperfusion injury,\textsuperscript{6–9} it is still unknown whether the beneficial effects of ACE inhibitors are attributable to a direct effect on cardiac myocytes.

In the present study, therefore, we focused on the direct molecular actions of an ACE inhibitor on cardiac myocytes. We have examined the following: (1) whether cilazaprilat, a nonsulfhydryl ACE inhibitor, directly protects cardiac myocytes against hypoxia/reoxygenation injury; (2) whether this protective action derives from an inhibition of angiotensin II synthesis or an accumulation of bradykinin; (3) whether this protective effect is mediated by prostaglandins, NO, or protein kinase C (PKC); and (4) whether cGMP is involved in the protective effect. We treated cultured rat neonatal cardiac myocytes with either modified Tyrode solution, cilazaprilat,
angiotensin II, bradykinin, or the angiotensin II type 1 receptor antagonist CV-11974 and subsequently exposed the cells to 5.5 hours of hypoxia followed by 1 hour of reoxygenation. In addition, we tested whether pretreatment with the kinin receptor antagonist Hoe 140, the NO synthase inhibitor N\textsuperscript{\text{3}}-monomethyl-L-arginine monooacetate (L-NMMA), the inducible NO synthase inhibitor aminoguanidine, the cyclooxygenase inhibitor indomethacin, or the PKC inhibitor staurosporine could block the beneficial effect of cilazaprilat. Furthermore, we measured the concentration of bradykinin in culture media after treatment with cilazaprilat and monitored the cGMP content in myocytes during hypoxia as well as the high-energy phosphates in myocytes during hypoxia/reoxygenation.

Methods

Materials

Cilazaprilat, Hoe 140, and CV-11974 were generous gifts from Nippon Rohg KK, Hoechst Marion Roussel AG, and Takeda Chemical Industries, Ltd, respectively. Angiotensin II and bradykinin were purchased from Pepptide Institute, Inc; L-NMMA from Calbiochem-Novabiochem International; aminoguanidine from Nacalai tesque Inc; and the remaining reagents from Sigma Chemical Co.

Culture of Neonatal Rat Cardiac Myocytes

Primary cultures of neonatal rat cardiac myocytes were prepared as previously described with some modifications. Briefly, hearts were removed from 1- to 2-day-old Wistar rats anesthetized by ether under aseptic conditions and placed in Ca\textsuperscript{2+}- and Mg\textsuperscript{2+}-free PBS. The hearts were washed with PBS, and the atria and aorta were discarded. The ventricles were minced with scissors into 1- to 3-mm\textsuperscript{3} fragments, and they were then enzymatically digested 4 times for 10 to 15 minutes at 37°C in a humidified incubator with 5% CO\textsubscript{2} air. The liberated cells were collected by centrifugation at 300g and incubated in 100-mm culture dishes (Falcon) for 60 minutes at 37°C in a humidified incubator with 5% CO\textsubscript{2} air. The nonadherent cardiac myocytes were harvested and seeded into 60-mm culture dishes (1\texttimes10\textsuperscript{6} cells/cm\textsuperscript{2}). The myocytes were incubated in Dulbecco’s modified Eagle’s medium (DMEM; Nissui Pharmaceutical Co) supplemented with 10% FBS (Bioserum Co). 5-Bromo-2’-deoxyuridine (BrDU; 100 \mu M/L) was added during the first 48 hours to inhibit proliferation of nonmyocytes. Using this method, we routinely obtained contractile myocyte-rich cultures with 90% to 95% myocytes, as assessed by immunofluorescence staining with a monoclonal antibody against b-myosin heavy chain. The myocytes were then incubated in DMEM containing 0.5% FBS without BrDU, and all experiments were done 36 to 48 hours after this incubation.

Preparation of Cardiac Nonmyocyte-Rich Culture

 Highly enriched cultures of cardiac nonmyocytes (hereafter called nonmyocyte culture) were prepared by 2 passages of the cells adherent to the culture dish during the preplating procedure. Until the second passage, cells were maintained in the same culture medium as above, except that 10% FBS was used and BrDU was not used. The nature of cells was determined by immunofluorescence staining with anti-rat factor VIII, anti-desmin, and anti-vimentin for identification of endothelial cells, smooth muscle cells, and fibroblasts, respectively. After the second passage, only 1% to 2% of cells were stained positively with anti-desmin or anti-factor VIII. More than 95% of cells were stained positively with anti-vimentin antibody, indicating that the nonmyocytes consisted of fibroblasts under our culture conditions.

Culture of Rat Aortic Endothelial Cells

Rat aortic endothelial cells (RAECs) were isolated from male Wistar rats (weight, 200 to 250 g) by the primary explant technique. The cells were cultured in DMEM supplemented with 10% FBS and 0.15 mg/mL endothelial cell growth supplement. RAECs were grown at 37°C in a humidified incubator with 5% CO\textsubscript{2} air and passaged with a split ratio of 1:5 using 0.05% trypsin-0.02% EDTA.

Assay of Creatine Kinase Release

Creatine kinase (CK) activity in culture media was measured before hypoxia, at the end of 5.5 hours of hypoxia, and after 1 hour of reoxygenation in all groups (Figure 1). CK activity and bradykinin concentration in culture media were measured as indicated and described in Methods.

Experimental Protocols

Figure 1 shows the experimental protocols. Before hypoxic exposure, cell medium was replaced by modified Tyrode solution (in mmol/L: NaCl 136.9, KCl 2.68, Na\textsubscript{2}HPO\textsubscript{4} \textsubscript{2}H\textsubscript{2}O 8.1, KH\textsubscript{2}PO\textsubscript{4} 1.47, CaCl\textsubscript{2} 0.9, MgCl\textsubscript{2} \cdot 6H\textsubscript{2}O 0.49; pH 7.4). The cardiac myocytes were transferred to an environmental chamber at 37°C in a humidified atmosphere flushed with 5% CO\textsubscript{2} and <1% oxygen (F-102, Iijima Electronics Co) in nitrogen for 5.5 hours and were then reoxygenated for 1 hour with DMEM.

To examine the role of angiotensin and bradykinin in hypoxia/reoxygenation injury, the myocytes were treated with the following before hypoxia/reoxygenation: (1) modified Tyrode solution (control); (2) cilazaprilat (10\textsuperscript{-8} to 10\textsuperscript{-5} mol/L); (3) CV-11974 (10\textsuperscript{-8} to 10\textsuperscript{-5} mol/L); (4) angiotensin II (10\textsuperscript{-9} to 10\textsuperscript{-8} mol/L); and (5) bradykinin (10\textsuperscript{-9} to 10\textsuperscript{-8} mol/L). Furthermore, to examine the mechanism of the cardioprotective effect of cilazaprilat, myocytes were treated with cilazaprilat (10\textsuperscript{-8} mol/L) before hypoxia/reoxygenation in the presence of (6) Hoe 140 (10\textsuperscript{-8} mol/L), (7) staurosporine (2\times10\textsuperscript{-9} mol/L), (8) L-NMMA (4\times10\textsuperscript{-4} mol/L), (9) aminoguanidine (5\times10\textsuperscript{-4} mol/L), (10) indomethacin (10\textsuperscript{-3} mol/L), and (11) methylene blue (10\textsuperscript{-5} mol/L).

Assay of Bradykinin Concentration

Bradykinin concentration in the culture media was measured during hypoxia as shown in Figure 1, according to a previously described method. Briefly, 0.1-mL samples of culture supernatant were acidified with 5 mL of 0.01 mol/L HCl and extracted twice with 20 mL of diethyl ether. The aqueous phase was taken to dryness with a rotary evaporator, and the dried samples were redissolved in 2.5 mL of 0.1 mol/L Tris-HCl buffer containing 0.2% gelatin, 0.1% neomycin, and 0.01 mol/L EDTA.
adjusted to pH 7.4. The incubation mixture for radioimmunoassay consisted of 0.1 mL of 0.01 mol/L 1,10-phenanthroline HCl, diluent buffer of 0.5 mL containing the unknown or standard bradykinin, 0.1 mL of antiserum diluted 1:600 with diluent buffer, and 0.1 mL of (\(^{125}\text{I}-\text{Tyr}\) )-bradykinin (\(\approx 8000\) cpm) dissolved in normal saline. The mixture was incubated in a polyethylene tube at 4°C for 24 hours, and dextran-coated charcoal was used to separate the free labeled antigen from that bound to antibody. Three replicate tubes containing only buffer, phenanthroline, and (\(^{125}\text{I}-\text{Tyr}\) )-bradykinin were incubated and treated with coated charcoal to determine the amount of labeled antigen that remained in the supernatant in the absence of antibody. The mean value of this measurement was subtracted from supernatant radioactivity after centrifugation of the antibody-containing tubes, and the resultant value was used to calculate the proportion of label bound to antibody.

**Measurement of cGMP in Cardiac Myocytes**

cGMP concentration in myocytes was measured after 1, 3, and 5.5 hours of hypoxia (Figure 1). Cardiac myocytes (2.7 \(\times\) \(10^6\) cells per dish) were treated with 0.25 mL of ice-cold 6% trichloroacetic acid and centrifuged at 1000 \(g\) for 10 minutes. The supernatant was extracted 3 times with 3 mL of diethyl ether saturated with water, and the aqueous phase was stored at 80°C. cGMP concentration in the supernatant was measured by radioimmunoassay. Briefly, 0.1 mL of dioxane-triethylamine mixture containing succinic anhydride succinylated cGMP was added to the supernatant (0.1 mL). After a 10-minute incubation, the reaction mixture was added to 0.8 mL of 0.3 mol/L imidazole buffer (pH 6.5). Succinyl cGMP tyrosine methyl ester (0.1 mL) iodinated with 125I (15,000 to 20,000 cpm in 0.1 mL of 0.3 mol/L imidazole buffer) was added to the assay mixture containing 0.1 mL of supernatant and 0.1 mL of diluted antisera, and the mixture was incubated at 4°C for 20 hours. A cold solution of dextran-coated charcoal (0.5 mL) was added to the mixture in an ice-cold water bath. The charcoal was spun down, and 0.5 mL of the supernatant was used for radioactivity in a gamma spectrometer. The amount of cGMP was normalized to protein content of cardiac myocytes assayed by the Lowry method.

**Measurement of ATP in Cardiac Myocytes**

The ATP content of myocytes was measured before hypoxia, after 3 or 5.5 hours of hypoxia, and after 1 hour of reoxygenation (Figure 1). Cardiac myocytes (2.7 \(\times\) \(10^6\) cells per dish) were treated with 0.25 mL of 0.6N ice-cold perchloric acid and centrifuged at 1000 \(g\) for 5 minutes at 4°C. The supernatant was neutralized with KOH to pH 5.0 for 5 minutes, and, after 10 minutes, was centrifuged at 8000 \(g\) for 5 minutes at 4°C to remove the KClO\(_3\). The supernatant was used for the assays. ATP was measured by high-performance liquid chromatography (LC-9A liquid chromatograph, Shimadzu) with a column of STR ODS-M (Shimadzu). Statistical Analysis

Data are expressed as mean\(\pm\)SEM of 6 samples derived from \(\approx\)6 separate experiments. Differences were analyzed by 2-way ANOVA combined with Scheffe’s test, and a \(P\) value of \(<0.05\) was considered to be statistically significant.

**Results**

**Effects of Cilazaprilat, CV-11974, Angiotensin II, and Bradykinin on Hypoxia/Reoxygenation Injury**

Treatment of myocytes with 5.5 hours of modified Tyrode solution followed by 1 hour of DMEM medium under normoxic conditions or hypoxia alone (5.5 hours) did not cause a significant release of CK into the myocyte culture media (data not shown). However, hypoxia followed by reoxygenation caused a significant increase in CK release, as shown in the control bar of Figure 2. Figure 2 also shows the effect of pretreatment with cilazaprilat, CV-11974, angiotensin II, and bradykinin on this reoxygenation-induced CK release. Cilazaprilat reduced CK release in a dose-dependent fashion to 36% of control at \(10^{-5}\) mol/L. Although CV-11974 is reported to block the effect of angiotensin II and III, it did not significantly inhibit CK release in the present study (Figure 2). In addition, there was no significantly additive increase in CK release after administration of angiotensin II. In contrast, bradykinin significantly lowered CK release in a dose-dependent manner, with \(10^{-2}\) mol/L bradykinin reducing CK release to 37% of control.

**Cardioprotective Mechanism of Cilazaprilat Against Hypoxia/Reoxygenation Injury**

Reoxygenation-induced CK release in the control and cilazaprilat-treated groups in the presence of Hoe 140, staurosporine, and amino-guanidine, indomethacin, and methylene blue on cilazaprilat-induced cardioprotection. Myocytes were treated with modified Tyrode buffer containing cilazaprilat (10\(^{-5}\) mol/L) in presence or absence of Hoe 140 (10\(^{-6}\) mol/L), staurosporine (2\(\times\)10\(^{-9}\) mol/L), L-NMMA (4\(\times\)10\(^{-4}\) mol/L), amino-guanidine (5\(\times\)10\(^{-4}\) mol/L), indomethacin (10\(^{-5}\) mol/L), or methylene blue (10\(^{-5}\) mol/L) before hypoxia/reoxygenation. CK release was measured as described in Methods. \(n\)=6. \(*P<0.05\), \(*\ast P<0.01\) vs control; \#\#P<0.01 vs cilazaprilat.
rosporine, L-NMMA, aminoguanidine, indomethacin, and methylene blue is shown in Figure 3. Cilazaprilat (10^{-5} mol/L) significantly reduced CK release compared with control. However, the protective effect of cilazaprilat was significantly inhibited by cotreatment with 10^{-6} mol/L Hoe 140 or 4×10^{-4} mol/L L-NMMA but not 2×10^{-9} mol/L staurosporine, 5×10^{-4} mol/L aminoguanidine, or 10^{-3} mol/L indomethacin. Cotreatment with 10^{-5} mol/L methylene blue partially blunted the cardioprotection by cilazaprilat.

Bradykinin Accumulation in Myocytes and Nonmyocytes During Hypoxia

Figure 4 show the time course of bradykinin concentration in the culture media and immunofluorescence stainings of myocytes, nonmyocytes, and RAECs. Hypoxia significantly increased bradykinin levels in the myocytes. Treatment of myocytes with 10^{-5} mol/L cilazaprilat significantly increased bradykinin production to 4.5 times that of control after 5.5 hours of hypoxia. In contrast, hypoxia did not enhance bradykinin production even in the presence of 10^{-6} mol/L cilazaprilat in nonmyocytes. Hypoxia also significantly increased bradykinin levels in RAECs, and 10^{-5} mol/L cilazaprilat significantly enhanced bradykinin levels to 2 times that of control after 5.5 hours of hypoxia.

cGMP Content in Cardiac Myocytes During Hypoxia

The time course of cGMP content change in the myocytes is illustrated in Figure 5. cGMP content in control cells did not
change appreciably during the 5.5 hours of hypoxia. However, cilazaprilat 10⁻⁵ mol/L markedly increased cGMP content with increased time of hypoxia. This augmentation of cGMP production by cilazaprilat was blunted by cotreatment with 4×10⁻⁴ mol/L L-NMMA or 10⁻⁵ mol/L methylene blue. In contrast, 5×10⁻⁴ mol/L aminoguanidine did not block the cilazaprilat-induced increase in cGMP content. Bradykinin (10⁻⁶ mol/L) significantly promoted cGMP production throughout the hypoxic period.

**ATP Content in Cardiac Myocytes During Hypoxia/Reoxygenation**

The time course of ATP content change in myocytes is shown in Figure 6. Exposure to 6.5 hours of normoxic culture conditions alone did not affect ATP content in the myocytes. In the control, hypoxia significantly lowered ATP content in a time-dependent manner, and reoxygenation induced a further decrease in ATP. Both cGMP production by cilazaprilat was blunted by cotreatment with 4×10⁻⁴ mol/L L-NMMA or 10⁻⁵ mol/L methylene blue. In contrast, 5×10⁻⁴ mol/L aminoguanidine did not block the cilazaprilat-induced increase in cGMP content. Bradykinin (10⁻⁶ mol/L) significantly promoted cGMP production throughout the hypoxic period.

**Discussion**

In the present study, we have demonstrated that an ACE inhibitor, cilazaprilat, shows remarkable protection against hypoxia/reoxygenation injury in a dose-dependent fashion. Recent reports demonstrated the existence of a local renin-angiotensin system within the myocardium and indicated that angiotensin II type 1 receptor antagonist improves ischemia-reperfusion injury. In the present study, however, pretreatment with the angiotensin II receptor antagonist CV-11974 or angiotensin II did not worsen CK release (Figure 2), which strongly suggests that cilazaprilat reduced myocardial hypoxia/reoxygenation injury independently of angiotensin II synthesis.

Our results also show that the cardioprotective effect of cilazaprilat is blocked by Hoe 140, which suggests that its effect is mediated by the bradykinin B₂ receptor (Figure 3). Furthermore, we demonstrated that bradykinin production during hypoxia was significantly increased by cilazaprilat (Figure 4). It has been reported that a local kinin-kallikrein system exists in the heart and that myocardial bradykinin levels are further enhanced by ischemia or ACE inhibitors through their inhibitory effect on the degradation of kinins.

It is still unclear which cells produce the bradykinin in the heart. One recent report suggested that the coronary vascular endothelium is the main source of the release of kinins. However, it is likely that myocytes also play a significant role because bradykinin levels were significantly enhanced by cilazaprilat after hypoxia in myocytes but not in nonmyocytes under our culture conditions (Figure 4). In addition, we have confirmed that nonmyocytes consist of 95% fibroblasts and 1% to 2% endothelial cells. Furthermore, the enhancement in bradykinin production by cilazaprilat in myocytes was greater than that in RAECs. Although these results were obtained from in vitro studies using neonatal cardiac myocytes, the present data strongly suggest that myocytes may be an important source of bradykinin and that ACE inhibitors may act directly on myocytes through a local kinin-kallikrein system, thereby contributing to cardioprotection in an autocrine/paracrine fashion. Indeed, recent data indicate that kallikrein activity can be detected in primary cultures of neonatal rat cardiocytes and in heart slices.

Previous observations have also demonstrated the existence of functional bradykinin B₂ receptors on cardiomyocytes, which are coupled to the activation of phospholipase C, the subsequent generation of inositol 1,4,5-triphosphate or diacylglycerol, an increase in cytosolic Ca²⁺ levels, and the activation of PKC. Elevated cytosolic Ca²⁺ can stimulate phospholipase A₂ and cyclooxygenase, as indicated by the production of prostaglandins. Furthermore, the increase in cytosolic Ca²⁺ through the B₂ receptor may also stimulate myocyte constitutive NO synthase (cNOS). In the present study, the cardioprotective effect of cilazaprilat was significantly blocked by L-NMMA but not by staurosporine, aminoguanidine, or indomethacin (Figure 3), therefore suggesting that the effect of cilazaprilat is not mediated by PKC, inducible NOS, or prostaglandins but rather by a cNOS-associated, bradykinin B₂ receptor–mediated pathway.

The present study also indicates that the protective effect of cilazaprilat is mediated by cGMP, because methylene blue significantly blocked this effect (Figure 3). In addition, cGMP content was significantly increased in hypoxic myocytes after treatment with cilazaprilat, and this augmentation of cGMP was blunted by cotreatment with L-NMMA and methylene blue but not aminoguanidine (Figure 5). The data therefore suggest that cGMP production in myocytes treated with cilazaprilat is mediated by a cNOS-NO–guanylate cyclase signaling pathway. It has been reported previously that cGMP can improve the energy state in the ischemic heart. Indeed, in the present study, cilazaprilat as well as bradykinin significantly preserved the ATP content of myocytes (Figure 6).

Although the role of cGMP in regulating myocardial contraction remains controversial, recent reports suggest that...
cGMP can regulate sarclemmal Ca$^{2+}$ influx through L-type Ca$^{2+}$ channels (I_{Ca,L}) by activation of a cGMP-stimulated phosphodiesterase 3B, or by cGMP-dependent protein kinase (PKG) and can reduce the myofilament response to Ca$^{2+}$ via activation of an endogenous cGMP-dependent protein kinase (cGMP-PK). Furthermore, cGMP has been recently reported to mediate the negative inotropic effect of NO. It is therefore tempting to speculate that NO production induced by cell-cell interactions modulates myocyte contractility and contributes to the energy-sparing effect, although we cannot exclude the possibility that other effects of NO, such as radical scavenging action, also contribute to cardioprotection.

In conclusion, the present study demonstrates that cell-cell interactions can protect isolated myocytes against hypoxia/reoxygenation injury, possibly as a result of bradykinin accumulation and the resultant production of NO by cNOS in hypoxic myocytes in an autocrine/paracrine fashion. NO may increase cGMP synthesis in myocytes, which may consequently modulate their contractility and may contribute to energy preservation and cardioprotection.

Acknowledgments

The authors are grateful to Dr Henry Fliss, Department of Cellular and Molecular Medicine, University of Ottawa, Canada, for the generous academic advice, and to Dr Shinni Fushiki, Department of Dynamic Pathology, Kyoto Prefectural University of Medicine, for the technical advice of immunohistochemical examination.

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* Circulation. 1999;99:817-822  
  doi: 10.1161/01.CIR.99.6.817

*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231  
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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