Novel Mechanism of Action for Hydralazine
Induction of Hypoxia-Inducible Factor-1 α, Vascular Endothelial Growth Factor, and Angiogenesis by Inhibition of Prolyl Hydroxylases

Helen J. Knowles, Ya-Min Tian, David R. Mole, Adrian L. Harris

Abstract—The vasodilator hydralazine, used clinically in cardiovascular therapy, relaxes arterial smooth muscle by inhibiting accumulation of intracellular free Ca\(^{2+}\) via an unidentified primary target. Collagen prolyl hydroxylase is a known target of hydralazine. We therefore investigated whether inhibition of other members of this enzyme family, namely the hypoxia-inducible factor (HIF)-regulating O\(_2\)-dependent prolyl hydroxylase domain (PHD) enzymes, could represent a novel mechanism of action. Hydralazine induced rapid and transient expression of HIF-1α and downstream targets of HIF (endothelin-1, adrenomedullin, haem oxygenase 1, and vascular endothelial growth factor [VEGF]) in endothelial and smooth muscle cells and induced endothelial cell-specific proliferation. Hydralazine dose-dependently inhibited PHD activity and induced nonhydroxylated HIF-1α, evidence for HIF stabilization specifically by inhibition of PHD enzyme activity. In vivo, hydralazine induced HIF-1α and VEGF protein in tissue extracts and elevated plasma VEGF levels. In sponge angiogenesis assays, hydralazine increased stromal cell infiltration and blood vessel density versus control animals. Thus, hydralazine activates the HIF pathway through inhibition of PHD activity and initiates a pro-angiogenic phenotype. This represents a novel mechanism of action for hydralazine and presents HIF as a potential target for treatment of ischemic disease. (Circ. 2004;95:162-169.)

Key Words: cardiovascular disease • iron chelation • ischemia • endothelial cells

Hydralazine is a vasodilator used to treat severe hypertension, congestive heart failure, myocardial infarction, and preeclampsia.\(^1,2\) It is thought to reduce peripheral resistance directly by relaxing the smooth muscle cell layer in arterial vessels. The precise mechanism of action remains unknown but potentially involves an altered Ca\(^{2+}\) balance in vascular smooth muscle cells, whereby inhibition of Ca\(^{2+}\) release from the sarcoplasmic reticulum\(^3\) prevents contraction mediated by Ca\(^{2+}\)-dependent ATPases, kinases,\(^4\) or ion channels.\(^5\) This disrupted Ca\(^{2+}\) balance is proposed to be secondary to either a membrane hyperpolarization event\(^6\) or an elevation of intracellular cGMP levels.\(^7\) However, no direct target of hydralazine has been identified explaining this vascular effect. During treatment of congestive heart failure, concomitant administration of hydralazine with isosorbide dinitrate prevents early development of nitrate tolerance and reduces long-term mortality.\(^8,9\) In this context, hydralazine is thought to inhibit activation of a membrane-associated oxidase responsible for the increased superoxide production that causes nitrate tolerance.\(^10\)

The range of physiological, cellular, and molecular actions of hydralazine might be explained if a primary target molecule(s) could be identified. One long-established target is protocollagen prolyl hydroxylase (CPH), a member of the Fe\(^{2+}\)-dependent and 2-oxoglutarate (OG)-dependent dioxygenase family. Hydralazine is thought to complex with enzyme-bound Fe\(^{2+}\), inhibiting CPH activity\(^11\) and preventing the posttranslational hydroxylation of collagen prolyl residues essential to form stable collagen fibers.\(^12\) Other members of this family include the regulators of hypoxia-inducible factor-α (HIF-α) subunits, prolyl hydroxylase domain (PHD) enzymes 1 to 3.\(^13,14\) Under normoxic conditions, these enzymes posttranslationally hydroxylate HIF-α, targeting it for interaction with the von Hippel–Lindau (pVHL) E3 ubiquitin ligase complex and rapid proteasomal degradation.\(^13,14\) The PHD enzymes have an absolute requirement for dioxygen and are therefore inactive under hypoxia, allowing HIF accumulation and activation of pathways regulating processes such as angiogenesis and metabolic adaptation.\(^15\)

We have investigated effects of hydralazine on the HIF system. We present evidence that hydralazine rapidly and transiently induces HIF-1α protein and downstream targets of HIF via inhibition of the PHD enzymes and stimulates specific endothelial cell (EC) proliferation in culture and rapid neo-angiogenesis in vivo. We have identified a novel target molecule for hydralazine and a potential mechanism of action for its therapeutic role clinically.

Original received June 9, 2003; resubmission received April 21, 2004; revised resubmission received May 14, 2004; accepted May 27, 2004.

From the Cancer Research UK Molecular Oncology Laboratory (H.J.K., A.L.H.), Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford; and the Henry Wellcome Building of Genomic Medicine (Y.-M.T., D.R.M.), University of Oxford, Roosevelt Drive, Oxford, UK.

Correspondence to Prof Adrian L Harris, Cancer Research UK Molecular Oncology Laboratory, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DS, UK. E-mail harrisa@cancer.ox.ac.uk

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Circulation Research is available at http://www.circresaha.org

DOI: 10.1161/01.RES.0000134924.89412.70
Cell Culture

Human umbilical vein EC (HUVEC), human dermal microvascular EC, and human vascular smooth muscle cells (HVS/MC) (Cambrex Bioscience, Wokingham, UK) were maintained in MCDB with 20% fetal bovine serum, L-glutamine (2 mM/L), heparin (5 IU/mL) and EC growth supplement (50 μg/mL). MDA 468 breast carcinoma cells were maintained in DMEM with 10% fetal bovine serum, L-glutamine, penicillin (50 IU/mL), and streptomycin sulfate (50 μg/mL). pVHL-deficient renal carcinoma cells (RCC4 VA) and VHL stable transfectants (RCC4 VHL) were cultured in DMEM and maintained in selection with 500 μg/mL G418. Hypoxic exposure (0.1% O2, 5% CO2, balance N2) was in a Heto-Holten CellHouse 170 (RS Biotech, Irvine, Scotland). Reagents were hydralazine HCl, CoCl2, DFO (Sigma, Poole, UK), and the proteasome inhibitor MG132 (Affiniti Research Products, Exeter, UK). Dimethylxalylglycine (MMOG) was a gift from C. Schofield (Dyson Perrins Laboratory, Oxford, UK).

Hydroxylated HIF-1α Antibody

Polycyonal antihydroxylated HIF-1α antiserum was raised in rabbit against the synthetic peptide CDLEMLAHypYIPMD (HIF-1α amino acids 558 to 569 with hydroxylated Pro569). Serum was tested for its ability to distinguish hydroxylated and native HIF-1α in RCC4 VHL cell extract under normoxia, hypoxia, or proteasomal inhibition.

Western Blotting

Cells were homogenized in lysis buffer (6.2 mol/L urea, 10% glycerol, 5 mM/L DTT, 1% SDS, protease inhibitors), lysate separated by 8% SDS-PAGE, and transferred to PVDF membrane. Primary antibodies were monoclonal antihuman (BD Transduction Laboratories, Lexington, Ky) or antismouse (Novus Biologicals, Littleton, Col) HIF-1α and anti-β-tubulin (Sigma). Immunoreactivity was visualized with HRP-linked goat antimouse serum and chemiluminescence.

Polymerase Chain Reactions

Total RNA was extracted in TRI Reagent (Sigma), reverse-transcribed (1st Strand cDNA Synthesis Kit; Roche Diagnostics, Mannheim, Germany) and polymerase chain reaction (PCR) amplified within the linear range using the Expand High Fidelity PCR System (Roche). PCR primers were: adrenomedullin 5’-TCTCTGCTGTTTGTGG-3’ and 5’-AGCG-TGGCTTAGA-3’; and haemoyxigenase-1 5’-TCTTCTCAGAAGAGCTGCA-3’ and 5’-AGCG-GTGAGACTGTGGAATCT-3’.

Vascular Endothelial Growth Factor and Endothelin-1 Enzyme-Linked Immunosorbsent Assay

Culture medium and tissue extracts were assayed using the Quantikine human vascular endothelial growth factor (VEGF) immunoassay or QuantiGlo human endothelin-1 immunoassay (R&D Systems). In the HIF-VHL capture assay, RCC4 VA cell extract was assayed in the presence of small amounts of the metabolizing enzyme N-acetyltransferase 2 may also contribute. The level of induction was concentration-dependent and decayed with time (Figure 1A), although doses >500 μM/L were toxic (data not shown). After decay of the response, a repeat 100-μM/L dose re-induced HIF-1α to a level comparable with the initial response (Figure 1B). This is consistent with instability of the active form of hydralazine in tissue culture (Figure 1B), as also occurs in blood plasma. Presence of small amounts of the metabolizing enzyme N-acetyltransferase 2 may also contribute. All cell lines tested demonstrated a comparable time course for HIF-1α induction, including smooth muscle cells and several cancer cell lines. In all cases, 50 μM/L hydralazine induced peak HIF-1α expression 1 to 2 hours after application, which returned to baseline by 16 to 24 hours (Figure 1C). Peak levels of induction were similar to that induced by hypoxia...
We therefore performed subsequent in vitro experiments with 50 μmol/L hydralazine, a nontoxic concentration that induces physiological levels of HIF-1α.

Hydralazine Induces HIF Target Genes in Culture

To determine whether hydralazine-induced HIF was transcriptionally active, we examined induction of downstream genes, focusing on those that modulate vascular tone and might be involved in vascular actions of hydralazine in vivo. In HUVEC, 50 μmol/L hydralazine induced adrenomedullin, haem oxygenase-1 (HO-1), and endothelin-1 (Et-1) mRNAs to a level approximating hypoxic induction (Figure 1D). Early expression peaked at 4 hours, complementing rapid induction of HIF-1α. Et-1 and HO-1 expression was maintained for 24 hours, suggesting potential positive feedback mechanisms regulating hydralazine-induced genes. At the protein level, significant Et-1 secretion was observed at 16 to 24 hours (Figure 1E, *P < 0.01 versus untreated control). 50 μmol/L hydralazine increased VEGF secretion from HVSMC and MDA468 (Figure 1F), although no effects were observed on either secreted or cell-bound VEGF in HUVEC (data not shown). This is consistent with reports that EC do not usually produce VEGF under normoxic or hypoxic conditions, either in culture or in vivo. Thus, hydralazine initiated rapid activation of the HIF transcriptional cascade in target cell lines in culture.

Hydralazine Inhibits PHD Enzyme Activity

Hydralazine (Figure 2A) complexes with free iron in solution and inhibits CPH activity by forming a putative labile complex with enzyme-bound iron at the active site. It might therefore inhibit the iron-dependent PHD enzymes via chelation of the active site iron moiety, stabilizing HIF in a similar manner to DFO. Indeed, exogenous free iron inhibited HIF-1α induction by equimolar concentrations of DFO and hydralazine (Figure 2B). We therefore assayed PHD activity in the presence of hydralazine. Dose-dependent inhibition of PHD enzyme activity was detected in HUVEC cell extract by analysis of HIF peptide-dependent degradation of 2-OG (Figure 2C, light bars). This dose-dependency complements the hydralazine-mediated induction of HIF-1α also observed in HUVEC (Figures 1A and 2C, dark bars), and the results from the HIF-VHL capture assay (Figure 2C, lower panel). It is clear that 50 μmol/L hydralazine is an insufficient dose to maximally inhibit PHD enzyme activity or maximally induce HIF-1α protein. However, it is nontoxic
and sufficient to induce physiological levels of HIF-1α in target vascular cells and induce downstream components of the pathway (Figure 1).

As further evidence that HIF-1α induction is caused by PHD inhibition, we analyzed the hydroxylation status of hydralazine-induced HIF-1α (Figure 2D). When PHDs are fully active, HIF-1α is hydroxylated and rapidly degraded. Any HIF-1α induced because of PHD inhibition should therefore be in the nonhydroxylated form. As shown in Figure 2D, hydralazine-induced HIF-1α was nonhydroxylated, demonstrating the same level of background antibody reactivity as in untreated control cells and during complete PHD inactivation (anoxia and MMOG). Also, hydralazine prevented hydroxylation of hypoxia-induced HIF-1α caused by PHD reactivation on reoxygenation (Figure 2D).

To eliminate other possible mechanisms of HIF-1α induction, we assayed effects of hydralazine in pVHL-deficient RCC4 VA cells that express HIF constitutively because of a defect in targeting the protein for degradation. Fifty micromoles per liter hydralazine did not increase basal HIF-1α levels (Figure 2E), indicating that it induces neither transcription nor translation but modulates HIF-1α degradation. Also, hydralazine did not interfere directly with the HIF–VHL interaction (data not shown). Together, these data suggest that hydralazine-mediated induction of HIF-1α can be explained directly by inhibition of PHD enzyme activity.

**Hydralazine Stimulates EC Proliferation**

Considering the vascular functions of hydralazine-induced genes, we hypothesized that hydralazine might stimulate EC proliferation. Fifty to 100 μmol/L hydralazine induced significant proliferation of HUVEC and HDMEC (Figure 3). Higher concentrations resulted in toxicity. This pro-proliferative effect was EC-specific. Hydralazine-induced EC proliferation (but not endogenous growth) was inhibited by the NO synthase inhibitor L-NAME (Figure 3 and data not shown). Both HIFα23 and Et-124 induce NO release from EC. Although not itself a growth factor, NO mediates autocrine-positive feedback of Et-1 expression25 and induces HO-1 transcription and mRNA stabilization,26 possible explanations for its apparent pro-proliferative effect and the prolonged induction of these mRNAs (Figure 1D). In concordance with Figure 1 data, EC proliferation was not inhibited by anti-VEGF neutralizing antibody at concentrations that inhibit proliferation mediated by 10 ng/mL VEGF (Figure 3 and data not shown).
In agreement with reports on the selective toxicity of iron chelators toward cancer cells,27 hydralazine concentrations that were pro-proliferative in EC and demonstrated no effect in HVS PMC significantly inhibited MDA468 cell growth (Figure 3). Unexpectedly, higher concentrations did not produce this effect. It is possible that the 250-µmol/L dose induced sufficient HIF-1α to compensate in some manner for toxic effects of iron chelation; however, because lower doses were generally used, this effect was not pursued.

**Hydralazine Induces HIF-1α and VEGF In Vivo**

In vivo analyses of hydralazine-mediated vasodilation describe substantial effects on vascular tone, blood flow, and tissue perfusion parameters.26–30 These effects show rapid onset after intravenous administration and are transient; for example, peak effects on normal murine tissue perfusion occur 1 to 2 hours after injection.28 This rapid and transient vascular response is strikingly similar to the hydralazine-induced HIF response we observed in culture. We therefore sought to determine whether HIF, and its downstream targets, are activated at early time points in vivo. Five milligrams per kilogram IV hydralazine28,29 stabilized HIF-1α protein in normal murine lung, heart, liver, and spleen (Figure 4A through 4F and Tables 1 and 2). This occurred 30 minutes after injection in lung and peaked at 1 hour in the majority of tissues tested. It is of note that we observed HIF-1α stabilization in organs of <50% of the mice. This was not caused by gender or age-related differences and may be a result of individual variation in response rate and/or to difficulties associated with preparation of tissue extracts for such a labile factor as HIF-1α. Despite this, significant HIF-1α induction was observed in 4 of 6 tissues (Table 1). We next assayed VEGF levels in tissue extracts and blood plasma. Combining all time points, VEGF was significantly increased in lung (*P<0.03) and liver (*P<0.04, Table 1), whereas VEGF induction in blood plasma was significant at 2 hours (*P<0.05; Table 2). It was therefore evident that hydralazine rapidly activates the HIF pathway in normal tissues in vivo. In parallel with the extensively reported effects on vascular tone, this suggests a potential role for components of this pathway in mediating the physiological response to hydralazine.

**Hydralazine Is Proangiogenic In Vivo**

Considering the pro-proliferative effect on EC, we hypothesized that hydralazine might induce angiogenesis in vivo. Daily 5 mg/kg hydralazine intravenously increased stromal cell infiltration into subcutaneously implanted sponges (8.9±1.4% infiltration versus 4.9±0.9%) in association with significantly increased blood vessel density (160±52 vessels/mm² versus 32±12, *P<0.02) at 6 days compared with controls (Figure 5A through 5C). No significant difference in either parameter was observed at 14 and 21 days (Figure 5D). The apparent reduced blood vessel density in the hydralazine-treated group at later time points was caused by 2 factors. First, the highest blood vessel concentration within the sponge always occurred at the leading edge of infiltration. As the degree of infiltration increased, the volume of the leading edge decreased, resulting in a reduced volume of tissue containing high-density vasculature. Second, at later time points, remodeling of the vasculature occurred to form fewer, larger vessels (compare Figure 5C and 5D). It was therefore evident that in this model, chronic administration of hydralazine accelerated the angiogenic response, resulting in an acute period of elevated tissue vascularization.

**Discussion**

We have demonstrated rapid and transient induction of HIF-1α by hydralazine in culture and in murine systems. No innate cell-type specificity was evident in hydralazine-mediated induction of HIF, although downstream consequences showed such effects. Hydralazine stimulated VEGF production in smooth muscle cells and cancer cell lines but not in EC. Conversely, it stimulated EC proliferation but not that of other cell types. It is recognized that EC do not generally induce VEGF,20,21 but VEGF production from the adjacent smooth muscle cell layer and other cells in vivo could modify EC function. Additionally, activation of the HIF transcriptional cascade upregulated a series of EC growth factors. Adrenomedullin,31 endothelin-1,32 and VEGF33 all induce EC proliferation and promote angiogenesis in
vivo. HO-1 protects EC function under oxidative stress by catalyzing conversion of haem to biliverdin, which is cytoprotective versus reactive oxygen species, and to CO, which mediates vasodilation.44 Additionally, all these molecules induce cGMP35–38 and are therefore candidates for the intermediate molecule(s) proposed to regulate the hydralazine-induced accumulation of cGMP initiating smooth muscle relaxation in vivo.7

We have demonstrated that rapid stabilization and activation of the HIF system also occurs in vivo using standard doses28,29 modeling the clinical effect.30,39 HIF levels were sufficient to elevate tissue and plasma VEGF significantly 2 hours after injection, in concordance with maximal effects on vascular tone seen at 2 to 4 hours in murine models28,29 and humans.30,39 Thus, the time course of induction of the HIF system in vivo is compatible with that of established vascular effects of hydralazine. It is also of note that the target organs showing HIF induction by hydralazine are the same as those in which it is induced by hypoxia, namely the liver, heart, lungs, spleen, and kidney.30,41 Added to the in vitro data, this suggests the HIF system as a candidate for the effector pathway mediating actions of hydralazine in vivo.

We have identified the HIFα-regulating PHD enzymes as molecular targets for hydralazine. Hydralazine inhibited PHD enzyme activity in cell-free assays and prevented hydroxylation (and subsequent degradation) of HIF-1α in cultured cells. This started at a 50 μmol/L concentration with only small inhibition of PHD activity using either cell extracts or recombinant proteins (10% inhibition by HIF–VHL capture assay). It is worth noting that the iron concentrations required for the assays could affect the chelation capacity of hydralazine, especially at lower doses. Although 5 μmol/L (capture assay) is within the upper range of cellular free iron concentration, the 50 μmol/L iron in the 2-OG degradation assay might affect chelation. However, a clear dose response was evident in the 2-OG degradation assay and, using standard doses, stabilization of HIF-1α has been observed in animals, suggesting hydralazine is a sufficiently powerful chelator to bind enzyme-associated iron under physiological conditions and reduce PHD activity.

It is of interest to consider whether rapid activation of the HIF system in vascular cells could mediate the clinical effects of hydralazine. We have obtained direct evidence of inhibition of PHD activity, induction of HIF-1α and downstream genes, and pro-proliferative effects of hydralazine on HUVEC. These effects were greater with doses >50 μmol/L, but these are not necessary for induction of HIF protein or proliferation. In vivo there is increasing evidence of an association between endothelial dysfunction, hypertension, and cardiovascular disease. Impaired EC-dependent vasorelaxation caused by loss of NO activity in vessel walls can be caused by factors including abnormal regulation of endothelial NOS and NO degradation by reactive oxygen species.42 There is no documented evidence linking hydralazine with endothelial NOS and, unlike other hydrazine derivatives, hydralazine cannot be oxidized to release NO directly.43 However, hydralazine-mediated HIF pathway activation in EC may release NO indirectly (eg, via Et-1) and genes such as VEGF also modulate vascular tone, potentially explaining its acute vasodilatory effect.

We have also identified a novel effect of hydralazine that could explain the chronic benefits of long-term administration. Hydralazine induced EC proliferation in vitro and in vivo, where chronic systemic administration at doses modeling the clinical administration initiated a pro-angiogenic phenotype. In myocardial ischemia patients, recombinant VEGF has been shown to improve perfusion and coronary vessel density.44 Similarly, adrenomedullin infusion enhances coronary blood flow and cardiac function.45 It also promotes re-endothelialization and is antiapoptotic and cardioprotective in transgenic mice.46 Although we have only measured VEGF levels in vivo, it is likely that this angiogenic effect is mediated by several different growth factors. This is supported by data that mice overexpressing VEGF alone develop inflammation and vascular leakage, in contrast to the leakage-resis-

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<tr>
<th>Time Postinjection</th>
<th>VEGF (pg/mL)</th>
<th>½ Hour (n=4)</th>
<th>1 Hour (n=9)</th>
<th>2 Hours (n=9)</th>
<th>All Timepoints (n=22)</th>
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<tr>
<td>Plasma</td>
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<td>Hydralazine</td>
<td></td>
<td>79.1±9.1</td>
<td>86.0±20.7</td>
<td>85.3±14.9</td>
<td>84.4±16.3</td>
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<tr>
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<td>74.3±6.4</td>
<td>78.3±9.0</td>
<td>71.3±14.0</td>
<td>74.7±11.0</td>
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<td>Significance</td>
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NS indicates not significant.
tant hypervascularity induced by HIF-1α overexpression. Induction of an angiogenic phenotype may therefore represent a novel mechanism whereby chronic hydralazine administration reduces mortality in congestive heart failure.

Hypoxia is fundamental to many ischemic diseases including stroke, pulmonary hypertension, cancer, myocardial ischemia, and chronic lung disease. HIF mediates the pathophysiological response to hypoxia in several such conditions and modulation of its activity has been proposed as a therapeutic approach. Such a strategy raises questions regarding possible effects of systemic activation of the HIF system, which may enhance erythropoiesis and elevate glycolytic and angiogenic capacity in areas besides the target organ. None of these side effects is described for hydralazine. This may be due to the transient nature of hydralazine effects, because of rapid metabolism of the active drug, making it difficult to detect and determine pharmacokinetic properties clinically. One side effect of hydralazine is a lupus erythematosus-like syndrome characterized by antinuclear autoantibodies and connective tissue lesions. This could possibly represent activation of HIF downstream genes, such as collagen and fibronectin, but is rare at the lower doses of hydralazine currently used. Target specificity and low chronic toxicity are key in the design of novel agents activating HIF. We have demonstrated that an agent acting through rapid, short-term activation of the HIF pathway is clinically viable, suggesting that intermittent activation of HIF with rapid metabolism of the activator can result in vascular specificity.

In summary, we have identified a potential novel mechanism for the therapeutic effect of hydralazine in cardiovascular therapy. First, that hydralazine transiently activates the HIF system by inhibiting PHD enzyme activity. Second, that hydralazine promotes angiogenesis. Development of novel agents activating HIF could therefore be feasible for treatment of ischemic disease, with the potential to monitor target specificity through plasma markers of HIF such as VEGF and endothelin-1.

Acknowledgments

The work was supported by the Wellcome Trust and Cancer Research (UK). The authors are grateful to Professor Peter J. Ratcliffe for many helpful discussions.

References


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Circ Res. 2004;95:162-169; originally published online June 10, 2004;
doi: 10.1161/01.RES.0000134924.89412.70
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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