ELF-1 Is a Transcriptional Regulator of the Tie2 Gene During Vascular Development

Antoinise Dube, Shelley Thai, John Gaspar, Susan Rudders, Towia A. Libermann, Luisa Iruela-Arispe, Peter Oettgen

Abstract—Vascular development requires the tightly coordinated expression of several growth factors and their receptors. Among these are the Tie1 and Tie2 receptors, which are almost exclusively endothelial cell–specific. The critical transcriptional regulators of vascular-specific gene expression remain largely unknown. The Ets factors are a family of evolutionarily conserved transcription factors that regulate genes involved in cellular growth and differentiation. We have recently shown that the Ets factor NERF is a strong transactivator of the Tie1 and Tie2 genes. To extend these studies, we have begun to identify the Ets factors that are expressed in developing blood vessels of the chicken chorioallantoic membrane (CAM), a highly vascular embryonic network. RNA was extracted from microdissected CAM blood vessels, and reverse transcriptase–polymerase chain reaction was performed using oligonucleotides encoding conserved amino acids within the Ets domain. One of the polymerase chain reaction fragments was subcloned and identified as the chicken homologue of the Ets factor ELF-1, cELF-1. ELF-1 is most closely related to the Ets factor NERF. In situ hybridization and immunohistochemistry demonstrate that cELF-1 is enriched in developing chicken blood vessels. cELF-1 is also a strong transactivator of the Tie1 and Tie2 genes and can bind to conserved Ets sites within the promoters of these genes. A complex of similar size forms when gel shifts are performed with cellular extracts derived from the CAM blood vessels, which is recognized by an antibody against cELF-1. In summary, ELF-1 belongs to a subset of Ets factors that regulate vascular-specific gene expression during blood vessel development. (Circ Res. 2001;88:237-244.)

Key Words: vascular development ■ angiogenesis ■ Tie2 gene ■ gene expression

Vasculogenesis, the development of new blood vessels, begins during the second week of normal human embryogenesis. The Tie1 and Tie2 genes are one family of endothelial-specific receptor tyrosine kinases that have been determined to be critical for vascular development.1 They are expressed predominantly on endothelial cells of the developing vasculature. Targeted disruption of Tie1 leads to the development of leaky blood vessels, resulting in edema and hemorrhage, whereas disruption of Tie2 leads to the formation of dilated blood vessels with abnormalities in sprouting and branching of the developing blood vessels, resulting in defects in capillary network formation and early embryonic death.2 Mutations in the Tie2 gene have been identified in humans, resulting in venous malformations.3 Although these receptors were described as being completely endothelial cell–specific, it has recently been shown that both receptors are expressed in up to 30% of undifferentiated hematopoietic stem cells and 10% of B cells, suggesting a possible role in hematopoiesis in addition to vasculogenesis.4,5

The Ets genes are a family of at least 30 members that function as transcription factors.6 All Ets factors share a highly conserved 80- to 90-amino acid–long DNA-binding domain, the Ets domain. Ets factors play a central role in regulating genes involved in development, cellular differentiation, and proliferation. Many genes specific to macrophages, B cells, and T cells are regulated by Ets factors. The role of Ets factors in the immune system has been substantiated by experiments in mice, where the genes encoding several Ets factors have been disrupted by homologous recombination. The PU.1 knockout is characterized by a lack of immune-system development.7 The Ets-1 knockout mice are characterized by T-cell apoptosis and increased terminal B-cell differentiation.8

Interestingly, the main regulatory elements found thus far in the Flt-1, Tie1, and Tie2 genes contain several conserved putative Ets-binding sites that are critical for the transcriptional activity of the promoters and enhancers of these genes.9–11 For example, a mutation of one Ets-binding site in the promoter of the Flt-1 gene leads to a 90% reduction in the basal activity of the promoter. Likewise, in transgenic animals in which LacZ expression is directed throughout the vasculature by the Tie2 promoter and enhancer, a mutation in

Original received July 27, 2000; revision received November 2, 2000; accepted November 14, 2000.
From the Cardiology Division (A.D., J.G., S.R., P.O.), Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Mass; New England Baptist Bone and Joint Institute (A.D., J.G., S.R., T.A.L., P.O.), Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Mass; and Department of Molecular Cell and Developmental Biology (S.T., L.I.-A.), University of California at Los Angeles, Los Angeles, Calif.
Correspondence to Peter Oettgen, Harvard Institutes of Medicine, 4 Blackfan Circle, Boston, MA 02115. E-mail joettgen@caregroup.harvard.edu
© 2001 American Heart Association, Inc.

Circulation Research is available at http://www.circresaha.org
an Ets-binding site in the core enhancer leads to a marked reduction in vascular-directed LacZ gene expression. We have recently identified conserved Ets-binding sites in the Tie1 and Tie2 promoters, which are necessary for vascular-specific gene expression.\textsuperscript{12,13} It is presently not known which of the Ets factors are critical for the transcriptional activity of these genes.

To determine which of the Ets transcription factors are expressed during blood vessel formation and may regulates vascular-specific genes during this process, we examined developing blood vessels in the chorioallantoic membrane (CAM) of the chicken and identified the chicken homologue of ELF-1 (cELF-1). Our results demonstrate that cELF-1 is a strong transactivator of the Tie1 and Tie2 genes, can bind to specific Ets sites within the Tie1 and Tie2 promoters, and is enriched in developing blood vessels, suggesting that it contributes to the transcriptional regulation of vascular development.

### Materials and Methods

#### Cell Culture

Human umbilical vein endothelial cells (HUVECs), human dermal microvascular endothelial cells, and human aortic endothelial cells (HAECs) were obtained from Clonetics. PY41 endothelial cells were a generous gift from Robert Auerbach (University of Wisconsin) and were grown as previously described.\textsuperscript{15,16} The murine yolk-sac endothelial cell line (C166) and the EOMA cell line were a generous gift from Richard Auerbach (University of North Carolina).\textsuperscript{14} The mouse yolk-sac endothelial cell line (C166) and the EOMA cells were a generous gift from Robert Auerbach (University of Wisconsin) and were grown as previously described.\textsuperscript{15,16}

#### RNA Extraction and Northern Blot Analysis

Total RNA was extracted from CAM blood vessels, cultured CAM endothelial cells, and blood derived from the CAM at different developmental stages, as previously described.\textsuperscript{17} Total RNA was electrophoresed and transferred onto a Nytran membrane. The filters were blocked in prehybridization solution and then hybridized with a cELF-1–specific probe. The cELF-1 cDNA fragment used to generate the probe is 700 bp in length and encodes the first 100 amino acids of the cELF-1 protein and 100 bp of the 5′ untranslated region. The size of the band detected by Northern blot analysis is 3.4 kb. To normalize for loading and transfer efficiency, the membranes were rehybridized with a probe for the 36B4 chicken housekeeping gene. The size of the band detected by Northern blot analysis is 1 kb.

#### Reverse Transcriptase–Polymerase Chain Reaction and Chicken \(\lambda\)-Phage Library Screen

To identify Ets factors that are expressed in the developing blood vessels of the chicken CAM, reverse transcriptase–polymerase chain reaction (RT-PCR) was performed using RNA extracted from the CAM blood vessels. cDNA was generated from 2 \(\mu\)g of total RNA by using random hexamer priming. Degenerate oligonucleotides corresponding to conserved regions within the Ets-DNA–binding domain were use as previously described.\textsuperscript{18} PCR fragments were subcloned, and fragments of the expected sizes were sequenced. A 5-day chicken yolk-sac library (Stratagene) was plated and screened with a partial cDNA fragment for cELF-1. Two full-length cDNA clones were isolated. For RT-PCR of the human and mouse endothelial cells, the ELF-1–specific primers used, which recognize both mouse and human ELF-1, were 5′-ATGGCTGCTGTTGTCCAAC-3′ and 5′-CCATGGAGAAGGCTTGGGG-3′, with an expected amplification product of 700 bp.

#### In Situ Hybridization

Whole-mount in situ hybridization on embryonic day 3 (E3) chick embryos and E10 CAMs was carried out as described by Wilkinson et al.\textsuperscript{20} The sense and antisense probes were derived from the same 700-bp fragment that was used for Northern blot analysis, which was subcloned into the PCRII vector (Invitrogen) containing both a T7 (sense) and an Sp6 (antisense) promoter. Briefly, embryos were fixed, dehydrated, and rehydrated through a methanol series and washed in PBS and PBS plus 0.1% Tween-20. Embryos were then permeabilized at room temperature. After color developed to the appropriate intensity, specimens were washed several times and then rehydrated through the graded methanol baths. Images of the embryos suspended in 80% glycerol were obtained using a 3CCD Toshiba camera on a Nikon SMZ-U dissecting microscope. Digoxigenin-labeled RNA probes were prepared per the manufacturer’s recommendations (Roche). The level of digoxigenin incorporation was assessed by using a dot-blot comparison to a standard (Roche).

#### Immunohistochemistry

Paraffin-embedded E4 and E5.5 chicken embryos were stained with a rabbit polyclonal anti–cELF-1 antibody. Sections were clarified with xylene and rehydrated through a decreasing gradient of ethanol. After several washes with ddH\(_2\)O and PBS, sections were treated with 0.1 mg/mL proteinase K in PBS. Hybridization with an anti–cELF-1 probe was performed overnight after blocking for 1 hour with 2% goat serum in PBS (PBS containing 0.05% Tween-20). Sections were then incubated with biotinylated anti-rabbit (Vector Laboratory) and fluorescein avidin DN (Vector Laboratory), consecutively. Immunostained sections were then analyzed by confocal microscopy. Nuclear staining was made possible with the addition of 0.1 mg/mL propidium iodide dissolved in 1:1 PBS/glycerol mounting medium.

#### DNA Transfection Assays

Cotransfections of 1.5 to 2 \(\times\)10\(^5\) endothelial cells or 293 HEK cells were performed using 1.75 \(\mu\)g of the reporter-gene construct DNA and 0.75 \(\mu\)g of the expression-vector DNA with Lipofectamine (Gibco BRL). The cells were harvested 16 hours after transfection and assayed for luciferase. Individual transfections were performed in duplicate and repeated independently in triplicate with similar results. Cotransfection of a second plasmid for determination of transfection efficiency was omitted, because potential artifacts with this technique have been reported\textsuperscript{21} and because many commonly used viral promoters contain potential binding sites for Ets factors.

#### In Vitro Transcription-Translation

Full-length chicken and human ELF-1 cDNA encoding the entire open-reading frames were inserted downstream of the T7 promoter into the Bluescript vector. Coupled in vitro transcription–in vitro translation reactions were performed with 1 \(\mu\)g of plasmid DNA using the TNT reticulocyte lysate kit (Promega) and T7 RNA polymerase as recommended by the manufacturer. The plasmid vector without an insert was used as a control.

#### Electrophoretic Mobility Shift Assay

DNA-binding reactions were performed as previously described.\textsuperscript{18,22} Briefly, 20-\(\mu\)L samples containing 2 \(\mu\)L of in vitro–translated products or cell extracts were incubated with a solution containing \(^32\)P-labeled double-stranded probes (30 000 cpm). Samples were incubated in the presence or absence of increasing amounts of cold competitor (5 or 50 ng) for 15 to 20 minutes at room temperature and run on a 4% polyacrylamide gel (acrylamide-bisacrylamide, 29:1) containing a buffer of 0.25\% TBE (22.5 mmol/L Tris borate and 0.5 mmol/L EDTA). Oligonucleotides used as probes for competition studies were as follows:

- Tie2 promoter oligonucleotide
  - 5′-TGCAAGGAAAACCGGAGAATTTAAC-3′
3′-ACGTTTCCTTTGTCCTTTTTCCTTGAATTG-5′
Tie1 P1
5′-ACTGGCTTCCTCCCTTTCCTGTCTC-3′
3′-TGACGAGAGGAAAGGAGGTCGTC-5′
Tie1 P2
5′-CCATCATTATCTCTTCCAGCAG-3′
3′-GGTAGAATTAGAAGGAGGTCGTC-5′
Tie1 P2 Mut1
5′-TGACCGAAGGAGGGAAAGGACAGAG-3′
3′-CCATCATTTAATCTTCCTCCCCAG-5′
Tie1 P2 Mut2
5′-GGTAGTAAAGGAGAAGGAGGGGTC-3′
3′-CCATCATTTAATCTTAATCCCCAG-5′
Tie1 P2 Mut1,2
5′-GGTAGTAAATTAGAAGGAGGGGTC-3′
3′-CCATCATTTAATCTTAATCCCCAG-5′

Results

Isolation of the cELF-1

In an effort to identify transcription factors belonging to the Ets family that are expressed during blood vessel development, we chose to examine the highly vascular chicken CAM. Vessels within the CAM undergo an exponential growth followed by a quiescent phase. Total RNA was extracted from blood vessels that were microdissected from 10-day-old CAMs. RT-PCR was performed with degenerate PCR primers corresponding to conserved regions of the Ets domain, allowing the identification of a partial DNA sequence for a member of the Ets-factor family. This fragment was used to screen a chicken yolk-sac cDNA library and isolate a cDNA clone encoding the full-length cELF-1. As shown in Figure 1, cELF-1 encodes a 617-amino acid–long protein with an expected molecular mass of 71.0 kDa. The highest degree of homology to ELF-1 exists in the DNA-binding domain (100%), with overall protein sequence homology of 77%. We have previously identified additional regions of homology between ELF-1 and a closely related Ets factor, NERF, in the transactivation domain. These 4 domains (A through D) are also highly conserved between human, murine, and chicken ELF-1.23 To demonstrate that translation of the cELF-1 generates a protein of the expected size, we performed in vitro transcription translation with 35S-methionine. As shown in Figure 2, translation of the cELF-1 and human ELF-1 cDNA fragments generated fragments of the expected size.

Expression Pattern of cELF-1 in the Chicken CAM

To determine the expression pattern of cELF-1 in the CAM, Northern blot analysis was performed using RNA derived from CAM blood vessels at different developmental stages. As shown in Figure 3A, cELF-1 is highly expressed in the CAM blood vessels. Because it has previously been shown that ELF-1 is expressed in T and B cells, we examined the expression of cELF-1 in fetal chicken blood at different stages of development. As expected, cELF-1 is also highly expressed in chicken blood (Figure 3B). To ascertain whether cELF-1 is expressed in the CAM blood vessels devoid of blood, RNA was extracted from CAM blood vessels flushed free of blood and from unflushed CAMs. Although flushing the blood vessels diminishes the expression of cELF-1, there is still significant expression of cELF-1 in the flushed vessels.

Figure 1. Complete protein sequence for cELF-1 compared with mouse (m) and human (h) counterparts. Dash (−) represents identity to amino acid, and underline (_) signifies amino acid not present in this position.

Figure 2. 35S-methionine in vitro–translated cELF-1 protein compared with human ELF-1. Molecular mass standard sizes in kilodaltons are shown on the left.
ELF-1 Is Expressed in a Subset of Human and Murine Endothelial Cells

We were somewhat surprised to find cELF-1 highly expressed in the CAM blood vessels, because we had previously examined ELF-1 expression in human endothelial cells and not detected it in either HUVECs or HAECs.13 To extend these studies, we examined additional murine and human endothelial cells for the expression of ELF-1. As shown in Figure 3D, ELF-1 is also expressed in the murine yolk-sac endothelial line C166, EOMA and PY41 endothelioma lines, and human dermal microvascular endothelial cells: PY41 and EOMA (endothelioma cell lines); C166, a murine yolk-sac endothelial cell line; human dermal microvascular endothelial cells (HDECs); HUVECs; HAECs; and 2 B-cell lines, HAFTL and A20.

Expression Pattern of cELF-1 in the Embryo

We have previously demonstrated that human ELF-1 is highly expressed in several fetal tissues, including the heart and liver, and, weakly, in the brain.23 To ascertain cELF-1 expression at different developmental stages, Northern blot analysis was performed with chicken fetal organs at different developmental stages. As shown in Figure 4, cELF-1 is strongly expressed in the fetal liver, in several later developmental stages in the heart, and in a temporal window in embryonic brain and limb development.

In Situ Hybridization of cELF-1 in the Developing Blood Vessels of the Chicken CAM

Having demonstrated strong expression of cELF-1 in the CAM blood vessels at different stages by Northern blot analysis, the expression of cELF-1 was examined by in situ hybridization to additionally define the expression pattern of cELF-1 during blood vessel development. As shown in Figure 5A (top), cELF-1 is expressed along the lining of the larger caliber blood vessels, with a punctate expression pattern in the smaller branching vessels. At higher magnification, strong expression of cELF-1 is demonstrated in these smaller-caliber branching vessels (Figure 5A, HP). Whole-mount in situ hybridization also confirmed strong expression in the developing heart at day 10 (Figure 5B).

Immunohistochemistry of cELF-1 in the Developing Chicken Embryo

To examine cELF-1 protein expression during chicken embryogenesis, with a particular focus on blood vessel development, immunohistochemistry was performed using an ELF-1 polyclonal antibody. Paraffin-embedded sections of E4 and E5.5 chicken embryos were incubated with anti–cELF-1, and immunocomplexes were detected with biotinylated antirabbit antibody followed by fluorescein avidin DN. Visualization of nuclei was performed with propidium iodide. As shown in Figure 6A, cELF-1 is highly expressed in the inner lining of the developing dorsal aorta (DA) of E4 chicken embryos. At higher magnification (Figure 6B), cELF-1 expression is also appreciated in a subset of blood cells within the lumen of the aorta, as would be expected, because ELF-1 has previously been shown to be expressed in a subset of hematopoietic cells. No expression could be detected with preimmune serum (Figures 6C and 6D). cELF-1 was also detected in smaller developing chicken blood vessels (Figure 3A).
6E) and in intersomitic vessels of the 4-day chicken embryo (Figure 6F). Because we detected high levels of cELF-1 expression in the developing chicken heart by whole-mount in situ hybridization, we also examined cELF-1 protein expression in the developing heart. At lower magnification (Figure 6G), expression is appreciated in the heart but not the surrounding lung. At higher magnification, cELF-1 expression is detected in the endocardium of the heart (Figure 6H). Interestingly, expression was also detected on the pericardial surface.

cELF-1 Can Transactivate the Tie1 and Tie2 Promoters

We have previously shown that one of the NERF gene isoforms, NERF2, is a strong transactivator of the Tie2 gene.13 Because ELF-1 is highly homologous to NERF2, we tested the ability of the chicken and human forms of ELF-1 to transactivate the Tie2 promoter. As shown in Figure 7A, cELF-1 was similar to human ELF-1 in its ability to transactivate the Tie2 promoter, in contrast to Ets-1 and Ets-2, which only weakly transactivate the Tie2 promoter. Because we have previously demonstrated that the Ets factor NERF is similarly able to transactivate the Tie1 gene, we were interested in testing whether the human or chicken ELF-1 could similarly transactivate the Tie1 promoter.12 As shown in Figure 7B, both chicken and human ELF-1 are strong transactivators of the Tie1 promoter. This suggests that both the Tie1 and Tie2 genes may be gene targets for ELF-1.

cELF-1 Can Bind to Ets Sites in the Tie1 and Tie2 Promoter

We have previously identified the Ets-binding sites that are necessary for transactivation by Ets factors with the Tie2
The ability of in vitro–translated human ELF-1 and cELF-1 to bind to the same Tie2 Ets sites was also examined. As shown in Figure 8A, both human and chicken ELF-1 form similar DNA-protein complexes with the Tie2 Ets sites (lanes 2 and 3). We then tested the ability of an ELF-1–specific antibody to interfere with the formation of these complexes. This antibody was able to interfere with complex formation of both human and chicken ELF-1. Furthermore, the appearance of an additional higher-mobility complex when the antibody was used in the presence of cELF-1 suggests the formation of a supershift (see arrow, lane 6). Because several Ets factors may potentially bind to the Tie2 Ets site in vivo, in the developing chicken, we performed gel-shift assays with the Tie2 Ets probe and cell extracts from the chicken CAM. As shown in Figure 8B, lane 3, a similar-sized complex is formed compared with the in vitro–translated cELF-1. When the ELF-1 antibody was added, it similarly resulted in the formation of a supershifted complex, suggesting that cELF-1 derived from the chicken CAM is the Ets factor that specifically binds to the Tie2 Ets site.

We also examined the ability of cELF-1 to bind to conserved Ets sites in the Tie1 promoter.9 There are 2 Ets-site doublets, P1 and P2, that are highly conserved in this promoter. We tested the ability of cELF-1 to bind to these Ets sites. As shown in Figure 8C, cELF-1 binds well to both of these Ets sites. To additionally demonstrate the specificity of binding to particular Ets sites, we tested the ability of various cold mutant oligonucleotides to interfere with binding of cELF-1 to the Tie1 P2 Ets sites (Figure 8D). When both of the Ets sites were mutated (Mut 1, 2), the oligonucleotides were unable to compete for binding. When the first Ets site was mutated, Mut 1, it competed only weakly with binding of cELF-1 to the Tie1 P2 probe. However, when only the second Ets site was mutated, it competed equally as well as the wild-type oligonucleotide,
suggesting that the first Ets site within this doublet is a higher-affinity binding site for cELF-1.

**Discussion**

The goal of this study was to identify transcription factors that regulate blood vessel development. In particular, we have identified a member of the Ets transcription-factor family, ELF-1, that is enriched in the developing blood vessels of the chicken embryo. ELF-1 was originally described as a regulator of T-cell–specific genes, including the interleukin-2 gene, interleukin-2 receptor, granulocyte-macrophage colony-stimulating factor, and CD4 genes.24–26 In addition, we and others have recently shown that ELF-1 is also expressed in B cells, where it regulates IgH gene expression.27 We have also recently isolated a novel member of the Ets gene family, NERF, which shares the highest degree of homology to ELF-1, and have shown that NERF and ELF-1 are also involved in the regulation of the B-cell–specific tyrosine kinase blk.23 This is the first report to demonstrate a role for ELF-1 in vascular-specific gene expression during blood vessel development in addition to its role in regulating genes of hematopoietic origin.

We have shown that ELF-1 can transactivate the Tie1 and Tie2 genes; therefore, these genes may serve as targets for ELF-1. The regulatory elements of both the Tie1 and Tie2 genes have been used to direct LacZ gene expression in a vascular-specific manner. Mutations in selected Ets sites in the regulatory regions of the Tie1 and Tie2 genes result in marked reductions in vascular-specific gene expression in vivo. Furthermore, with certain mutations, in addition to a reduction in overall vascular-specific gene expression, the LacZ-directed gene expression is reduced more in certain vascular beds than in others.12 This would suggest that certain Ets factors may be more important for the regulation of vascular-specific gene expression in certain vascular beds. With regard to expression of human and chicken ELF-1, for example, both are expressed only briefly in the fetal brain and not at all in adult brain, whereas both are strongly expressed in the fetal and adult heart, supporting a role for ELF-1 in differentiation of cardiac progenitor cells.23,28 This suggests that certain transcription factors may be critical for both the normal development of hematopoietic cells and blood vessels and that there may be a common stem-cell precursor for both lineages. The most striking defects were a disorganized array of capillaries and absence of normal vitelline blood vessel formation. Although the larger vitelline blood vessels were not present, a smaller network of interconnecting vessels did exist. The architecture of these vessels revealed normal-appearing endothelial cells as well as the smooth muscle cells or pericytes that constituted the outer lining of the blood vessels.

In conclusion, the results of these studies provide substantial support for the role of the Ets factors in vascular development and vascular-specific gene expression in addition to their known role in hematopoiesis. The present study provides strong evidence for a dual role for ELF-1 in hematopoiesis and vascular development and, in particular, in regulating the gene expression of the Tie2 gene.

**Acknowledgments**

This study was supported by National Institutes of Health grants RO1/HL63008 and KO8/CA71429 to P.O. and an American Heart Association Grant in Aid to L.I.

**References**


ELF-1 Is a Transcriptional Regulator of the Tie2 Gene During Vascular Development
Antoinise Dube, Shelley Thai, John Gaspar, Susan Rudders, Towia A. Libermann, Luisa Iruela-Arispe and Peter Oettgen

Circ Res. 2001;88:237-244
doi: 10.1161/01.RES.88.2.237

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/88/2/237

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/