Sympathetic Innervation Promotes Arterial Fate by Enhancing Endothelial ERK Activity

Luc Pardanaud, Laurence Pibouin-Fragner, Alexandre Dubrac, Thomas Mathivet, Isabel English, Isabelle Brunet, Michael Simons, Anne Eichmann

Rationale: Arterial endothelial cells are morphologically, functionally, and molecularly distinct from those found in veins and lymphatic vessels. How arterial fate is acquired during development and maintained in adult vessels is incompletely understood.

Objective: We set out to identify factors that promote arterial endothelial cell fate in vivo.

Methods and Results: We developed a functional assay, allowing us to monitor and manipulate arterial fate in vivo, using arteries isolated from quails that are grafted into the coelom of chick embryos. Endothelial cells migrate out from the grafted artery, and their colonization of host arteries and veins is quantified. Here we show that sympathetic innervation promotes arterial endothelial cell fate in vivo. Removal of sympathetic nerves decreases arterial fate and leads to colonization of veins, whereas exposure to sympathetic nerves or norepinephrine imposes arterial fate. Mechanistically, sympathetic nerves increase endothelial ERK (extracellular signal-regulated kinase) activity via adrenergic α1 and α2 receptors.

Conclusions: These findings show that sympathetic innervation promotes arterial endothelial fate and may lead to novel approaches to improve arterialization in human disease. (Circ Res. 2016;119:607-620. DOI: 10.1161/CIRCRESAHA.116.308473.)

Key Words: arterial–venous endothelial differentiation ■ embryonic development ■ ERK ■ sympathetic nervous system ■ VEGF signaling

It is well known that arterial endothelial cells are different from venous or lymphatic ones, but the origin of these differences remains unclear. Until the late 1990s, it had been assumed that arterial–venous (AV) differences arise as a consequence of different hemodynamic forces within arteries and veins. In 1998, EphrinB2 and its receptor EphB4 were discovered as the first specific molecular markers of arterial and venous endothelial cells, respectively.1 Since then, the signaling pathway specifying arterial endothelial identity has been shown to require vascular endothelial growth factor (VEGF) signaling through VEGF receptor 2 (VEGFR2) and neuropilin 1 (Nrp1), leading to extracellular signal-regulated kinase (ERK) activation and expression of delta-like ligand 4 (Dll4), which signals through Notch receptors to induce expression of EphrinB2.2–7 Transcription factors Foxc1, Foxc2, and Sox17 contribute to arterial specification by interacting with VEGF and Notch signaling and inducing arterial-specific genes Dll4 and Hey2.8–9 In veins, the transcription factor Coup-TFII is thought to suppress Nrp1 function and downstream Notch signaling, and endothelial Coup-TFII knockout mice show ectopic expression of arterial markers in embryonic veins.10 Knockout of arterial-specific genes, such as Alk1, Nrp1, Dll4, and Notch1, leads to vascular remodeling defects and AV shunt formation, ultimately causing embryonic lethality.11–16 These studies show that artery- and vein-specific genes are required for normal vascular patterning and support a genetic basis for specification of AV identity.

Despite increased knowledge about arterial fate specification, how this process is driven in normal development and how it can be stimulated in peripheral artery disease remains incompletely understood. Genetic deletion of plex and loss of downstream ERK signaling in zebrafish embryos completely prevents expression of arterial-specific genes and arterial branching.17,18 Whereas enhancing ERK activity in mice or in cultured endothelial cells in vitro enhances expression of arterial marker genes and promotes arterial branching.19 These data identify ERK signaling as a critical driver of arterial differentiation.

Original received February 2, 2016; revision received June 21, 2016; accepted June 24, 2016. In May 2016, the average time from submission to first decision for all original research papers submitted to Circulation Research was 14.93 days.

Correspondence to Dr Luc Pardanaud, INSERM U970, Paris Center for Cardiovascular Research (PARCC), 75015 Paris, France. E-mail luc.pardanaud@inserm.fr; or Dr Anne Eichmann, INSERM U970, Paris Center for Cardiovascular Research (PARCC), 75015 Paris, France. E-mail anne.eichmann@inserm.fr

© 2016 American Heart Association, Inc.

Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.116.308473
ERK activation is driven by VEGF signaling in embryonic endothelial cells, with arterial differentiation occurring in angioblasts exposed to higher VEGF concentrations, whereas less exposed cells acquire venous fate.\(^{4,7,20}\) The morphogen sonic hedgehog induces VEGF expression in zebrafish embryos.\(^{21}\) Angioblasts close to sonic hedgehog-expressing tissues receive high VEGF concentrations and subsequently differentiate into arterial cells that form the dorsal aorta. Angioblasts located further away differentiate as veins but can be converted into arterial cells by sonic hedgehog or VEGF overexpression.\(^{22}\) In mouse embryos, VEGF released from peripheral sensory nerves induces arterial differentiation of primitive vessels in the skin, and genetic deletion of Vegf from nerves prevents arterial differentiation.\(^{22}\) Whether other factors besides VEGF contribute to developmental arterial differentiation is unknown. VEGF expression is regulated by hypoxia,\(^{23}\) and its expression is downregulated in arteries after birth,\(^{24}\) strongly suggesting that additional factors must contribute to maintenance of arterial fate in postnatal and adult tissues.

Furthermore, it remains unknown if all endothelial cells within a given artery are engaged in or committed to arterial differentiation. Arterial cells can dedifferentiate and contribute to venous intersomitic vessels in zebrafish embryos.\(^{25}\) Conversely, some venous endothelial cells differentiate into coronary arteries or into lymphatic endothelial cells.\(^{26,27}\) These observations show that normal development entails a switch in AV identity of at least some endothelial cells, but the factor(s) regulating this switch remain largely unknown.

We had previously shown in quail-chick chimera grafts that differentiated arterial cells can contribute to veins.\(^{28}\) Here we use this assay to identify factors driving arterial differentiation. We graft arteries of quail embryos at different developmental stages into the coelom of a chick embryo at embryonic day 2 (E2),\(^{100}\%\) of arterial endothelial cells colonize host arteries but not veins, whereas before E11, \(\approx40\%\) of the grafted cells colonize veins. This switch in AV colonization occurs in the absence of any effects on cell number, suggesting that it reflects acquisition of arterial fate by a subset of previously undetermined endothelial cells. Because E11 corresponds to the onset of arterial innervation by sympathetic nerves in avian embryos,\(^{30,33}\) we investigated the role of sympathetic innervation and found that adrenergic signaling via \(\alpha_1\) and \(\alpha_2\) receptors activates endothelial ERK signaling, thereby enhancing arterial endothelial marker expression and promoting arterial colonization. These data reveal catecholamines as novel players in arterial endothelial fate decisions.

**Methods**

**Animals**

Chick (Gallus gallus, JA57) and quail (Coturnix coturnix japonica) eggs were incubated at 38°C until they developed the appropriate stages. Aortae, carotids, femorals, jugular veins, and arteries of the choioallantoic membrane (CAM) were dissected together with their wall and cut into rings (50–100-\(\mu\)m long) before grafting or culture. For Western blotting and quantitative polymerase chain reaction (qPCR) aortae were immediately frozen after dissection. Adventitia and sciatic nerves were isolated from E15–E21 chick embryos. To remove the adventitia from quail embryos, vessels were dipped in PBS without Ca\(^{2+}\)-Mg\(^{2+}\), pinned at one end onto a black support, and dissected using a fine pair of forceps and a sharpened microscalpel. After the dissection, the tissues were placed in PBS+Ca\(^{2+}\)-Mg\(^{2+}\).

**Coelomic Grafs**

Indian ink was diluted 1:1 in PBS+Ca\(^{2+}\)-Mg\(^{2+}\) and injected beneath E2 chick host blastodiscs (18–24 somites). Coelomic grafts were performed as described.\(^{32}\) The freshly prepared or overnight-cultured explants were introduced into the coelom at the level of somite 15 to 20 (Figure 1A). The hosts were euthanized 2 days later, fixed in Bouin’s fluid, and processed for serial paraffin sections. Five hundred sixty-six grafts were analyzed in this study.

**Immunohistochemistry**

Quail hemopoietic 1 antibody (QH1),\(^{29}\) quail cell marker antibody (QCPN), and the macrophage marker LEP100\(^{33}\) were obtained from Developmental Studies Hybridoma Bank. QH1/QCPN immunohistochemistry was done as described\(^{32}\) and followed by a glycemalum nuclear staining. QH1/von Willebrand factor (DakoCytomation)/LEP100 triple immunostaining required 15 minutes pretreatment with 0.025% trypsin at 37°C. QH1 was developed with Alexa 350 goat anti-mouse IgM (Invitrogen) and LEP100 with Alexa 488 goat anti-mouse IgG (Invitrogen). von Willebrand factor staining was amplified with biotinylated anti-rabbit and streptavidin Cy3 (GE Healthcare, Amersham). VE-Cadherin (kind gift from T. Schultheiss) was used after heat-induced antigen retrieval and developed with Alexa555 goat anti-rabbit Ig (Invitrogen).

**Quantification of Endothelial Migration Into Host Arteries and Veins**

The number of QH1\(^+\) cells reaching host arteries and veins was counted manually on every fourth section (\(x25\) objective, final magnification \(x110\)). A positive cell was scored when it showed a glycemalum-positive nucleus and membrane QH1 staining. 3D reconstructions of serial sections were made using the free-d software\(^{34}\) to determine which great vessels the cells integrated (mostly aorta and cardinal vein, omphalomesenteric artery and vein, subclavian and brachial arteries, umbilical vein, segmental arteries and veins). In rare cases, cells were found in the limb bud, and there we considered that all endothelial cells at the periphery belonged to the venousplexus, whereas arterial cells were centrally located and connected to the brachial artery. In the perineural vascular plexus, QH1\(^+\) cells were only quantified if we could trace their connection with a known artery or a vein. If we could not classify the arterial or venous identity of a donor cell, it was not counted. The total number of cells per graft was set at 100%, and the percentage of grafted cells present in host arteries and veins was calculated.

Statistical analyses were done using unpaired 2-tailed nonparametric t tests. P values were \(^*P<0.05\), \(^{*}P<0.01\), or \(^{*}P<0.001\).

**Organotypic Cultures**

Vessel rings were placed onto a semisolid medium (50% agar, 40% DMEM, 5% fetal calf serum [Invitrogen], 5% antibiotics [Gibco]) with or without drugs (Table I) for one night at 37°C, 5% CO\(_2\). For associations, vessels and tissues were placed together on the semisolid medium.
Figure 1. Method to test endothelial arterialization. A, Summary of the quail-chick grafting procedure: pieces of quail aorta (Ao) are dissected and grafted in the coelom (Coe) of an E2 chick embryo (right drawing). B, Four hours after the operation, a QH1+ endothelial cell (arrowhead) is found to be migrating from the QCPN+ graft (G) into the host mesoderm. bar=210 μm. C and D, Transverse (Continued)
Figure 1 (Continued). sections of E4 host chicks 48 h after grafting. C, Numerous QH1+ endothelial cells (brown, arrows) have colonized the aorta (Ao) through a ventral branch (*) connected to the graft (G). Two QH1+ endothelial cells reached the subclavian artery (S, arrow), whereas 2 others are in the cardinal vein (CV). D, The endothelium of the umbilical vein (*) and a branch (arrow) are partly QH1+. bar=40 μm in C and D. E and F, 3D reconstructions of grafted endothelial cell distribution in hosts. E, E15 aorta graft: The QH1+ endothelial cells (green dots) leave the graft (G) and colonize the ventral aortic endothelium (Ao) and a ventral aorta branch (arrow). Only 3 QH1+ cells reach the umbilical vein (arrowheads). F, E8 aorta graft: QH1+ endothelial cells are distributed between the aorta (Ao) and its ventral branches (arrows and veins (arrowhead). G, Histogram of arterial and venous colonization during ontogeny. The red columns show the percentage of QH1+ endothelial cells that colonize host arteries; the blue columns represent the complementary percentage of QH1+ cells in host veins. QH1+ cells from older aorta (E11-post hatching) almost exclusively colonize chick arteries while they reach both arteries and veins when isolated from quail younger than E11. **P<0.001, by comparison with E15 column. Error bars: SEM, n=number of samples. Top, Schematic illustration of the results: after E11, the migration is restricted to arteries (——), but before E11, grafts of quail aortic endothelial cells can migrate toward chick arteries and veins (+-+). H, Quantification of the total number of emigrating QH1+ endothelial cells in E15 and E8 grafts. Unpaired 2-tailed nonparametric t tests in G and H. S, somite; Ao indicates aorta; BW, body wall; C, caudal; G, graft; LCV, left cardinal vein; M, mesonephros; R, rostral; and RCV, right cardinal vein.

Endothelial Cell Isolation
E8 and E15 aortae (16–25 per experiment) were longitudinally sectioned and treated with collagenase (Sigma) for 30 minutes at 37°C. Endothelial cells were flushed out with a micropipette, placed in DMEM with 20% fetal calf serum, centrifuged, and resuspended in 100 μL of the medium plus 0.1% sodium heparin. Ten microliter aliquots were cultured for 4 hours to verify the presence of QH1+ endothelial cells. Twenty microliter aliquots were placed overnight in culture drops for aggregate formation and grafting.

Glyoxylic Acid Treatment
Freshly dissected quail aortae were longitudinally sectioned and incubated for 30 minutes at room temperature in PBS with 2% glyoxylic acid (Sigma) and 10% sucrose (wt/vol), pH 7.5.35 Vessels were placed smooth muscle layer up on a glass slide, dried for 30 minutes at room temperature, and stretched for 4 minutes at 100°C on a heated plate. Slides were mounted in Entellan (Merck) and imaged with a fluorescent microscope (Olympus; excitation wavelength 350 nm, emission wavelength 461 nm).

Denervation
Quail embryonic aortae were placed in 6-hydroxy-dopamine solution (Sigma, 300 μM in distilled water) during 3h30 at 37°C and rinsed in PBS+Ca++Mg++ before grafting.

For surgical denervation, the neural tube and notochord were removed from E12 quail embryos as described.36 The resulting embryos lacked the peripheral nervous system. Aortae were isolated from operated quail embryos between E11 and E16 (n=5) and grafted into E2 chick coeloms.

Western Blotting
Samples were lysed in NP40 lysis buffer (LifeTechnologies) containing protease inhibitor set I (Millipore), 5 mmol/L orthovanadate, and 2 mmol/L sodium pyrophosphate, then briefly sonicated, incubated for 15 minutes at 4°C and centrifuged 15 minutes at 13000 rpm. Supernatants were immediately frozen.

Proteins (2.5 μg/sample) were separated using SDS-PAGE (NOVEX Nupage 4% to 12%; LifeTechnologies) and transferred to a nitrocellulose membrane (Amersham Protran Premium 0.45 μM; GE Healthcare Lifesciences). All gels and transfers were run in double; one membrane was incubated with anti-Phospho-p44/42 MAPK (p-ERK, Cell Signaling 4370) and the other with anti-p44/42 MAPK (total ERK, Cell Signaling 9102). Membranes were blocked in TBST containing 5% nonfat dry milk for 1 hour at room temperature and then incubated overnight at 4°C with primary antibody diluted in blocking buffer (1:1000). Membranes were washed in tris buffer saline-tween 20 (TBST) and then incubated with horseradish peroxidase (HRP)–conjugated secondary antibody (1:1000 in blocking buffer, Pierce, 31460). Bands were developed using Supersignal west Femto substrate (ThermoFisher). Membranes were imaged and bands quantified using the GE Healthcare ImageQuant LAS-4000 system.

Quantitative PCR Analysis
Total RNA was extracted and purified using NucleoSpin RNA XS kit (Macherey-Nagel). RNA concentrations were measured on a Nanodrop and retrotranscribed using SuperScriptIII (LifeTechnologies). We performed real-time qPCR reactions in duplicate on a T100 real-time thermal cycler (BIORAD).

For vascular endothelial growth factor (VEGF) treatment, aorta segments were incubated for 20 hours in E15 aorta media (R&D Systems) supplemented with 20 ng/mL VEGF-A (R&D Systems) for HUAECs. Cells are treated for 20 hours with 100 nM VEGF-A.

Quantification of the total number of emigrating QH1+ endothelial cells in E15 and E8 grafts. Unpaired 2-tailed nonparametric t tests in G and H. S, somite; Ao indicates aorta; BW, body wall; C, caudal; G, graft; LCV, left cardinal vein; M, mesonephros; R, rostral; and RCV, right cardinal vein.

HUEVC/HUAEC Experiments
Humoral umbilical vein endothelial cells (HUEVCs) and human umbilical artery endothelial cells (HUAECs; Cell Application) were cultured in EGM2-BulletKit (Lonza) supplemented with 100 ng/mL VEGF-A (R&D Systems) for HUAECs. Cells are treated for 20 hours with phenylephrine (30 nM), m-3M3FBS (5 μM), and KT5720 (1 μM). When added, ERK inhibitor was used at 10 μM.

Results
We grafted aorta fragments microdissected from postnatal day 15 quails into the coelomic cavity of 2-day-old chick embryos (Figure 1A). When placed into this location, vessel fragments attached to the coelomic angle (Figure 1B) and graft-derived endothelial cells colonized chick vessels. Endothelial cells originating from the grafted vessel were identified using the QH1 antibody specific for quail endothelial and hematopoietic cells.
Figure 2. Extracellular signal-regulated kinase (ERK) dependence of arterial differentiation.

A. Western blot of P-ERK and total ERK levels at E8 and E15. Avian ERK migrates at 44 kD. Each lane represents one aortic sample. Quantification of 9 samples per age shows a statistically significant increase in P-ERK levels in E15 versus E8 aorta. **P<0.01, unpaired t test.

B. Quantitative polymerase chain reaction (qPCR) of arterial and venous marker genes at E8 and E15. Note increase of arterial and decrease of venous gene expression levels at E15. *P<0.05, Mann–Whitney U test.

C. Effects of ERK inhibitor U0126 on E15 aorta and jugular vein grafts. U0126 decreased arterial colonization and had no effects on veins. **P<0.001, Student t test by comparison with E15 column. Error bars: SEM, n=number of samples.
Figure 3. Sympathetic innervation promotes arterial differentiation. A–E, Glyoxylic acid staining of sympathetic nerves in the aortic vessel wall. No detectable staining at E8 (A); the first fibers are visible at E10 (B, arrowhead); and fibers form a network at E15 (C). D, When the adventitia is removed from E15 aorta, the neural fibers disappear. E, Chorioallantoic membrane (CAM) arteries are not innervated. (Continued)
and additional endothelial markers, including von Willebrand factor and VE-Cadherin (Figure 1C–1F; Online Figure I). Some QH1+ cells formed hematopoietic stem cells in the ventral floor of the aorta or LEPI00+ macrophages (Online Figure I), but these were easily distinguished from the endothelial cells and not quantified. We quantified only QH1+ endothelial cells colonizing host arteries and veins 48 hours after grafting on serial sections and 3D reconstructions (Figure 1C–1F).

Strikingly, the vast majority (>90%) of QH1+ cells emigrating from postnatal day 15 aorta grafts were found in host arteries, whereas only a small percentage of cells (<10%) colonized host veins (Figure 1C, 1E, and 1G). QH1+ cells were mainly found in the aorta (Figure 1C and 1E) and more rarely in the subclavian, brachial, segmental, and mesonephric arteries. Similar experiments done with embryonic aorta grafts showed that arterial endothelial cells mainly colonize host arteries when isolated from embryos older than E11, that is, roughly two thirds of the 16-day quail embryonic incubation period (Figure 1G). Before E11, around 40% of the grafted cells also colonized veins (Figure 1D, 1F, and 1G) where they lose arterial marker expression.28

The overall number of endothelial cells originating from grafted E15 or E8 aortae was similar (Figure 1H), indicating that the assay was not selecting for a particular population of cells. Rather, it seemed that E11 aortic cells had experienced an extrinsic signal, prohibiting colonization of host veins and driving them toward arterial fate. This assay, therefore, allows functional testing of the capacity of an endothelial cell to adopt an arterial fate.

Because ERK activation is known to drive arterial fate,4,19 we assessed levels of activated ERK in aorta fragments and isolated endothelial cells from E8 and E15 embryos by Western blot. P-ERK levels were significantly higher in E15 than when compared with E8 aorta (Figure 2A; Online Figure II). Furthermore, in line with enhanced ERK activity, qPCR showed that expression of arterial markers *Dlk1*, *EfnB2*, and *Nrp1* increased in the aorta between E8 and E15, whereas venous markers *Apx* and *CoupTFII* decreased (Figure 2B).

To functionally assess involvement of ERK in arterial fate decisions, we tested effects of the ERK inhibitor U0126 using overnight culture of arterial fragments on a semisolid medium supplemented with the drug. Although E15 fragments cultured without drug behaved just like native E15 fragments and colonized mainly host arteries, exposure of E15 quail aortae to 100 nM U0126 effectively decreased arterial fate, and >80% of the grafted cells colonized host veins (Figure 2C). By contrast, treatment of E15 jugular veins with U0126 had no effect, and the vast majority of cells derived from these vessels colonized host veins (Figure 2C). These data show that late embryonic and postnatal quail aortic endothelial cells maintain their arterial identity in a foreign environment in an ERK-dependent manner. Almost all endothelial cells have acquired arterial identity at E11, whereas before that stage, >40% of the grafted arterial cells have the capacity to switch to venous identity.

A major event occurring around E11 is the onset of innervation of the aortic vessel wall by sympathetic nerves.30,31 Glyoxylic acid staining to detect catecholamine released by perivascular nerve terminals35 showed that E8 aortae were negative, and E10 aortae showed few nerve terminals, and E15 aortae displayed abundant positive staining (Figure 3A–3C), demonstrating that aortic innervation correlated with acquisition of arterial fate. Abundant sympathetic innervation was also observed on E15 carotid and femoral arteries (Online Figure III). Surgical removal of the adventitia abolished glyoxylic acid staining, indicating that staining was specific for sympathetic nerves (Figure 3D). Furthermore, in contrast to the aorta, arteries in the E15 CAM, an extraembryonic membrane that supplies the embryo with oxygen and nutrients, displayed lack of sympathetic innervation (Figure 3E).

To test the function of sympathetic innervation in acquisition of arterial identity, we used a series of loss and gain of function approaches. When E15 aortic endothelial cells are isolated from the vessel wall and cultured overnight, they fail to exhibit artery-only colonization, and >30% colonize veins (Figure 3F). In these conditions, arterial markers were downregulated (Figure 3G). Likewise, when noninnervated E15 CAM arteries were grafted, >40% of cells colonized veins (Figure 3F), and the expression of arterial markers was decreased in comparison to aortae isolated from the same embryos (Figure 3H). The surgical removal of the adventitia from innervated E15 aortae also led to the loss of arterial identity (Figure 3F).

We next ablated innervation using chemical and surgical protocols. Chemical denervation was done using 6-hydroxydopamine.37 Glyoxylic acid staining showed that a 3h30 treatment of isolated aortic fragments depleted sympathetic nerves on the arterial vessel wall (Figure 3I), and grafting experiments showed that the absence of nerves induced a significant reduction in arterial fate and colonization of veins by >10% of the grafted denervated aortic endothelial cells from E15 embryos.

Figure 3 (Continued). F. Colonization of arteries and veins by endothelial cells from E15 aortae isolated from their vessel wall, in CAM arteries, and E15 aortae without adventitia. *P<0.001, Student t test by comparison with E15 column. Error bars: SEM, n=number of samples. G and H, qPCR comparison of arterial marker expression; G, Between endothelial cells freshly isolated from E15 aortae (black columns) and isolated endothelial cells cultured overnight in control medium (gray columns); **P<0.01, Mann–Whitney U test. H, Between E15 aortae (black columns) and E15 CAM arteries (gray columns); each aorta is compared with CAM arteries of the same embryo. *P<0.05, **P<0.001, paired Student t test. I, Pharmacological 6-hydroxy-dopamine solution (6HDOPA) denervation destroys nerve fibers on E15 aortae. J, Surgical denervation of a 15 somite-stage quail embryo by resection (*) of the neural tube and notochord between the fifth somite and the caudal region (white arrowheads). Rostrally, the neural tube is left (arrow). K, Glyoxylic acid staining after the surgery shows the destruction of nerve fibers at the surface of an E15 aorta. L, Arterial colonization after chemical and surgical denervation in E15 and posthatching aorta. *P<0.05, **P<0.01, Student t test by comparison with E15 column or posthatching. Error bars: SEM, n=number of samples. M, Effects of E8 aorta coculture with E15 chick sciatic nerve on arterial colonization. ***P<0.001, Student t test by comparison with E8 column. Scale bars =150 μm in A–E, I, and K, and 385 μm in J. H indicates head.
Figure 4. α1-Adrenoceptor signaling promotes arterial fate. A, Norepinephrine (NE) induces arterialization of E8 aortae and E13-15 chorioallantoic membrane (CAM) arteries in a dose-dependent manner. *P<0.05, **P<0.01, and ***P<0.001, Student t test by comparison with untreated (0) column. Error bars: SEM, n=number of samples. B, Quantitative polymerase chain reaction (qPCR) analyses of ADRA1 expression in E8 and E15 aortae. ADRA1A expression levels at E8 were set as 1. Note that all 3 ADRA1 isoforms are expressed (Continued)
For surgical denervation, we removed the neural tube and notochord after the fifth somite level from E2 quail embryos, which leads to development of embryos devoid of most neural crest derivatives, including peripheral nerves46 (Figure 3J). Operated embryos survived up to E11. The surgery successfully ablated vascular innervation as attested by the dramatic decrease of fluorescence after glyoxylic acid staining (Figure 3K and legend to colonizations of veins by >10% of endothelial cells from grafted denervated aortae (Figure 3L). Similar results were obtained with denervated carotid and femoral arteries (Online Figure IV).

To test effects of gain of innervation, E8 aortic fragments were cocultured for one night with catecholamine-producing E15 sciatic nerves and grafted into chick hosts. More than 90% of grafted cells acquired arterial fate as attested by colonization of host arteries (Figure 3M). Taken together, these data show that acquisition of arterial identity by almost all grafted arterial endothelial cells occurs concomitantly with sympathetic innervation of the aortic vessel wall, that removal of sympathetic innervation induces partial loss of arterial identity, and that exposure to nerve-derived signals drives arterial fate, together demonstrating that sympathetic innervation promotes arterial fate of endothelial cells.

To characterize the mechanisms through which sympathetic nerves drive arterial fate, we focused on norepinephrine, the principal neurotransmitter produced by sympathetic ganglia. Exposure of E8 aortae to the PLC agonist m-3M3FBS (1 μM) induced arterialization, whereas exposure of E15 aorta to PLC antagonist (U73122, 20 μM) decreased colonization of host arteries by 10% (Figure 4D), supporting involvement of PLC in arterial fate decisions downstream of sympathetic innervation.

Phenylephrine also induced arterial fate in isolated aortic endothelial cells, indicating that it can directly affect the endothelium (Figure 4D). To test conservation of adrenergic signaling on arterial differentiation in human endothelial cells, we treated HUVECs with phenylephrine and the PLC agonist m-3M3FBS, which increased expression of arterial markers EFNB2, DLL4, and JAGGED1 to levels comparable to those seen in untreated HUAEs (Figure 4E). When the ERK inhibitor U0126 was added to the treatments, the increase of arterial markers was abolished or greatly diminished (Figure 4F). Exposure of HUAEs to phenylephrine and PLC agonist also increased arterial marker expression in an ERK-dependent manner; interestingly this effect was seen only when cells were simultaneously treated with phenylephrine or PLC agonist and VEGF, whereas phenylephrine or PLC agonist or VEGF alone had no effect (Online Figure V and data not shown). Thus, α1 adrenergic activation promotes arterial marker expression in human endothelium, supporting an evolutionary conserved effect of sympathetic innervation on arterial fate decisions.

In addition to α1 receptors, norepinephrine activates α2 receptors,42,43 which are encoded by ADRA2A, ADRA2B, and ADRA2C genes that are all expressed by embryonic aorta, with ADRA2A and C being downregulated and ADRA2B upregulated in E15 compared with E8 aorta (Figure 5A). α2 receptors promote vasoconstriction by inhibition of adenylate cyclase pathway44 (Figure 5B). Adenylyl cyclase activates PKA, which phosphorylates Raf on serine 259, thereby inhibiting its activity.45 Because Raf activation is an obligate upstream regulator of ERK activation,46 we reasoned that α2 receptor migrating could trigger arterial differentiation via inhibition of adenylate cyclase and PKA-dependent ERK inhibition (Figure 5B).

We tested this model functionally using pharmacological pathway agonists and antagonists. α2 receptor activation of E8 aorta fragments or isolated aortic endothelial cells with the α2 agonist BHT (10 μM) induced arterial colonization of >90% of grafted cells (Figure 5C; Online Table I).
Figure 5. Arterialization is also induced by α2-adrenoceptors. A, Quantitative polymerase chain reaction (qPCR) analyses of ADRA2 receptors in E8 and E15 aorta. Note decreased expression of ADRA2A and ADRA2C and increased expression of ADRA2B between E8 and E15. *P<0.05, **P<0.01, Mann–Whitney U test. B, Model of A2AR-mediated arterialization via inhibition of the adenylate cyclase/PKA pathway that leads to extracellular signal-regulated kinase (ERK) inhibition. The different drugs used to test the model are noted in green. C, Left, BHT933, an ADRA2 agonist, as well as inhibitors of adenylate cyclase (SQ22536) and PKA (KT7520), induce arterial fate in E8 aortae. The effect of (Continued)
Inhibition of downstream adenylate cyclase and PKA with SQ22536 (500 nM) and KT7520 (10-4 mol/L), respectively, also arterialized >90% of grafted E8 aortic endothelial cells (Figure 5C; Online Table I). Combined inhibition of PKA and ERK completely abolished the arterIALIZing effect, supporting ERK dependence of the pathway (Figure 5B and 5C). Neither inhibition of adenylate cyclase nor PKA or combined PKA and ERK inhibition affected overall number of endothelial cells originating from the grafted E8 aorta (Online Figure VI), indicating that treatments affected cell fate rather than cell number. Finally, increasing cAMP levels by activating adenylate cyclase with Forskolin (10 μM) or PKA activation induced the loss of arterial identity in E15 aortae, as shown by >20% of cells colonizing host veins (Figure 5C; Online Table I).

Because α2 receptor activation also induced arterial fate in isolated aortic endothelium (Figure 5C), we reasoned that it could directly activate arterial differentiation and tested this in cultured HUVECs. α2 agonists and inhibitors of adenylate cyclase or PKA enhanced expression of arterial marker genes EFNB2, JAGGED1, and DLL4 in an ERK-dependent manner (Figure 5D and 5E). Similar findings were obtained with VEGR-treated HUAECs (Online Figure V).

**Discussion**

We here use a functional assay to study AV endothelial fate decisions. The assay monitors colonization of chick arterial and venous vasculature by grafted quail endothelial cells. We show that after E11, almost 100% of aortic endothelial cells colonize host arteries but not veins, whereas before E11, ~40% of the grafted cells also colonize veins. Because this change in behavior occurs in the absence of any effects on cell number, we interpret this artery-specific colonization behavior to reflect acquisition of arterial fate by endothelial cells (Figure 6). In support of this model, E15 artery-only colonization correlates with increased expression of arterial marker genes, downregulation of venous genes, and increased ERK phosphorylation when compared with E8 artery and vein colonization. Several interesting observations can be made using this model: first, acquisition of arterial fate by close to 100% of cells occurs before birth and is, thus, independent of oxygen tension that modifies VEGF levels, demonstrating that factors other than VEGF contribute to arterial differentiation. Second, although we and others have shown that shear stress is critical for AV differentiation,14-18 fate decisions in this model occur in a nonperfused environment within the coelom and, thus, are independent of flow. Third, the decision to colonize host arteries or veins must be based on expression of adhesion molecules or other molecular mediators that remain to be defined. Finally, the assay shows that until E11, ~40% of endothelial cells in the aorta are not determined toward arterial fate. Thus, despite expression of arterial markers Dll4, ephrinB2, and Nrp1, a significant proportion of these endothelial cells are still able to switch to venous fate. Previous studies had shown heterogeneity in arterial and venous marker expression in mouse and zebrafish arteries,51-53 and a similar result had been reported previously in mouse embryonic endothelial cells treated with VEGF, where only 50% of cells could be induced to express ephrinB2.22 Although it remains unknown why endothelial cells respond differentially to arterIALIZing factors, we show here that exposure to catecholamines derived from sympathetic nerves and the adrenal gland is sufficient to induce arterIALIZation of the entire aortic endothelial cell population.

We show that arterial fate acquisition at E11 occurs concomitantly with sympathetic innervation of the aortic vessel wall, that removal of sympathetic innervation induces partial loss of arterial identity, and that exposure to catecholamines drives arterial fate, together demonstrating that sympathetic innervation promotes arterial differentiation of endothelial cells. Mechanistically, we show that catecholamines promote arterial fate via effects on endothelial ERK activation, with contributions from both α1 and α2 receptors (Figure 6).

Catecholamines have been previously shown to contribute to collateral artery growth and arteriogenesis in hindlimb ischemia in mice35,55; this was attributed to trophic effects of adrenergic signaling on arterial smooth muscle cells and mobilization of bone marrow–derived hematopoietic cells that contribute to hindlimb revascularization. Our data extend these findings by revealing potential direct effects of catecholamines on arterial endothelial cells themselves. Exposure of isolated E8 arterial endothelial cells to norepinephrine or adrenoreceptor agonists induces virtually all of them to colonize host arteries, and treatment of HUVECs enhances expression of arterial marker genes. Developmentally, innervation of the arterial vessel wall and acquisition of arterial identity all occur at E11, and release of plasma catecholamines in chick embryos peaks during the last trimester of the incubation period,56 suggesting that circulating catecholamines may contribute to arterIALIZation as well. This might also explain why removal of innervation of the vessel wall induces only a partial loss of arterial identity, although we cannot exclude that other factors present in the vessel wall might contribute. Three lines of evidence suggest that effects on arterIALIZation are independent of effects on vasoconstriction: first, CAM arteries are arterIALIZED by norepinephrine treatment but do not vasoconstrict in response to catecholamines57; second, norepinephrine or adrenoreceptor activation can directly affect endothelium; and finally, doses of norepinephrine required to arterIALIZize endothelial cells are lower than those required to mediate vasoconstriction.30,31
Thus, low-dose vasoconstrictors might enhance arterialization in settings of ischemic disease. These data extend the neurovascular link between the peripheral nervous system and the arterial vasculature by demonstrating direct potential effects of catecholamines on endothelial fate decisions. Chronologically, during embryonic development, VEGF derived from sensory nerves or other sources promotes arterial differentiation of immature vessels. The newly specified arteries recruit smooth muscle cells that induce noradrenergic differentiation of sympathetic progenitors and promote extension and growth of sympathetic nerve fibers along the arteries and eventually arterial innervation. Innervation occurs during late gestation in avian embryos, which are fully motile at birth, whereas it occurs postnatally in mice, which remain immature and acquire full motility only after postnatal day 10. Catecholamine release in turn contributes to maintenance of arterial fate, revealing a positive feedback loop between nerve-induced arterial function and arterial morphogenesis.

Acknowledgments
We thank Ferdinand LeNoble and Jean-Sébastien Silvestre for comments.

Sources of Funding
This work was funded by Inserm and Agence Nationale pour la Recherche (Gandhi). A. Dubrac was supported by an American Heart Association (AHA) postdoctoral fellowship.

Disclosures
None.

References

Figure 6. Model for sympathetic nerve action on arterial differentiation. A, Grafted arterial cells contribute to 60% arterial and 40% venous cells before the onset of sympathetic innervation at E11, whereas after E11, they contribute only to arteries. B and C, In blood vessels, norepinephrine (NE) is released by sympathetic fibers surrounding the arterial wall and also from the adrenal glands that secrete NE in the blood stream, where it is directly in contact with endothelial cells. C, NE binds to α1AR and α2AR adrenoreceptors at the surface of smooth muscle and endothelial cells (red box). α1AR1 activates extracellular signal-regulated kinase (ERK) pathway by stimulating phospholipase C. α1AR2 inhibits the adenylyl cyclase/PKA pathway that represses ERK activity. ERK induction leads to the acquisition of arterial fate.


Novelty and Significance

What Is Known?
- Arterial and venous endothelial cells differ in morphology, function, and gene expression profile.
- Vascular endothelial growth factor and extracellular signal-regulated kinase (ERK) are known to drive arterial gene expression.

What New Information Does This Article Contribute?
- We show that sympathetic innervation promotes arterial differentiation.
- Adrenergic α1 and α2 receptors activate endothelial ERK signaling.

Arterial endothelial cells are distinct from those found in veins and lymphatic vessels. Understanding how arterial fate is acquired during development and maintained in adult vessels may lead to novel approaches to improve arterialization in human diseases. We developed a functional assay in avian chimeras, allowing us to monitor and manipulate arterial fate in vivo. We found that sympathetic innervation promotes arterial endothelial fate. Our results show that sympathetic nerves promote arterial differentiation independent of effects on vasoconstriction via adrenergic α1 and α2 receptor activation of endothelial ERK signaling, thereby enhancing arterial endothelial gene expression. These data reveal catecholamines as novel players in arterial endothelial fate decisions.
Sympathetic Innervation Promotes Arterial Fate by Enhancing Endothelial ERK Activity
Luc Pardanaud, Laurence Pibouin-Fragner, Alexandre Dubrac, Thomas Mathivet, Isabel English, Isabelle Brunet, Michael Simons and Anne Eichmann

_Circ Res._ 2016;119:607-620; originally published online June 27, 2016;
doi: 10.1161/CIRCRESAHA.116.308473

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2016 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

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/119/5/607

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2016/06/27/CIRCRESAHA.116.308473.DC1

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/
**Supplemental Material**

**Online Table I**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Activity</th>
<th>Concentration</th>
<th>Previously tested in Chicken</th>
<th>Result on aortic EC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norepinephrin (Sigma)</td>
<td>Adrenoceptor ligand</td>
<td>10^{-9}M to 10^{-8}M</td>
<td>E17-21 embryo 1</td>
<td>Promotes arterial fate</td>
</tr>
<tr>
<td>Phenylephrine (Sigma)</td>
<td>α1 adrenoceptor agonist</td>
<td>10^{-9}M to 10^{-8}M</td>
<td>E17-21 embryo 1</td>
<td>Promotes arterial fate</td>
</tr>
<tr>
<td>Prazosin (Sigma)</td>
<td>α1 adrenoceptor antagonist</td>
<td>1, 10 and 100nM</td>
<td>E17-21 embryo 1</td>
<td>Inhibits arterial fate</td>
</tr>
<tr>
<td>BHT933 (Sigma)</td>
<td>α2 adrenoceptor agonist</td>
<td>10^{-9}M to 10^{-8}M</td>
<td>E17-21 embryo 1</td>
<td>Promotes arterial fate</td>
</tr>
<tr>
<td>m-3M3FBS (R&amp;D Systems)</td>
<td>Phospholipase C agonist</td>
<td>1 mM, 10 mM and 50nM</td>
<td>E6 retinas 2</td>
<td>Promotes arterial fate</td>
</tr>
<tr>
<td>Forskolin (Sigma)</td>
<td>Adenyl cyclase activator</td>
<td>1 and 10mM</td>
<td>CAM arteries 4</td>
<td>Inhibits arterial fate</td>
</tr>
<tr>
<td>SQ22536 (Sigma)</td>
<td>Adenyl cyclase antagonist</td>
<td>0.5, 1 and 10mM</td>
<td>E14 astrocytes 5</td>
<td>Promotes arterial fate</td>
</tr>
<tr>
<td>8CPT-2Me-cAMP (Sigma)</td>
<td>cAMP activator</td>
<td>200mM</td>
<td>E10 retinas 6</td>
<td>Inhibits arterial fate</td>
</tr>
<tr>
<td>KT5720 (Sigma)</td>
<td>PKA inhibitor</td>
<td>0.1 and 1μM</td>
<td>E15 astrocytes 7</td>
<td>Promotes arterial fate</td>
</tr>
<tr>
<td>U0126 (Sigma)</td>
<td>ERK signaling inhibitor</td>
<td>0.1, 1 and 10mM</td>
<td>E2 embryos 5</td>
<td>Inhibits arterial fate</td>
</tr>
</tbody>
</table>


2. Chen SK, Ko GY, Dryer S.E. Somatostatin peptides produce multiple effects on gating properties of native cone photoreceptor cGMP-gated channels that depend on circadian phase and previous illumination. *J Neurosci.* 2007; 27:12168-12175.


**Online figure I**

A) Identification of quail endothelial cells emigrating from the graft (G) in the host using QH1/vWF/LEP100 immunostaining. QH1+/vWF+ endothelial cells (arrows) can be discriminated from QH1+/LEP100+ macrophages (arrowhead). Ao=aorta. B) VE-CADH/QH1 staining identifies quail endothelial cells both in the graft (arrows) and migrating in the host (arrowhead). C) A cluster of round QH1+ hematopoietic progenitors (arrowheads) that buds from the ventral endothelium of the aorta (Ao) is visible among QH1+ endothelial cells (arrows). Hematopoietic cells were not counted in this study. G=graft. Bars=50µm (A), 60µm (B), 40µm (C).

**Online figure II**

Western blot of isolated endothelial cells from E8 and E15 quail aorta labeled with anti-P-ERK and total ERK. Each lane represents one sample of cells isolated from 16-25 aortae. Quantification (bottom) shows that P-ERK increases in isolated endothelial cells between E8 and E15 while total ERK does not vary.

**Online figure III**

Glyoxylic acid stains sympathetic nerves in E15 carotid (top) and femoral artery (bottom) vessel walls. Bar=60µm

**Online figure IV**

Arterial colonization following surgical denervation of E15 carotid and femoral arteries. **P<0.01, ***P<0.001, Student’s t test by comparison with E15 carotids or aortae.

**Online figure V**

Drugs that induce α1 adrenergic pathway stimulate the expression of arterial markers in HUAECs cultured in the presence of VEGF (A). Their effects are inhibited when U0126 is added (B). The α2 adrenergic pathway also increases the arterial markers (C) that are partly down-regulated in presence of U0126 (D). n=6 in A-D.
*P<0.05, **p<0.01, Mann-Whitney U test, by comparison with untreated HUAEC.

**Online figure VI**
Quantification of the total number of emigrating QH1⁺ endothelial cells in untreated or treated E8 aortae with inhibitors of the α2 adrenergic pathway or ERK: no significant difference is observed.
Online figure I

A

B

C

QH1/WVF/LEP100

VE CADH/QH1

QH1/Glyceomalun
Online figure II

E8 EC  E15 EC
P-ERK

tot-ERK

P-ERK / tot-ERK

Ao E8  Ao E15

n=3  n=3
Online figure IV

% of QH+ EC found in arteries

- E15 Carotids
- + Surgical denerv.
- E15 Aortae

n=7  n=7  n=17  n=8
Online figure V

A

Relative gene expression (compared to HUVEC+VEGF)

JAGGED1  |  EFNB2  |  DLL4

HUVEC  |  HUVEC+PE (30nM)  |  HUVEC+3MSFBS (5uM)  |  HUVEC

B

Relative gene expression (compared to HUVEC+VEGF)

JAGGED1  |  EFNB2  |  DLL4

HUVEC  |  HUVEC+PE (30nM)  |  HUVEC+3MSFBS (5uM)  |  HUVEC

C

Relative gene expression (compared to HUVEC)

JAGGED1  |  EFNB2  |  DLL4

HUVEC  |  HUVEC+BHT903 (0.1uM)  |  HUVEC+KT77520 (1uM)  |  HUVEC+SQ22536 (1uM)  |  HUVEC

D

Relative gene expression (compared to HUVEC+VEGF)

JAGGED1  |  EFNB2  |  DLL4

HUVEC  |  HUVEC+BHT903 (0.1uM)  |  HUVEC+KT77520 (1uM)  |  HUVEC+SQ22536 (1uM)  |  HUVEC