Protection Against Myocardial Infarction and No-Reflow Through Preservation of Vascular Integrity by Angiopoietin-Like 4

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Background—Increased permeability, predominantly controlled by endothelial junction stability, is an early event in the deterioration of vascular integrity in ischemic disorders. Hemorrhage, edema, and inflammation are the main features of reperfusion injuries, as observed in acute myocardial infarction (AMI). Thus, preservation of vascular integrity is fundamental in ischemic heart disease. Angiopoietins are pivotal modulators of cell–cell junctions and vascular integrity. We hypothesized that hypoxic induction of angiopoietin-like protein 4 (ANGPTL4) might modulate vascular damage, infarct size, and no-reflow during AMI.

Methods and Results—We showed that vascular permeability, hemorrhage, edema, inflammation, and infarct severity were increased in angptl4-deficient mice. We determined that decrease in vascular endothelial growth factor receptor 2 (VEGFR2) and VE-cadherin expression and increase in Src kinase phosphorylation downstream of VEGFR2 were accentuated after ischemia-reperfusion in the coronary microcirculation of angptl4-deficient mice. Both events led to altered VEGFR2/VE-cadherin complexes and to disrupted adherens junctions in the endothelial cells of angptl4-deficient mice that correlated with increased no-reflow. In vivo injection of recombinant human ANGPTL4 protected VEGF-driven dissociation of the VEGFR2/VE-cadherin complex, reduced myocardial infarct size, and the extent of no-reflow in mice and rabbits.

Conclusions—These data showed that ANGPTL4 might constitute a relevant target for therapeutic vasculoprotection aimed at counteracting the effects of VEGF, thus being crucial for preventing no-reflow and conferring secondary cardioprotection during AMI. (Circulation. 2012;125:140-149.)

Key Words: no-reflow ■ acute myocardial infarction ■ endothelial cells ■ vascular integrity ■ hypoxia

Rapid restoration of adequate myocardial reperfusion in obstructed infarct arteries is a key determinant of short- and long-term outcomes for patients with acute myocardial infarction (AMI). Restoration of blood supply in cardiac ischemic tissue provides oxygen and nutrients to the starved myocardium and thus limits the extent of AMI, but it also induces microvascular dysfunction, inflammation, and oxidative damage. Increased vascular permeability is an important
Vascular damage also contributes to inadequate myocardial perfusion (the "no-reflow" phenomenon), which is observed in 30% of patients and is associated with a worse prognosis at follow-up and higher incidence of death. Prevention of vascular leak and edema improves flow during reperfusion post-AMI. Enhancing myocardial flow therefore represents a pertinent strategy to improve ongoing therapies that might limit infarct size, ameliorate cardiac function, and prolong survival. Treatments that minimize microvascular damage should be prioritized to protect the injured myocardium as recommended by guidelines set by the American College of Cardiology/American Heart Association. However, pharmacological treatment that targets microvascular dysfunction and inhibits the no-reflow phenomenon after reperfusion is not available.

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Angiopoietins are major regulators of angiogenesis and vascular leakage through binding to the Tie2 receptor. Angiopoietin-1 promotes vessel maturation, whereas angiopoietin-2 destabilizes interactions between the cells of the vascular wall responsible for endothelial barrier disruption and vascular leakage. Angiopoietin-like proteins (ANGPTL1–7) share structural and functional properties with angiopoietins but do not bind to the Tie2 receptor. Angiopoietin-like protein 4 (ANGPTL4) is a secreted 55-kDa protein that is processed in 20-kDa and 35-kDa forms. It is a dual-function protein: an inhibitor of lipoprotein and angiogenesis in vivo. In humans, ANGPTL4 possesses several variants, including the inactive E40K variant, which is associated with significantly lower plasma levels of angiogenic properties and inhibits the no-reflow phenomenon after reperfusion is not available.

ANGPTL4 is a relevant therapeutic approach to confer secondary cardioprotection in AMI.

Materials and Methods

The experiments were performed in accordance with the guidelines of the French Ministry of Agriculture. This study conforms to the standards of INSERM (the French National Institute of Health) in accordance with European Union Council Directives (86/609/EEC). All experiments were performed blindly, meaning that the experimenter was blind to the mouse genotype.

Animals and Genotyping

Agnptl4<sup>−/−</sup>LacZ/LacZ mice in which the agnptl4 locus was replaced by a lacZ reporter gene were generated and genotype was determined by the polymerase chain reaction of tail genomic DNA. Eight to 12-week-old agnptl4<sup>−/−</sup>LacZ/LacZ and agnptl4<sup>−/−</sup>LacZ/LacZ knockout male mice, intercrossed in C57Bl/6 mice for >8 generations, were subjected to myocardial infarction protocols or used as control in basal conditions.

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Myocardial Ischemia-Reperfusion Experiments

Ischemia-reperfusion protocol was performed on agnptl4<sup>−/−</sup>LacZ/LacZ and agnptl4<sup>−/−</sup>LacZ/LacZ mice or rabbits by using a standard technique described in the online-only Data Supplement Materials. Mice and rabbits randomly received either vehicle or human recombinant 55-kDa full-length ANGPTL4 (rhANGPTL4 10 µg/kg IV).

Modified Miles Assay

Male agnptl4<sup>−/−</sup>LacZ/LacZ and agnptl4<sup>−/−</sup>LacZ/LacZ mice were anesthetized with the use of pentobarbital. Under basal conditions, mice were subjected to coronary occlusion for 45 minutes and intravenously injected with 1% Evans blue (200 µL) before the 4 hours of reperfusion. At euthanization, mice were perfused through the aorta with citrate buffer, pH4. Blood, dye, and buffer exited through an opening in the right atrium. Evans blue was eluted for 18 hours at 70°C in 1 mL of formamide. After centrifugation, absorbance at 620 nm was measured by using a spectrophotometer. Extravasated Evans blue (ng) was determined from a standard curve and normalized to tissue weight (g).

Immunofluorescence Study and Confocal Analysis on Cryosections

Immunofluorescence staining was performed as previously described, and confocal analysis on cryosections is detailed in the online-only Data Supplement Materials.

Immunoprecipitation and Immunoblotting Analyses

For in vivo samples, mice subjected to 45 minutes of ischemia followed by 4 hours or 18 hours of reperfusion, were anesthetized, injected into tail vein with 1 mmol/L Na<sub>3</sub>VO<sub>4</sub> and 2 mmol/L H<sub>2</sub>O<sub>2</sub>, and dissected to remove the left ventricle. For human umbilical artery endothelial cell experiments, 40,000 cells/cm<sup>2</sup> were seeded in 20-kDa and 35-kDa forms. It is a dual-function protein: an inhibitor of lipoprotein and angiogenic properties. In vivo, ANGPTL4 possesses several variants, including the inactive E40K variant, which is associated with significantly lower plasma levels of angiogenic properties and inhibits the no-reflow phenomenon after reperfusion is not available.

We show here that ANGPTL4 expression is induced in response to myocardial infarction in humans and mice. The goal of the present study was to analyze the role that ANGPTL4 might play in this context. Indeed, ANGPTL4 is expressed by cardiomyocytes, but also in ECs in response to hypoxia, and it could modulate vascular permeability. In a model of myocardial ischemia-reperfusion, we show that angptl4-deficient mice display (1) increased size of myocardial infarcts, (2) increased no-reflow, (3) decreased vascular integrity through Src-dependent dissociation of the VEGFR2/VE-cadherin complex and subsequent destabilization of endothelial adherens junctions, and (4) increased hemorrhage and inflammation. In addition, we show that recombinant human ANGPTL4 (rhANGPTL4) has therapeutic potential in reducing no-reflow, hemorrhage, and infarct size after myocardial infarction in mice and in a nonrodent species (rabbit). These data therefore provide evidence that vasculoprotection through ANGPTL4 is a relevant therapeutic approach to confer secondary cardioprotection in AMI.

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Myocardial Ischemia-Reperfusion Experiments

Ischemia-reperfusion protocol was performed on agnptl4<sup>−/−</sup>LacZ/LacZ and agnptl4<sup>−/−</sup>LacZ/LacZ mice or rabbits by using a standard technique described in the online-only Data Supplement Materials. Mice and rabbits randomly received either vehicle or human recombinant 55-kDa full-length ANGPTL4 (rhANGPTL4 10 µg/kg IV).

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Isolation of Cardiomyocytes and Viability Assay

Cardiac myocytes were isolated as described in the online-only Data Supplement Materials. Mice and rabbits randomly received either vehicle or human recombinant 55-kDa full-length ANGPTL4 (rhANGPTL4 10 µg/kg IV).

Modified Miles Assay

Male agnptl4<sup>−/−</sup>LacZ/LacZ and agnptl4<sup>−/−</sup>LacZ/LacZ mice were anesthetized with the use of pentobarbital. Under basal conditions, mice were injected into the tail vein with 1% Evans blue (200 µL) and euthanized 4 hours later. After ischemia-reperfusion, mice were subjected to coronary occlusion for 45 minutes and intravenously injected with 1% Evans blue (200 µL) before the 4 hours of reperfusion. At euthanization, mice were perfused through the aorta with citrate buffer, pH4. Blood, dye, and buffer exited through an opening in the right atrium. Evans blue was eluted for 18 hours at 70°C in 1 mL of formamide. After centrifugation, absorbance at 620 nm was measured by using a spectrophotometer. Extravasated Evans blue (ng) was determined from a standard curve and normalized to tissue weight (g).

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Isolation of Cardiomyocytes and Viability Assay

Cardiac myocytes were isolated as described in the online-only Data Supplement Materials and incubated in an anaerobic chamber containing a humidified atmosphere of 5% CO<sub>2</sub> and 95% N<sub>2</sub> for 3 hours. Experimental medium was changed to serum-free, glucose-
free. Cardiac myocyte survival was measured by staining cells with trypan blue (Sigma).

**Statistical Analyses**

All data were analyzed with the use of nonparametric tests. Overall comparisons were performed either by using the Kruskal-Wallis test (1 factor, 3 groups) or the Friedman test (2 factors, 2 groups). Individual comparisons were examined with a Mann-Whitney test. The use of Bonferroni correction allowed the adjustment for multiple comparisons. Differences were considered significant with *P<0.05.

**Results**

**Early Postischemic Vascular Integrity Is Altered in angptl4lacZ/LacZ Mice**

Angptl4lacZ/LacZ mice in which the angptl4 locus was replaced by a lacZ reporter gene were generated. We first analyzed vascular permeability after 45 minutes of ischemia and 4 hours of reperfusion in the heart of angptl4lacZ/LacZ and angptl4lacZ/+ mice. Histological analyses showed that tissue damage was equivalent in both groups at this early time point (data not shown). In contrast, angptl4lacZ/LacZ mice displayed an increased vascular leakage of Evans blue dye compared with angptl4lacZ/+ mice 4 hours after ischemia (117.5 ± 8.7 versus 67.8 ± 17 μg/mL, P < 0.05), whereas no significant difference was observed under basal conditions (Figure 1A).

Importantly, angptl4lacZ/LacZ mice did not exhibit functional cardiac defects (online-only Data Supplement Table I) or abnormal vascular morphology, as examined by CD31 and NG2 staining (which was used to label endothelial cells and pericytes, respectively) under basal (online-only Data Supplement Figure 1A and 1B) and ischemic conditions (online-only Data Supplement Figure 1C and 1D).

Then, fluorescent microspheres were injected to label sites of vascular leakage. Under basal conditions, fluorescein isothiocyanate beads did not extravasate in either group (Figure 1B and 1C, online-only Data Supplement Figure 1A and 1B). After 45 minutes of ischemia and 4 hours of reperfusion, fluorescein isothiocyanate beads were not observed in angptl4lacZ/+ mice (Figure 1D and online-only Data Supplement Figure 1C), whereas extravasation of fluorescent microspheres was detected in angptl4lacZ/LacZ mice, indicating that endothelial integrity was altered (see asterisks in Figure 1E and online-only Data Supplement Figure 1D).

The stability of adherens junctions is critical for the maintenance of the endothelial permeability and integrity. Hence, we sought to investigate VE-cadherin distribution at endothelial adherens junctions under basal conditions and after ischemia-reperfusion. In nons ischemic myocardium from both genotypes, VE-cadherin linear signal labeling a dense vascular network was observed (Figure 1B and 1C). After ischemia-reperfusion, a heterogeneous pattern of VE-cadherin staining was observed; both intense and linear signals were adjacent to thinner signals in angptl4lacZ/+ mice (arrows and arrowheads, respectively, in Figure 1D and 1E). In angptl4lacZ/LacZ mice, ischemia-reperfusion injury induced more severe damage at endothelial junctions, which were mainly disrupted as shown by more systematic discontinuous VE-cadherin staining (Figure 1E, open arrowheads). Three-dimensional reconstruction from confocal images stained with anti–VE-cadherin (Figure 1F and 1G) or with anti-CD31 and anti-NG2 (online-only Data Supplement Figure IE and IF) antibodies further confirmed disrupted EC junctions and extravasated fluorescein isothiocyanate beads in angptl4lacZ/LacZ coronary vessels.

These observations suggested that coronary vascular integrity was severely disrupted and junction disassembly more
frequent in angpt1/LacZ/LacZ mice in comparison with angpt1/LacZ/+ mice after ischemia/reperfusion, thereby leading to increased vascular permeability.

Postischemic Decrease in the Expression of VEGFR2 and VE-Cadherin Combined to Increase Src Kinase Phosphorylation Downstream of VEGFR2 in angpt1LacZ/LacZ Mice

In the vasculature, VEGFR2 and VE-cadherin form complexes that are transiently dissociated on VEGF binding to VEGFR2. During myocardial infarction, ischemia promotes VEGF expression that leads to vascular permeability and edema. We therefore investigated if disassembly of the VEGFR2/VE-cadherin complex might constitute the mechanism responsible for increased junctional disruption in angpt1/LacZ/LacZ mice after ischemia-reperfusion injury.

Using real-time quantitative reverse transcription polymerase chain reaction methodology, the expression of vegfr2 and ve-cadherin mRNA were quantified in the left ventricle in angpt1/LacZ/+ and angpt1/LacZ/LacZ mice under control conditions or after 4 or 18 hours of reperfusion. As shown in online-only Data Supplement Figure IIA, vegfr2 (left) and VE-cadherin (right) mRNA expression was similar in both groups under basal conditions. After 4 hours of reperfusion, the decrease in vegfr2 expression was more prominent in angpt1/LacZ/LacZ mice in comparison with control mice (66.5% versus 44.6% for vegfr2, P<0.05). VE-cadherin mRNA expression was unchanged in angpt1/LacZ/LacZ mice in comparison with control mice. Vegfr2 mRNA downregulation was maintained after 18 hours of reperfusion (decrease of 60±4% for angpt1/LacZ/LacZ mice versus 10±5% for angpt1/LacZ/+ mice, P<0.05). VEGFR2 and VE-cadherin protein levels were strongly affected at 18 hours of reperfusion as shown by Western blot analyses undertaken with the use of total extracts from left ventricles (online-only Data Supplement Figure IIB).

Src signaling transduces VEGF-mediated permeability by dissociating the VEGFR2/VE-cadherin complex. To further determine the mechanism leading to early postischemic junction alteration in angpt1/LacZ/LacZ mice, Src kinase signaling downstream of VEGFR2 was analyzed under control conditions and after ischemia-reperfusion. Left-ventricle lysates were immunoprecipitated for VEGFR2 followed by immunoblotting for VEGFR2, VE-cadherin, Src, and phospho-Src (Figure 2). Transient destabilization of VEGFR2/VE-cadherin complexes was observed at 4 hours of reperfusion and was restored after 18 hours of reperfusion in angpt1/LacZ/+ mice whereas VE-cadherin remained dissociated from VEGFR2 in angpt1/LacZ/LacZ mice at both time points (Figure 2A, top). In addition, immunoblotting showed an increased phosphorylation of Src kinase after 18 hours of reperfusion in angpt1/LacZ/+ in comparison with angpt1/LacZ/+ mice (Figure 2A, middle and bottom, and 2B).

These results showed that a decrease in the expression of VEGFR2 and VE-cadherin combined with an increase in the phosphorylation of Src kinase downstream of VEGFR2 lead to dissociation of the VEGFR2/VE-cadherin complex. Both events are responsible for massive disorganization of VE-cadherin in endothelial adherens junctions in angpt1/LacZ/LacZ mice after ischemia-reperfusion.

Infarct Size, No-Reflow, and Postischemic Inflammation Are Increased in angpt1LacZ/LacZ Mice

We next hypothesized that alteration of vascular integrity in angpt1/LacZ/LacZ mice might be translated to abnormal myo-
cardial reperfusion and damage to heart tissue at 48 hours of reperfusion. Indeed, infarct size was increased in angptl4LacZ/LacZ mice in comparison with angptl4LacZ/H11001 mice (47% versus 36%, P<0.01) (Figure 3A). In addition, no-reflow was more important in the angptl4LacZ/LacZ group in comparison with the angptl4LacZ/H11001 mice if expressed as a percentage of the area at risk (19% versus 11%, P<0.05) (online-only Data Supplement Table II).
Necrosis, hemorrhage, and edema were also quantified (score 1–3) on hematoxylin and eosin–stained sections from infarcted hearts of both genotypes (Figure 3B and 3C). In accordance with increased infarct size, tissue necrosis was increased in \(\text{angptl4}^{+/\text{LacZ/LacZ}}\) in comparison with control mice (2.5±0.6 versus 1.2±0.2) (Figure 3D). Similarly, assessment of hemorrhage and edema revealed more severe tissue injury in \(\text{angptl4}^{+/\text{LacZ/LacZ}}\) mice (2.3±0.6 versus 1.1±0.2 and 2.3±0.2 versus 0.8±0.2, respectively; Figure 3D). The post-ischemic inflammatory response was also analyzed in both genotypes (Figure 3E to 3G). Macrophage density was significantly higher in infarcted areas in \(\text{angptl4}^{+/\text{LacZ/LacZ}}\) mice, whereas no statistical difference was observed between the 2 groups in noninfarcted areas (Figure 3G).

We then analyzed vascular density in the core infarct area and in the periphery by the use of CD31 staining (Figure 3H and 3I). A similar diminished microcapillary density was quantified in both genotypes in the central infarcted areas in comparison with the periphery. No difference was quantified between both genotypes in both areas (Figure 3I).

Transmission electron microscopy study was undertaken to assess tissue injury at the ultrastructural level. Analyses of reperfused infarcted areas did not show structural differences in cardiomyocytes between the 2 groups (see C1–3 in online-only Data Supplement Figure IIIA and IIIB). In contrast, large edematous areas with few inflammatory cells were observed in \(\text{angptl4}^{+/\text{LacZ/LacZ}}\) mice, whereas inflammatory cells had already invaded the edematous region in \(\text{angptl4}^{+/\text{LacZ/LacZ}}\) mice (see E in online-only Data Supplement Figure IIIc and IIID). Polynuclear neutrophils, macrophages (\(\text{M}\Phi\)), lymphocytes (L), and fibrinogen deposits (F) were observed only in \(\text{angptl4}^{+/\text{LacZ/LacZ}}\) mice (online-only Data Supplement Figure 3D). Taken together, these data indicated increased vascular alterations that correlated with increased inflammatory infiltrates in \(\text{angptl4}^{+/\text{LacZ/LacZ}}\) mice.

In addition, hypoxic activation of \(\text{angptl4}\) mRNA has been reported in cardiomyocytes in vitro, probably mediated by hypoxia-inducible factor 1.24 Using in situ hybridization, we demonstrated \(\text{angptl4}\) mRNA expression in vivo in cardiomyocytes and in ECs in cardiac samples from patients who died of AMI (online-only Data Supplement Figure IV). As in humans, in situ hybridization performed in mice subjected to ischemia-reperfusion showed that \(\text{angptl4}\) mRNA expression was induced in cardiomyocytes and in ECs (online-only Data Supplement Figure VA and VB). LacZ staining undertaken on hematoxylin and eosin–stained and CD31-immunostained sections and on whole mount from \(\text{angptl4}^{+/\text{LacZ/LacZ}}\) mice further confirmed expression in cardiomyocytes and ECs after ischemia-reperfusion injury (online-only Data Supplement Figure VC to VF, see arrowheads and arrows, respectively). We therefore tested the hypothesis that ANGPTL4 might affect cardiomyocyte survival in hypoxic conditions. Mouse cardiomyocytes from both genotypes were isolated and subjected to an in vitro survival assay. No difference in cardiomyocyte survival was observed in vitro between both groups in normoxia or hypoxia, suggesting that ANGPTL4 does not have a direct effect on cardiomyocytes (Figure 3K), thereby further suggesting it targets ECs.

Recombinant ANGPTL4 Stabilizes the VEGFR2/VE-Cadherin Complex in Response to VEGF

We next investigated if rhANGPTL4 might rescue the more severe ischemia-reperfusion–induced cardiac damage in \(\text{angptl4}^{+/\text{LacZ/LacZ}}\) mice. When injected in \(\text{angptl4}^{+/\text{LacZ/LacZ}}\) mice at 10 \(\mu\text{g/kg}\) body weight 5 minutes before ischemia-reperfusion, rhANGPTL4 reduced infarct size to 41±4% in comparison with 67±7% when mice were injected with vehicle \((P<0.05; \text{Figure 4A})\). Remarkably, the infarct size of \(\text{rhANGPTL4}\)-injected \(\text{angptl4}^{+/\text{LacZ/LacZ}}\) mice was indistinguishable from that of control \(\text{angptl4}^{+/\text{LacZ/+}}\) mice (47±2%) injected with vehicle, showing that ANGPTL4 could rescue the knockout phenotype in mice.

We then sought to define in vitro the molecular pathways responsible for rhANGPTL4-induced cardioprotection by testing if rhANGPTL4 could confer protection of disassembly of the VEGFR2/VE-cadherin complex. Confluent human umbilical artery endothelial cells were stimulated for 5 minutes with VEGF alone or with rhANGPTL4. Cell lysates were immunoprecipitated for VEGF2 followed by immunoblotting for VEGFR2, VE-cadherin, Src, and phospho-Src. The preexisting VEGFR2/VE-cadherin observed in control conditions was rapidly disrupted within 5 minutes of VEGF stimulation. This complex was protected from dissociation in cells treated with both VEGF and rhANGPTL4 (Figure 4B, left, and 4C). Src kinase and phospho-Src immunoblotting revealed that VEGF-mediated destabilization of the VEGFR2/VE-cadherin complex was correlated with an increased phosphorylation of Src downstream of VEGFR2 that was reduced in cells treated with both VEGF and rhANGPTL4 (Figure 4B, right, and 4C).

Assessment of the Therapeutic Cardioprotective Effects of rhANGPTL4 in a Nonrodent Species

We next hypothesized that attenuation of vascular alterations by rhANGPTL4 may lead to the enhancement of endothelial barrier function, which ultimately could protect ECs from ischemia-induced reperfusion injury. Myocardial ischemia-reperfusion does not induce massive no-reflow in mice under these conditions, so we sought to analyze the therapeutic potential of ANGPTL4 in a nonrodent species. We chose an open-chest model of myocardial ischemia-reperfusion in rabbits, because the no-reflow phenomenon had been well established in this model.31

We undertook intravenous injection of 10 \(\mu\text{g/kg}\) rhANGPTL4 or vehicle, 5 minutes before ischemia-reperfusion. Infarct size (percentage area at risk) was 57±5% in the control group and 34±7% in the rhANGPTL4-treated group \((P<0.01; \text{Figure 5A})\). Then, the zone of no-reflow was studied. When expressed as a percentage of the area at risk, it was 41±2% in the control group and 19±6% in the rhANGPTL4-treated group \((P<0.05; \text{Figure 5B})\). More importantly, when expressed as a percentage of the infarct size, it was 73±4% in the control group and 55±7% in the rhANGPTL4-treated group \((P<0.05; \text{Figure 5C})\). Histological analyses showed that myocardial infarction comprised a core of necrosis and huge hemorrhage within interstitial spaces in the control group (Figure 5D). In the rhANGPTL4-
preservation of vascular integrity that reduced infarct size, hemorrhage, and no-reflow, thereby conferring cardioprotection.

Discussion

On AMI, HIF proteins, the major transcription factors involved in the regulation of responses to hypoxia, are rapidly activated and induce VEGF-A expression that participates to the angiogenic response. However, VEGF-A also causes vascular permeability and edema, resulting in extensive injury. We showed here that angiptl4 mRNA, which was previously shown to be induced by hypoxia in ECs and in cardiomyocytes in vitro, and in critical hindlimb ischemia and stroke, as well, was also expressed in cardiac tissue from patients who died of AMI. In the present study, we provide evidence that ANGPTL4 mediates protection from postischemic tissue damage through preservation of the integrity of the vascular EC barrier that limits no-reflow and the extent of AMI.

In pathological ischemic conditions, increased permeability, which is controlled by endothelial junction stability, is responsible for altered vascular integrity. VE-cadherin, which constitutes the major component of the adherens junctions between ECs, is required in vivo in the postnatal vasculature to maintain the integrity and barrier function of the endothelium. Yang et al showed that myocardial VE-cadherin is significantly decreased in the ischemic myocardium, suggesting that microvascular integrity is damaged by ischemia-reperfusion. VE-cadherin associates with VEGFR2 and regulates permeability. Indeed, systemic VEGF-A injection, thereby activating VEGFR2, induces dissociation of the VEGFR2/VE-cadherin complex. Here, we showed durable dissociation of VEGFR2/VE-cadherin complexes and altered VE-cadherin distribution in angptl4 mice that caused disrupted adherens junctions and decreased EC barrier function after AMI. Gene and protein expression analyses revealed (1) more prominent diminished VEGF and VE-cadherin levels, and (2) prolonged decrease in vegfr2 mRNA levels in angptl4 knockout mice subjected to AMI. In the heart, decreased levels VEGFR2/VE-cadherin complexes in ECs in response to ischemia might participate in junction disruptions and altered endothelial integrity after AMI in angptl4 mice. Src is an essential molecule required for promoting the disruption of EC contacts and paracellular permeability. We here provide evidence for enhanced Src kinase phosphorylation leading to more severe destabilization of the VEGFR2/VE-cadherin complex in angptl4 mice subjected to AMI. Other members of the angiopoietin family also have a role in the regulation of vascular permeability. Angiopoietin-1 phosphorylates Tie2 and phosphatidylinositol 3-kinase, inducing activation of the GTPase Rac1, which is needed to maintain cell–cell adhesion and which also activates mDia, resulting in sequestration of Src. Whether regulation of Src/mDia or Rac1 signaling pathways by ANGPTL4 affects intracellular VE-cadherin distribution deserves further investigation.

Preservation of the microcirculatory network and, to a lesser extent, preservation of hemorrhage by rhANGPTL4 limited the extent of no-reflow. This phenomenon is the result of incompletely characterized anatomic changes of the coronary microcirculation in which ANGPTL4 might play a crucial role through its vasculoprotective effect. In addition, reperfused myocardial infarction is associated with cellular...
infiltration and the acute inflammatory response.41 A critical point in postischemic therapy is containment of the deleterious, persistent, and expanding inflammatory response.42 We showed here that altered vascular integrity in angptl4LacZ/LacZ mice might suppress a point of control that participates in limiting the postinfarction inflammatory response and expansion of the infarcted area.

Our results showing disorganization of endothelial adherens junctions in angptl4LacZ/LacZ mice suggest that ANGPTL4 could promote the endothelial barrier function at multiple levels. We showed that endothelial barrier integrity was perturbed in angptl4LacZ/LacZ mice, and that rhANGPTL4 could restore some degree of integrity and protect mice and rabbits during ischemia-reperfusion. In addition, ANGPTL4 modulates the balance between circulating triglyceride-rich lipoproteins, very-low-density lipoproteins and chylomicrons (as well as their uptake) by inhibiting endothelial lipase and lipoprotein lipase anchored at the surface of ECs.43,44 In angptl4LacZ/LacZ mice, which display decreased circulating levels of triglycerides, loss of inhibition of lipoprotein lipase could lead to increased lipolysis products such as oxidized fatty acids that induce EC inflammation.45 This could then participate in altered endothelial barrier integrity46 and, therefore, endothelial function.47 Elucidation of the precise mechanisms linking ANGPTL4 activity, triglyceride uptake, and potential fatty acid-induced oxidative stress25 needs to be definitively addressed. Nevertheless, markers of oxidative stress such as fkp53 and gsta3 were not differentially regulated in angptl4LacZ/LacZ mice in comparison with control mice during ischemia-reperfusion (data not shown), showing that this is not likely to be the case. Alternatively, ANGPTL4-mediated dysregulation of VEGF-B control of endothelial uptake of fatty acids may also be involved.48

Finally, whereas cardiomyocytes are primarily recognized as the therapeutic target of myocardial ischemia, few studies have focused on the importance of heart vessels in this context. Clinical efforts are underway to block VEGF-A–mediated leak in patients after AMI49 or stroke.50 The present findings show that ANGPTL4 counteracts the increase in permeability observed in reperfused AMI. This justifies the search for combined strategies that will have a significant impact on reducing tissue injury and improving the coronary microcirculation and the no-reflow phenomenon, thereby improving AMI therapy.

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References


**CLINICAL PERSPECTIVE**

The most effective way to limit myocardial infarct (MI) size is the earliest reperfusion of the ischemic myocardium. Although primary percutaneous coronary intervention achieves epicardial coronary artery reperfusion, paradoxical abnormal myocardial perfusion persists in 5% to 50% of MI patients despite the lack of angiographic evidence of mechanical vessel obstruction, a condition known as no-reflow. Reperfusion of the ischemic myocardium can induce reperfusion injury, ie, microvascular dysfunction and leaky vessels that promote edema and leukocyte infiltration. In these patients, the no-reflow phenomenon is a strong predictor of poor clinical outcome and of 5-year mortality. New strategies aimed at preventing no-reflow therefore represent a major medical need. We previously demonstrated that angiopoietin-like 4 (ANGPTL4) inhibits vascular permeability. Here, we showed induced ANGPTL4 expression in cardiac tissue from MI patients and in a mouse model. By the use of ANGPTL4 knockout mice, we showed that MI size was increased, and, whereas cardiomyocyte survival was not affected per se, major loss of integrity of coronary vessel network was evidenced. The knockout phenotype was rescued by administering human recombinant ANGPTL4, which counteracted ischemia-induced vascular endothelial growth factor signaling and disruption of endothelial cell junctions, thereby leading to subsequent protection of the coronary capillary network and reduction of infarct size. ANGPTL4 also reduced infarct size in a nonrodent species, ie, in rabbit, by preserving vascular integrity and reducing no-reflow. Therefore, ANGPTL4 represents a promising pharmacological and therapeutic approach, either alone or in combination with other strategies, for cardioprotection during acute MI by targeting the no-reflow phenomenon.
Protection Against Myocardial Infarction and No-Reflow Through Preservation of Vascular Integrity by Angiopoietin-Like 4
Ariane Galaup, Elisa Gomez, Rachid Souktani, Mélanie Durand, Aurélie Cazes, Catherine Monnot, Jérémie Teillon, Sébastien Le Jan, Claire Bouleti, Gaëlle Briois, Josette Philippe, Sandrine Pons, Valérie Martin, Rana Assaly, Philippe Bonnin, Philippe Ratajczak, Anne Janin, Gavin Thurston, David M. Valenzuela, Andrew J. Murphy, George D. Yancopoulos, Renaud Tissier, Alain Berdeaux, Bijan Ghaleh and Stéphane Germain

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Supplemental material

Supplemental methods

Mice myocardial ischemia-reperfusion experiments: Mice were anesthetized by an intraperitoneal injection of sodium pentobarbital. To assess infarct size and for immunohistochemistry (IHC), ultrastructural, biochemical or expression studies, male angptl4\(^{LacZ/+}\) and angptl4\(^{LacZ/LacZ}\) mice were reperfused during either 4 h, 18 h or 48 h after ischemia. For rescue study mice randomly received either vehicle or human recombinant 55 kDa full-length ANGPTL4 \(^1\) (10 µg/kg i.v.) 5 min before the 45 min occlusion step followed by 48 h-reperfusion. The area at risk was identified by Evans blue staining at 48 h after ischemia, and the infarct area was identified by 2,3,5-triphenyltetrazolium chloride (TTC) staining. The area at risk was identified as the non blue region and expressed as a percentage of the left ventricle weight. The infarcted area was identified as the TTC-negative zone and expressed as a percentage of the area at risk. To measure no-reflow, the chest was reopened and thioflavine S (4%; 1.5 mL/kg) was infused through the left atrium four hours after the onset of reperfusion. The hearts were then perfused retrogradely with Alcian blue (0.5%) and cut into slices. Slices were photographed using UV light to identify the region of no-reflow. The areas of infarct and risk zone were determined as defined above. Ultrastructural analyses were performed on a Hitachi H-9500 electron microscope.

Immunofluorescence study and confocal analysis on cryosections: Mice subjected to ischemia and 4 h of reperfusion were anesthetized with ketamine and xylazine injected intraperitoneally. FITC-beads (20 µL) were injected into the femoral vein as previously described \(^2\). The chest was opened rapidly, and the vasculature was perfused for 2 min at a pressure of 120 mmHg with 1% paraformaldehyde. The heart was then placed into 1%
paraformaldehyde for 1 h at room temperature, rinsed with PBS and frozen for cryostat sectioning. ECs, pericytes and adherens junctions were identified with rat anti-CD31 (BD Pharmingen), rabbit anti-NG2 (Chemicon) and rat anti-VE-Cadherin (personal gift from E. Dejana, IFOM) antibodies, respectively. Confocal sections were imaged on a Leica SP5 microscope (Leica Microsystems GmbH) using a 63x (NA= 1.4) oil objective. An increment of 0.6 µm between each section was used. 3D reconstruction of the different structures was obtained using the LABELVOXEL and the SURFACEGEN modules in Amira 5.2.1 software (Visage Imaging GmbH).

**Immunoprecipitation and immunoblotting analyses**: Proteins were extracted on ice in 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% DOC, 0.5% NP-40, 10% glycerol, 1 mM β-glycerophosphate, 1 mM NaF, 2.5mM Na pyrophosphate, 1 mM Na3VO4 and a cocktail of protease inhibitors (Calbiochem). Lysates were split for immunoprecipitation and for total extracts immunoblottings. For immunoprecipitation, extracts were precleared for 60 min with protein A–agarose beads, incubated overnight with anti–VEGFR-2 (Cell signaling), and the immunocomplexes were collected on protein A–agarose beads for 3 h. Proteins were eluted by boiling for 10 min in reducing laemmLi sample buffer. Samples were analyzed by SDS-PAGE followed by Western blotting on nitrocellulose membrane. Anti-VEGFR2 (Cell signaling), anti-VE-Cadherin (Santa Cruz), anti-Src kinase family (Cell signaling), anti-phospho Src family Tyr- 416 (Cell signaling) antibodies were used. Signal was revealed by Attophos chemiluminescence (Promega) and band intensity was quantified by Quantity One 1-D Analysis Software (Biorad).

**Isolation of cardiomyocytes and viability assay**: Under anesthesia, the heart was removed from the chest and was cannulated. The heart was perfused for 4 min with tyrode buffer
Perfusion was switched to an enzyme solution ([mM] NaCl 113 ; KCl 4.7 ; KH$_2$PO$_4$ 0.6 ; Na$_2$HPO$_4$ 0.6 ; HEPES 10 ; MgSO$_4$ 1.2 ; NaHCO$_3$ 12 ; KHCO$_3$ 10 ; taurine 30 ; phenol red 0.032 ; glucose 5.5 ; with pH adjusted to 7.46 with NaOH 1N) containing 0.1 mg/mL liberase blendzyme IV, (Roche diagnostics) and 0.14 mg/mL trypsin (Sigma). When hearts became swollen and turn slightly pale, the atria and aorta were removed; the left ventricle were cut into small pieces and gently triturated. Cell suspension was transferred into a stopping buffer ([mM] NaCl 113 ; KCl 4.7 ; KH$_2$PO$_4$ 0.6 ; Na$_2$HPO$_4$ 0.6 ; HEPES 10 ; MgSO$_4$ 1.2 ; NaHCO$_3$ 12 ; KHCO$_3$ 10 ; taurine 30 ; phenol red 0.032 ; glucose 5.5 ; CaCl$_2$ 0.0125 ; with pH adjusted to 7.46 with NaOH 1N). Extracellular calcium was added incrementally up to 1.0 mM. All cells studied were rod-shaped, had clear cross-striations and lacked any visible vesicles on their surfaces.

**Rabbit experiments:** New Zealand rabbits (2.5-3.0 kg) were anesthetized using zolazepam, tiletamine and pentobarbital (all 20-30 mg/kg i.v.). The animals were intubated, mechanically ventilated and a left thoracotomy was performed. A suture was passed beneath a major branch of the left coronary artery through a short propylene tubing to form a snare. Rabbits then randomly received either vehicle or human recombinant 55 kDa full-length ANGPTL4 (10 µg/kg i.v.). Five minutes after, coronary artery occlusion (CAO) was induced during 30-min by pulling the snare through the tubing. Reperfusion was subsequently induced by releasing the snare. The chest was then closed in layers. Four hours after the onset of reperfusion, the chest was reopened and thioflavine S (4%; 1.5 mL/kg) was infused through the left atrium. Rabbits were then sacrificed using pentobarbital followed by potassium chloride. After excision, the hearts were perfused retrogradely with Alcian blue (0.5%) and cut into slices.
Slices were photographed using UV light to identify the region of no-reflow. The areas of infarct and risk zone were determined as in mice.

**Ultrasound analysis of cardiac parameters:** Mice were subjected to ultrasound measurements using an echocardiograph (Vivid 7, GE Medical Systems Ultrasound) equipped with a 12-MHz linear transducer.

**Real-time quantitative PCR analysis (RT-qPCR):** Mice subjected to ischemia and 4 h or 18 h reperfusion were anesthetized, perfused for 2 min at a pressure of 120 mmHg with PBS and dissected to remove left ventricle. Total RNA was isolated by extraction with TRIzol (Invitrogen). Reverse transcription, quantitative PCR (in triplicate) and analysis were performed as previously described \(^3\). The following primers were used: mouse *ve-cadherin*, 5'-CACTGCACATCTACGGCTACG -3' and 5'- CAGTCGTTGAGGAAGTCATAATCG -3'; mouse *vegfr2* gene was detected using pre-designed primer pairs (QuantiTect primer assays; QIAGEN). mRNA expression level was normalized to the housekeeping gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Fold changes were calculated using the comparative Ct method.

**In situ hybridization (ISH) and immunohistochemistry (IHC) analyses:** Paraffin blocks of human infective endocarditis, congenital heart disease and myocardial infarcts were obtained from the Pathology Department of Georges Pompidou European Hospital, Paris, France. The presence of infarcted areas was assessed on standard HE staining and adjacent slides were used for ISH and IHC analyses. To assess *angptl4* mRNA expression in mice, male *angptl4lacZ/+* and *angptl4LacZ/LacZ* mice were reperfused during either 48 h after 45 min
ischemia. ISH using human or mouse angptl4 probes, LacZ staining and IHC immunolabellings anti-CD45, -Mac3, and-CD31 were performed as previously described \(^4\).
Supplemental Tables

<table>
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<tr>
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<th>angptl4^{LacZ+}</th>
<th>angptl4^{LacZ/-}</th>
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<tbody>
<tr>
<td>cardiac output (ml.min^{-1})</td>
<td>6.04 (± 1.43)</td>
<td>6.09 (± 0.98)</td>
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<td>left ventricular internal diameter in diastole (LVIDd, cm)</td>
<td>0.37 (± 0.03)</td>
<td>0.37 (± 0.03)</td>
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<tr>
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<td>0.08 (± 0.02)</td>
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<td>0.06 (± 0.01)</td>
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<td>left ventricle septum in systole (LVSc, cm)</td>
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<td>shortening fraction (SF, %)</td>
<td>36 (± 6.4)</td>
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<tr>
<td>Heart rate (bpm)</td>
<td>453 (± 44)</td>
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Galaup et al, Table I
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<th>No-reflow (% area at risk)</th>
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<tr>
<td>angptl4 \textsuperscript{LacZ/+}</td>
<td>11 (± 2)</td>
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<tr>
<td>angptl4 \textsuperscript{LacZ/ lacZ}</td>
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Galaup \textit{et al.}, Table II
Galaup et al, Supplemental figure 1
Galaup et al, Supplemental figure 2
Galaup et al, Supplemental figure 3
B

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<tr>
<th>Case n°</th>
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<td>1</td>
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<tr>
<td>2</td>
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<tr>
<td>3</td>
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<tr>
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<tr>
<td>6</td>
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<tr>
<td>7</td>
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<tr>
<td>8</td>
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Galaup et al, Supplemental figure 4
Supplemental Table Legends:

**Supplemental Table 1:** Measures of cardiac parameters assessed by ultrasound examinations performed on $\text{angptl}4^{\text{LacZ/+}}$ and $\text{angptl}4^{\text{LacZ/LacZ}}$ adult mice. Data are representative of 6 mice.

**Supplemental Table 2:** Extent of the no-reflow zone expressed as a percentage of the area at risk in $\text{angptl}4^{\text{LacZ/+}}$ mice compared to $\text{angptl}4^{\text{LacZ/LacZ}}$ mice subjected to ischemia and 48h reperfusion; *p<0.05 (B). Data are representative of 6 mice.

Supplemental Figures Legends:

**Supplemental Figure 1:** Confocal microscopic images of CD31 and NG2 immunoreactivities performed on frozen cardiac sections under basal conditions (A and B) or after 45 min ischemia-4 h reperfusion (C and D) in $\text{angptl}4^{\text{LacZ/+}}$ (left panel) and $\text{angptl}4^{\text{LacZ/LacZ}}$ adult mice (right panel). Scale bar=20 $\mu$m (A to D). Three dimensional-image rebuilt from confocal images showing extravasation of FITC-beads (green) from hearts subjected to ischemia-reperfusion stained with anti-CD31 (red) and anti-NG2 (blue) antibodies. Scale bar=2 $\mu$m (E and F). Data are representative of at least 3 experiments.

**Supplemental Figure 2:** Expression of $\text{vegfr2 mRNA}$ (left), $\text{ve-cadherin mRNA}$ (right) measured by RT-qPCR, in left ventricles isolated from $\text{angptl}4^{\text{LacZ/+}}$ and $\text{angptl}4^{\text{LacZ/LacZ}}$ mice in basal conditions (CTL) or subjected to 45 min ischemia and 4 h or 18 h reperfusion. RT-qPCR were performed in triplicate (A). Immunoblots of left ventricle of $\text{angptl}4^{\text{LacZ/+}}$ and $\text{angptl}4^{\text{LacZ/LacZ}}$ mice in basal conditions or subjected to 45 min ischemia and 4 h or 18 h reperfusion using VEGFR2, VE-Cadherin, and actin antibodies (B). Bar graphs show
VEGFR2 and VE-Cadherin protein expression levels normalized to actin (left and right, respectively). Bar graphs represent mean± SEM of 3 experiments. Differences were considered significant with *, p<0.05.

**Supplemental Figure 3:** Transmission electron micrographs of infarcted hearts from angptl4\(^{LacZ/+}\) and angptl4\(^{LacZ/LacZ}\) mice (left and right panels, respectively). Cardiomyocytes are marked by « C1 to 4 », edema by « E », macrophages by « Mφ », lymphocytes by « L », fibrinogen deposits by « F », vessels by « V » and pericytes by « Pc ». Cardiomyocytes in infarcted areas are shown in A and B. At higher magnification, analysis of edema (C&D) revealed the infiltrating macrophages and lymphocytes in angptl4\(^{LacZ/LacZ}\) mice. Pericytes are closely apposed to endothelial cells in angptl4\(^{LacZ/+}\) vessels (E) whereas basement membrane from angptl4\(^{LacZ/LacZ}\) vessels are edematous (arrow in F). Data in A–F are representative of at least 4 mice. Scale bar=5 µm in A, B and D. Scale bar=2 µm in C, E and F.

**Supplemental Figure 4:** *In situ* hybridization analysis of angptl4 mRNA levels in infective endocarditis (1 and 2), congenital heart disease (3 to 5) and AMI (6 to 10) in humans (A and B). At higher magnification, angptl4 mRNA after AMI was identified in both endothelial cells (top) and cardiomyocytes (bottom) around altered necrotic ischemic areas (N) (C). Scale bar=80 µm in A; 40 µm in C (upper panel) and 20 µm in C (upper panel).

**Supplemental Figure 5:** Histological analyzes performed in wild-type mice subjected to 45 min ischemia-48h reperfusion. H&E-stained sections showing infarcted area around areas of necrosis shown with arrowheads (A). *In situ* hybridization analysis of angptl4 mRNA levels in adjacent slides showing murine angptl4 mRNA expression in cardiomyocytes and endothelial cells (arrows) and showing necrosis areas (arrowheads) (B). β-galactosidase
staining recapitulating the *bona fide* expression pattern of the *angptl4* gene in the ischemic area of the heart of *angptl4*\(^{LacZ/LacZ}\) mice subjected to 45 min ischemia/48 h reperfusion (C to F). β-galactosidase staining in cardiomyocytes, arrowheads (C) and ECs, arrows (D and E). Double-labeling was performed using an anti-CD31 antibody in order to confirm endothelial cells identity (E). Whole-mount β-galactosidase staining showing staining downstream the coronary artery ligation (asterisk), atria were removed prior to β-galactosidase staining (F). Data are representative of at 4 mice. Scale bar=100 µm in A and B, 50 µm in C to E, 1000 µm in F.
Supplemental References:


