Attenuates Postinfarct Remodeling by Inducing Angiogenesis Through Heterocellular Signaling

Xin Zhao,* Poornima Balaji,* Ronald Pachon, Daniella M. Beniamen, Dorothy E. Vatner, Robert M. Graham, Stephen F. Vatner

Objective—Stimulation of cardiac α₁A-adrenergic receptors (α₁A-AR) has been proposed for treatment of heart failure, since it increases myocardial contractility. We investigated a different mechanism, induction of angiogenesis.

Approach and Results—Four to 6 weeks after permanent coronary artery occlusion, transgenic rats with cardiomyocyte-specific α₁A-adrenergic receptor overexpression had less remodeling than their nontransgenic littermates, with less fibrosis, hypertrophy and lung weight, and preserved left ventricular ejection fraction and wall stress (all P<0.05). Coronary blood flow, measured with microspheres, increased in the infarct zone in transgenic rats compared with nontransgenic littermates (1.4±0.2 versus 0.5±0.08 mL min⁻¹ g⁻¹; P<0.05), which is consistent with angiogenesis, as reflected by a 20% increase in capillary density in the zone adjacent to the infarct. The question arose, how does transgenic overexpression of a gene in cardiomyocytes induce angiogenesis? We identified a paracrine mechanism, whereby vascular endothelial growth factor-A mRNA and protein were increased in isolated transgenic cardiomyocytes and also by nontransgenic littermate cardiomyocytes treated with an α₁A-agonist, resulting in angiogenesis. Conditioned medium from cultured cardiomyocytes treated with an α₁A-agonist enhanced human umbilical vein endothelial cell tubule formation, which was blocked by an anti-vascular endothelial growth factor-A antibody. Moreover, improved cardiac function, blood flow, and increased capillary density after chronic coronary artery occlusion in transgenic rats were blocked by either a mitogen ERK kinase (MEK) or a vascular endothelial growth factor-A inhibitor.

Conclusion—Cardiomyocyte-specific overexpression of the α₁A-adrenergic receptors resulted in enhanced MEK-dependent cardiomyocyte vascular endothelial growth factor-A expression, which stimulates angiogenesis via a paracrine mechanism involving heterocellular cardiomyocyte/endothelial cell signaling, protecting against remodeling and heart failure after chronic coronary artery occlusion. (Arterioscler Thromb Vasc Biol. 2015;35:2451-2459. DOI: 10.1161/ATVBAHA.115.305919.)

Key Words: alpha adrenergic receptors ■ angiogenesis ■ heart failure ■ myocardial infarction ■ myocytes, cardiac ■ vascular endothelial growth factor

Cardiac-specific α₁A-adrenergic receptor (α₁A-AR) stimulation has been proposed as a therapeutic strategy for heart failure, as it increases myocardial contractility, and blockade of the α₁A-AR exacerbates heart failure. We investigated a different mechanism, namely that cardiomyocyte-specific overexpression of the α₁A-AR induces angiogenesis through a paracrine mechanism, which could be therapeutically beneficial for heart failure, particularly that due to chronic myocardial infarction (MI). This is because, in the presence of permanent coronary artery occlusion (CAO), even cardioprotective interventions are destined to fail as they do not increase blood flow distal to the CAO, even to that observed in hibernating myocardium. This is required to limit ischemic damage to the myocardium. In fact, despite the hundreds, if not thousands, of studies identifying molecular pathways protecting the heart, there has been little clinical translation of these findings, and even approaches to enhance myocardial regeneration have met with uneven success. In the permanent absence of blood flow to the ischemic heart and in the absence of preformed collateral channels, almost any intervention is destined to fail. Although several transgenic models have shown cardioprotection in the setting of ischemia/reperfusion, the key is that in reperfusion models, blood flow is restored after a relatively short duration of ischemia, generally from 15 minutes to an hour.
For these reasons, we investigated here whether \( \alpha_{1A} \)-AR overexpression also limits cardiac remodeling after chronic MI resulting from permanent CAO. Specifically, we examined the effects of permanent CAO on the development of remodeling and heart failure in a rat model with 40-fold cardiomyocyte overexpression of the \( \alpha_{1A} \)-AR. Our hypothesis was that if we observed protection from remodeling after 4 weeks of permanent CAO in the transgenic rats, then there must have been some sustained blood flow to the ischemic myocardium, resulting in preservation of left ventricular (LV) ejection fraction and wall stress (Figure 1). Indeed, we did observe that the transgenic \( \alpha_{1A} \)-AR rat heart was protected from remodeling after permanent CAO, and because, unlike the dog and hamster, the rat has few preformed collateral vessels, we also examined the extent to which protection against remodeling is mediated by angiogenesis. Accordingly, we measured coronary blood flow to the ischemic zone and quantified the percentage of viable myocardium within this zone, as well as the development of newly formed coronary vessels and Ki67-positive myocytes. Because overexpression of the \( \alpha_{1A} \)-AR was restricted to cardiomyocytes, we then determined the mechanism underlying neoangiogenesis in the transgenic hearts. Given that microarray studies, verified by quantitative polymerase chain reaction, identified vascular endothelial growth factor-A (VEGF-A) as the sole angiogenesis gene upregulated in transgenic myocytes, we focused on this factor and also examined mitogen ERK kinase (MEK)/extracellular-signal-regulated kinase (ERK) signaling, which has been found to be activated with \( \alpha_{1A} \)-AR stimulation and to induce VEGF-A mediated angiogenesis.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

### Results

#### Attenuated Cardiac Remodeling in Transgenic Rats After Chronic MI

At 4 to 6 weeks post MI, compared with nontransgenic littermates (NTLs), transgenic rats showed significantly less fibrotic tissue deposition in the areas adjacent (25% less) and remote (33% less) to the ischemic zone (Figure 2A). Compared with baseline, the amount of cellular hypertrophy post MI was significantly less in the transgenic group in both the adjacent (573±14 mm\(^2\)) and remote zones (485±34 mm\(^2\)) versus those seen in the NTLs (652±24 mm\(^2\) in the adjacent zone and 582±18 mm\(^2\) in the remote zone; \( P < 0.05 \); Figure 2B). Consistent with these findings, the ratio of LV weight/tibial length was reduced by 16% in transgenic rats compared with NTLs (Figure 2C; \( P < 0.05 \)) and the ratio of lung weight/tibial length was also lower in transgenic

### Figure 1

**A**, Schematic mechanism for reduced remodeling after chronic myocardial infarction in \( \alpha_{1A} \)-adrenergic receptor \( (\alpha_{1A} \)-AR) transgenic (TG) rats. Angiogenesis induced through the MEK (mitogen ERK kinase)/VEGF pathway provides blood flow to the ischemic zone, despite permanent coronary artery occlusion, to allow cardiomyocyte survival, reduce remodeling, and preserve cardiac function. This resulted in preserved left ventricular (LV) ejection fraction **B** and wall stress **C**. Results are expressed as mean±SEM; \( n=4 \) to 5 per group; \( P < 0.05 \) vs nontransgenic littermates (NTLs).
**Increased Angiogenesis in Transgenic Rats With Upregulation of VEGF-A**

Myocardial blood flow was studied with microspheres injected at 1 day, 1 week, 2 weeks, and 4 weeks post MI. During the 4-week period of permanent CAO, both groups showed a gradual recovery of blood flow within the ischemic zone. However, the rate of recovery was significantly faster and greater in transgenic rats when compared with their NTLs (Figure 3A). At 4 weeks post MI, the blood flow in the central ischemic, adjacent, and remote zones was consistently higher in TG group compared with NTL rats ($P<$0.05; Figure 3B).

Capillary density was increased by 21% in the zone adjacent to the infarct in transgenic rats compared with NTLs at 4 to 6 weeks after MI ($P<$0.05; Figure 3C). This was consistent with the finding of more viable tissue within the ischemic zone in transgenic rats (Figure 2E), secondary to collateral blood flow through angiogenesis.

Determination of angiogenic genes using microarray analysis revealed 6 upregulated angiogenesis-related genes in cardiomyocytes of transgenic rats. Of the 6 genes, only VEGF-A mRNA was validated by quantitative polymerase chain reaction to be significantly increased compared with NTLs (Figure 3D). In addition, we found a 3-fold increase in VEGF-A protein levels in transgenic mouse cardiomyocytes compared with NTLs ($P<$0.05; Figure 4A).

**Activation of the α_{1A}-AR in Cardiomyocytes-Induced Angiogenesis via a Paracrine Mechanism**

In isolated mouse cardiomyocytes, VEGF-A expression was found to be upregulated at both the protein and mRNA levels.
in transgenic mouse cardiomyocytes compared with those in NTLs (Figure 4A and 4B). VEGF-A levels also increased significantly after stimulation of NTL cardiomyocytes with α<sub>1A</sub>-AR agonist, A61603 (25 nmol/L; Figure 4A and 4B). A61603-mediated upregulation of VEGF-A mRNA was abolished by pretreatment of cardiomyocytes with the α<sub>1A</sub>-AR antagonist, prazosin (1 µmol/L; Figure 4B), indicating that the upregulation of VEGF-A is mediated by the α<sub>1A</sub>-AR.

To evaluate the underlying cellular mechanism of the α<sub>1A</sub>-AR–mediated angiogenic effect, we used a Matrigel culture system using human umbilical vein endothelial cells. Tubule formation was increased after treating human umbilical vein endothelial cells with conditioned medium collected either from cultured transgenic cardiomyocytes or from A61603-treated NTL cardiomyocytes, but it was completely abolished by pretreatment with an anti-VEGF-A antibody (Figure 4C and 4D) but not by pretreatment with control IgG (data not shown). This suggests that the growth factor, VEGF-A, is required for endothelial cell growth and organization into tubules, after its release from cardiomyocytes on activation or with overexpression of the α<sub>1A</sub>-AR. Thus, the α<sub>1A</sub>-AR seems to program an angiogenic response within the myocardium that enhances endothelial cell organization through a paracrine mechanism involving heterocellular signaling.

### Table 1. Cardiac Function at Baseline and at 4- to 6-wk After MI

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>4- to 6-wk MI</th>
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<tbody>
<tr>
<td>Heart rate, bpm</td>
<td>NTL 387±6.3</td>
<td>354±13</td>
</tr>
<tr>
<td></td>
<td>TG 358±11</td>
<td>329±7.6</td>
</tr>
<tr>
<td>Mean arterial pressure, mm Hg</td>
<td>NTL 112±14</td>
<td>113±12</td>
</tr>
<tr>
<td></td>
<td>TG 108±8.9</td>
<td>111±6.2</td>
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<tr>
<td>LV systolic pressure, mm Hg</td>
<td>NTL 143±3.9</td>
<td>143±3.9</td>
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<tr>
<td></td>
<td>TG 135±5.0</td>
<td>142±9.1</td>
</tr>
<tr>
<td>LV ejection fraction, %</td>
<td>NTL 71±0.5</td>
<td>38±1.1</td>
</tr>
<tr>
<td></td>
<td>TG 87±1.2*</td>
<td>63±1.8*</td>
</tr>
<tr>
<td>LV end-diastolic diameter, mm</td>
<td>NTL 6.9±0.3</td>
<td>9.6±0.3</td>
</tr>
<tr>
<td></td>
<td>TG 6.7±0.3</td>
<td>7.8±0.1*</td>
</tr>
<tr>
<td>LV systolic wall stress, dynes/cm²</td>
<td>NTL 36±6.3</td>
<td>185±21</td>
</tr>
<tr>
<td></td>
<td>TG 16±3.5*</td>
<td>94±9.3*</td>
</tr>
</tbody>
</table>

n=4–5 at baseline and n=8–9 at 4- to 6-wk MI. LV indicates left ventricle; MI, myocardial infarction; NTL, nontransgenic littermate; and TG, transgenic. *P<0.05 vs NTL.

Diminished Myocardial Remodeling in Transgenic Rats Was Abolished by Inhibition of the MEK-VEGF-A Pathway

U0126, a MEK inhibitor, or SU5416, a VEGF receptor inhibitor, was administered throughout the 4 weeks post MI period in both NTLs and transgenic rats. Table 2 compares the effects of the MEK and VEGF receptor inhibitors in NTLs and transgenic rats with those observed in NTLs and transgenic rats given only vehicle. At 4 weeks post MI, the preserved cardiac function and attenuated remodeling observed in vehicle-treated transgenic rats, when compared with their vehicle-treated NTLs, were abolished after treatment with either inhibitor. Thus, with inhibitor treatment, cardiac function, reflected by ejection fraction, and LV wall stress were now similar in the transgenic animals and their NTLs (Table 2). Also, scar size was not significantly different between U0126- or SU5416-treated NTL and transgenic hearts at 4 weeks post MI (Figure 5A). Compared with vehicle-treated transgenic hearts post...
In the present investigation, we demonstrated that overexpression of α₁A-AR in cardiomyocytes protected the heart from the adverse effects of remodeling and heart failure that occurs after permanent CAO (Figure 1). In the rat model of heart failure induced by permanent CAO, others have reported that LV ejection fraction falls to levels of 40% to 45% after heart failure develops. In contrast, LV function was better preserved with less remodeling after CAO, in terms of fibrosis, myocyte hypertrophy, LV weight/tibial length and lung weight/tibial length, and scar size, in the transgenic rats. Although cardiac α₁A-AR stimulation has been proposed previously for the treatment of heart failure on the basis of its increased inotropic properties, and because α₁A-AR blockade exerts an adverse effect in heart failure, the results of the current investigation provide an additional novel mechanism that mediates α₁A-AR protection against the remodeling and the development of heart failure that occurs after permanent CAO, that is, α₁A-AR–stimulated angiogenesis.

Because with complete CAO, it is difficult for modulators of apoptosis or preconditioning to work without blood flow to keep the tissue alive, we concluded that the chronic protection must have ensued because of angiogenesis in the transgenic rats. This provided collateral blood flow to the central ischemic zone.
and adjacent zones after CAO and restored blood flow to the ischemic myocardium, resulting in less cell death with more viable tissue within the ischemic zone and within the central infarct area. We documented the recovery of myocardial blood flow in the central ischemic zone and the zone adjacent to the infarct using the microsphere method, which is one of the only ways to measure regional myocardial blood flow after permanent CAO. It is important to appreciate that additional myocardial blood flow was necessary for improved function of the heart after chronic CAO. Previous studies have shown that overexpression of any α-receptor subtype in the heart can induce angiogenesis, and there are relatively few myocyte-specific angiogenic factors.30 Whereas others have suggested vasoconstriction,25,26 Studies have found different results on which subtypes of α-receptors are predominant23,24 and whether there are differences between regulation of right ventricle versus LV or normal versus heart failure.27 Although all of these points are interesting and important, they really do not bear on the major findings of this present article.

We found that overexpressed α1A-receptors in myocytes induced angiogenesis both in vivo and in vitro and that the additional myocardial blood flow was necessary for improved function of the heart after chronic CAO. Previous studies have shown that α-adrenergic receptor inhibition rather than stimulation in brain and hindlimb endothelial cells induced angiogenesis,29 which is opposite to our results in the heart. To our knowledge, there is no extant evidence showing that overexpression of any α-receptor subtype in heart can induce angiogenesis, unless that model also affected angiogenesis directly29 or through a pathway that induced angiogenesis, for example, elaboration of an associated growth factor.30

We next determined the angiogenic factor that mediates the increase in blood flow and in capillary density in the α1A-AR transgenic rat model with permanent CAO. Microarray analysis revealed enhanced expression of 6 angiogenic growth factor genes in transgenic versus NTL cardiomyocytes. However, only enhanced VEGF-A expression in

| Table 2. Cardiac Function at 4-wk After Myocardial Infarction Treated With Vehicle, U0126, or SU5416 |
|-----------------|-----------------|-----------------|-----------------|
|                 | NTL             | TG              |                 |
| Heart rate, bpm |                 |                 |                 |
| Vehicle         | 397±12          | 333±13*         |                 |
| U0126           | 409±24          | 350±11*         |                 |
| SU5416          | 425±31          | 397±18          |                 |
| Mean arterial pressure, mm Hg |                 |                 |                 |
| Vehicle         | 89±5.5          | 100±3.1         |                 |
| U0126           | 72±3.3          | 80±2.2*         |                 |
| SU5416          | 71±4.2          | 74±2.7          |                 |
| LV systolic pressure, mm Hg |                 |                 |                 |
| Vehicle         | 102±5.6         | 112±3.2         |                 |
| U0126           | 87±3.7          | 94±3.0          |                 |
| SU5416          | 91±5.2          | 88±3.3          |                 |
| LV ejection fraction, % |                 |                 |                 |
| Vehicle         | 39±2.3          | 55±1.1*         |                 |
| U0126           | 37±0.9          | 40±1.8          |                 |
| SU5416          | 33±2.1          | 27±1.0          |                 |
| LV end-diastolic diameter, mm |                 |                 |                 |
| Vehicle         | 10±0.4          | 8.6±0.4*        |                 |
| U0126           | 11±0.2          | 11±0.5          |                 |
| SU5416          | 9.7±0.3         | 9.5±0.6         |                 |
| LV systolic wall stress, dynes/cm² |                 |                 |                 |
| Vehicle         | 193±22          | 137±11*         |                 |
| U0126           | 218±9.3         | 221±12          |                 |
| SU5416          | 229±4.2         | 236±7.6         |                 |

n=4. LV indicates left ventricle; NTL, nontransgenic littermate; and TG, transgenic.

*P<0.05 vs NTL.
transgenic cardiomyocytes could be verified by quantitative polymerase chain reaction (Figure 3C). VEGF-A promotes endothelial proliferation and migration resulting in tubule formation,31–33 and as a proangiogenic factor, it is known to be activated by hypoxia and has an important role in angiogenesis and in reducing hypoxic cellular damage.32,34

Because the promoter used in the transgenic model was cardiac myocyte specific, there was no overexpression of \( \alpha_{1A} \)-AR in the vessels or endothelial cells, and therefore, we postulated a paracrine mechanism, whereby angiogenesis was induced in the current myocyte-specific transgenic model through a cross talk between the cardiomyocytes and endothelial cells, also known as heterocellular signaling, which has been described for the interaction between one cell type and another in the same organ.35–38 Although heterocellular signaling has been described between several cell types, the current investigation focuses on cross talk between cardiomyocytes overexpressing the \( \alpha_{1A} \)-AR and endothelial cells to induce angiogenesis. This paracrine mechanism seems to involve transmission of a signal from one cell type to an adjacent cell via the elaboration by the former of a secreted factor—in this case, VEGF-A secretion one cell type to an adjacent cell via the elaboration by the former of a secreted factor—in this case, VEGF-A secretion.

Recent studies have suggested that MEK/ERK signaling is essential for VEGF-regulated endothelial cell proliferation.18,44,45 The MEK/ERK pathway has been found to be upregulated in hearts with cardiomyocyte-specific \( \alpha_{1A} \)-AR overexpression15,16 and is also activated with in vitro receptor stimulation of cardiomyocytes expressing native levels of the \( \alpha_{1A} \)-AR.41 In this study, inhibition of the MEK pathway with U0126 abolished the attenuated cardiac remodeling observed after MI in transgenic rats by not only increasing LV wall stress and decreasing LV contractility but also by decreasing capillary density. In agreement with our findings, previous studies have suggested that MEK/ERK signaling is essential for VEGF-regulated endothelial cell proliferation.14,44,45 The MEK/ERK pathway has also been shown to mediate preconditioning in the CAO/reperfusion model.41 However, as noted above, the molecular mechanism is not effective in the absence of blood flow.

There is another point that needs to be considered. Recently, Ye et al.46 reported that VEGF plays a role in cardiomyocyte differentiation from induced pluripotent stem cells. Although there is substantial evidence that the increases in blood flow are sufficient to explain the preservation of myocytes, it is possible that an effect on cardiomyocyte differentiation contributes. We measured Kif67-positive myocytes and found that there was a significant increase in labeling in the transgenic rats versus NTLs in the ischemic zone,52 suggesting the induction of new myocytes and preservation of older myocytes. It is difficult to distinguish which is the chicken versus the egg, but it is conceivable that the increased blood flow because of angiogenesis permitted this to occur.

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Figure 5. Responses of nontransgenic littermates (NTLs) and transgenic (TG) rats to 4 weeks of coronary artery occlusion (CAO) and treatment with vehicle (10% DMSO), the MEK (mitogen ERK kinase) inhibitor, U0126 (400 \( \mu \)g/kg per day), or the vascular endothelial growth factor (VEGF) receptor inhibitor, SU5416 (25 mg/kg per day). A, Scar size was significantly smaller in the vehicle-treated TG vs NTL hearts; a difference that was no longer observed after inhibitor treatment. B, Coronary blood flow in the ischemic zone after 4 weeks of CAO was significantly higher in TG vs NTL hearts, and the increase in the TG hearts was abolished with either U0126 or SU5416 treatment for 4 weeks. C, Capillary density, normalized to NTLs, was increased in the adjacent zone of TG after 4 weeks of CAO, and this increase was abolished with either U0126 or SU5416 treatment for 4 weeks. D, In NTL cardiomyocytes, A61603 increased VEGF-A mRNA and protein levels, and these increases were prevented when the \( \alpha_{1A} \)-adrenergic receptor agonist was combined with U0126. Results are expressed as means±SEM; n=4 to 6; *P<0.05 vs NTL+vehicle; †P<0.05 vs A61603- treated NTL cardiomyocytes.
In summary, the results of this investigation suggest a novel therapeutic target for improved clinical outcomes by limiting post-MI loss of myocardium and, hence, remodeling and heart failure as a result of α1A-AR-MEK/VEGF-A-mediated angiogenesis, an effect that also protects the function of marginally ischemic myocardium adjacent to the infarct. Protection against remodeling (less fibrosis and myocyte hypertrophy) also protects the nonischemic zone after chronic CAO, a mechanism, unlike the adjacent zone, not dependent on increased blood flow. Another novel finding was that overexpression of cardiomyocytes-specific α1A-AR was able to induce angiogenesis, a mechanism that might be available for other myocyte proteins and might promote a new line of research in the angiogenesis field. Although cardiac α1A-AR stimulation has been proposed as a potential treatment for heart failure based on its ability to augment inotropy, this is the first time that the α1A-AR has been linked to the enhanced expression of a potent proangiogenic factor, VEGF-A, a factor that also induces angiogenesis in response to ischemia. This mechanism improving perfusion to ischemic myocardium changes the paradigm explaining α1A-AR’s salutary action in heart failure, from simply increasing inotropy, which also increases myocardial oxygen consumption, to increasing inotropy while improving perfusion, which protects myocardial oxygen consumption.

Acknowledgments
We thank Grace Lee, Dr Yimin Tian, and Dr Chumbo Wang for their technical support related to the histology and quantitative polymerase chain reaction analyses.

Sources of Funding
This study was supported by National Institute of Health grants (5R01HL119464, 3P01HL069020, 6T32HL069752, 6R01HL093481, 5R01HL106511, and 1R01HL124282), University of New South Wales Goldstar Award (RG14231), R.T. Hall Estate, National Health and Medical Research Council Program Grant (1074386), and Australian Research Council Stem Cells Australia SR110001002.

Disclosures
None.

References


**Significance**

This investigation demonstrates, at several levels, a novel mechanism for α1A-adrenergic receptor–mediated protection from remodeling and heart failure after permanent coronary artery occlusion, that is, angiogenesis. First, stimulation of the α1A-adrenergic receptors in cardiomyocytes has not been demonstrated previously to induce angiogenesis. Moreover, the fact that this occurs through heterocellular signaling from myocytes to coronary vessels will open new areas of investigation into the mechanisms of myovascular coupling and potentially will lead to new therapeutic modalities. This is not only significant at the basic science level but also from a clinical perspective because cardiomyocyte α1A-adrenergic receptor stimulation has been proposed for the treatment of heart failure based on its action to increase myocardial contractility. Given the current findings, there is perhaps a more important reason to support cardiac α1A-adrenergic receptor stimulation for treatment of heart failure, particularly when it is of myocardial ischemic cause, that is, improvement of myocardial blood flow through angiogenesis.
Overexpression of Cardiomyocyte α1A-Adrenergic Receptors Attenuates Postinfarct Remodeling by Inducing Angiogenesis Through Heterocellular Signaling

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Arterioscler Thromb Vasc Biol. 2015;35:2451-2459; originally published online September 3, 2015;
doi: 10.1161/ATVBAHA.115.305919

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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METHODS AND MATERIALS

**Animals** Seventy one TG Sprague-Dawley male rats with 40-fold cardiomyocyte-specific overexpression of the α1A-AR\(^1\), thirty TG FVB mice with 66-fold cardiomyocyte specific α1A-AR overexpression\(^2, 3\) and their respective NTLs were housed on a 12-hour light-dark cycle with standard chow and water. Development of the transgenic rats and mice was performed with approval of the St. Vincent’s Hospital Animal Ethics Committee, whereas the breeding and the experiments performed at both Rutgers-New Jersey Medical School and Victor Chang Cardiac Research Institute were approved by their IACUC and all animals were maintained in accordance with the guidelines for the Guide for the Care and Use of Laboratory Animals (National Research Council, 8th Edition 2011). The *in vivo* and histology studies were performed in TG rats, whereas the *in vitro* cell culture experiments were studied in TG mice.

**Myocardial infarction** Coronary artery ligation was performed as previously described\(^1\). Briefly, male α1A-TG rats and their NTLs, weighing between 300-450g, were treated with buprenorphine (0.05mg/kg s.c.) and cefazolin (50mg/kg i.m.), and were then anesthetized with sodium pentobarbital (60mg/kg i.p.). Rectal temperature was monitored and body temperature maintained at 37°C with an automatic heating lamp. After intubation of the trachea, rats were ventilated with a tidal volume of 0.5ml/kg at a rate of 90 breaths per minute, a left thoracotomy was performed at the fourth intercostal space and myocardial ischemia was induced by permanent ligation of the left anterior descending coronary artery (LAD). After completion of the ligation, the chest was closed and buprenorphine (0.05mg/kg s.c.) was given as post-operative medication. For the MEK inhibitor, U0126 (catalog# U-6770, LC Labs, MA), which was previously used at a different dosage of 1-400µg/kg/day via osmotic pumps\(^4, 6\), To ensure the full blockade of
MEK, a dose of 400µg/kg/day was chosen to be delivered via an osmotic pump subcutaneously from the day of LAD occlusion continuously for 4 wk. SU5416 (catalog# S8442, SigmaAldrich, MO), which has been tested at 25mg/kg per day before to fully block VEGF \(^6\text{-}^9\), was administered in both NTL and TG rats with same dose, i.p. for 4 wk.

**Echocardiography** At 4-6 wk after myocardial infarction, rats were anesthetized by injection of sodium pentobarbital, (60mg/kg i.p.). Transthoracic echocardiography was performed as previously described\(^{10}\). The animal body temperature was carefully monitored and maintained as close to 37°C as possible during the entire procedure. Using a VisualSonics Vevo 770 ultrasound system, LV internal dimensions were measured in systole and diastole using leading-edge methods and guidelines of the American Society of Echocardiography\(^{11}\). LV systolic function was estimated using the Vevo 770 software for calculation.

**Myocardial blood flow assessment** At 1 day, 1 wk, 2 wk and 4 wk after MI, myocardial blood flow was assessed using the microsphere technique. Briefly, TG rats and their NTLs were catheterized using sterile surgical technique as described previously\(^{12}\). An 8-inch in length and 0.023 in diameter (internal) fluid filled catheter (Braintree Scientific RPT 037) was inserted via the right carotid artery into the left ventricle, and a second catheter of the same size was inserted into the left femoral artery. After recovery from anesthesia, the rats received approximately 1,000,000 stable-isotope labelled color microspheres (Gold; BioPAL, Worcester, MA; catalog# C-10H10,15µm diameter) that were injected into the LV via an intraventricular catheter. A reference blood sample was collected simultaneously at a rate of 0.5mg/ml for 2 minutes. On completion of the protocol, animals were euthanized with a saturated KCl solution and the hearts collected. The LV was isolated and sliced in the short axis into 2 mm thick rings. The center of the ischemic zone was dissected and demarcated as the
infarct zone, whereas the tissue within 1.5mm distance from the edge of the infarct zone was collected and considered as the adjacent zone, and tissue within non-ischemic zone and directly opposite to the ischemic zone was considered as the remote zone. All individual heart samples, reference blood samples and both kidney samples were sent to the Biophysics Assay Laboratory (BioPAL, Worcester, MA) for microsphere analysis. The analysis with similar blood flow in both kidneys demonstrated adequate mixing of the microspheres. In addition, the statistical differences also confirm the accuracy.

**Tissue preparation for capillary density** To measure vessel density, heparinised animals were euthanized with an overdose of pentobarbital (120mg/kg i.p.) and then the ascending aorta was dissected and cannulated for perfusion of a vasodilation solution and a fixation solution. Briefly, 10ml of 1 x Dulbecco’s Phosphate-Buffered Saline (DPBS) with heparin (100U/ml), 10ml of vasodilation solution [1 x DPBS with adenosine (100mM) and sodium nitroprusside (10mM) and bovine serum albumin (0.05% (wt/vol)], and 10ml of fixation solution [(4% (wt/vol) paraformaldehyde solution)] were infused consecutively at a constant pressure of 80mmHg.

**Histology staining** Hearts were collected from TG and NTL rats and fixed in 10% formalin. After embedding in paraffin, LV rings were sectioned at 5µm thickness and processed for the following:

- Interstitial fibrosis - Tissue sections were stained with Picro-Sirius Red (PSR) to identify interstitial fibrosis deposition. The percentage of total fibrosis was quantified using ImagePro-Plus software, as previously described.

- Scar size/viable tissue - Using PSR staining, scar size was assessed by quantification of the fibrotic area and calculated as a percentage of the whole myocardial area. Viable tissue was identified as the non-fibrotic area, which was quantified and expressed as the percentage of the ischemic zone.
Ki67 positive myocytes - Using Ki67 (Life Technologies, catalog#18-0192Z) and WGA double immunostaining, Ki67 positive myocytes were quantified and expressed as the percentage of total cardiomyocytes within the ischemic zone\textsuperscript{31}.

Cell size - Cardiomyocyte size was determined using rhodamine-conjugated wheat germ agglutinin (WGA), which stains all cell membranes, allowed for discrimination of myocytes and non-myocytes based on their size and phenotype. The circumference of each cardiomyocyte was traced and quantified using ImagePro-Plus software.

Capillary density - Using isolectin staining (isolectin IB4-Alexa448, catalog# I21414 Invitrogen), capillaries were identified as single endothelial cell layer with a diameter less than 25\textmu m. Capillary density was quantified at 40x magnification as absolute number per unit myocardial area.

**Angiogenic gene screening** Microarray analysis was performed on cardiomyocytes isolated from TG and NTL rats to determine changes in gene expression of angiogenic markers\textsuperscript{32,33}. Upregulation of angiogenic genes was further confirmed by quantifying their mRNA levels using quantitative real-time PCR (qPCR).

**Cardiomyocyte isolation** Cardiomyocytes from both TG rats and 8-12 week old male mice and their corresponding NTLs were isolated using a modified Langendorff perfusion system. Briefly, the heart was first perfused with perfusion buffer (NaCl 120.4mmol/L; KCl 14.7mmol/L; KH2PO4 0.6mmol/L; Na2HPO4 0.6mmol/L; MgSO4\textcdot 7H2O 1.2mmol/L; NaHCO3 4.6mmol/L; Na-HEPES 10mmol/L; taurine 30mmol/L; BDM10mmol/L; glucose 5.5mmol/L) for 4min at 37°C at 3-4ml/min and then with enzyme buffer (perfusion buffer 50ml, containing collagenase II (catalog# LS004176, Worthington, NJ) 2.0mg/ml) for 12-15min. At the end of the digestion procedure, live myocytes were collected.
**RNA extraction** Cardiomyocytes were homogenized in Trizol (1ml/5 x 10⁶ cells; Roche, Switzerland) and chloroform (0.2ml) was added. After vortexing and incubating at room temperature for 10min, samples were centrifuged at 12,000rpm at 4°C for 15min. Isopropanol (1.5ml) was added and the resulting mixture incubated at room temperature for 10min before centrifugation at 12,000rpm for 15min. The supernatant was removed and the pellet was washed with 1ml 75% ethanol. An additional centrifugation was performed at 10,000rpm at room temperature for 10 min and the resulting RNA pellet was dried and resuspended in nuclease free water. RNA quality was tested using NanoDrop (Thermo Scientific, DE) for the following criteria: concentration >1.0µg/µl; A₂₆₀/₂₈₀ >2.0. RNA integrity was confirmed by electrophoresis, with a 28S:18S ratio of 2:1 being considered satisfactory for microarray gene analysis.

**Tube formation assay** Cardiomyocytes isolated from 8-12 week old male NTL or TG mice were cultured in the presence or absence of the α₁A-AR agonist, A61603 (25 nM). After 48 hours, the conditioned medium from cardiomyocytes was collected and centrifuged at 1000rpm for 10 minutes followed by filter sterilization using 100µm syringe filters. The conditioned medium was then aliquoted and stored at -80°C until further use. For the tube formation assay, human umbilical vein endothelial cells (HUVECs) (catalog# CRL-1730™, ATCC, Manassas, VA) were used. Matrigel (reduced growth factor, BD Bioscience) was placed in 96-well tissue culture plates and allowed to polymerize at 37°C for 30 min. HUVECs at a density of 5000 cells/well were then plated on Matrigel in the presence of either unconditioned medium or the cardiomyocyte conditioned medium. For VEGF-A inhibition, the cardiomyocyte conditioned medium was pre-treated for 1 hour with either a control IgG or anti-VEGF-A neutralizing antibody (catalog# sc-152, Santa Cruz) before being added to HUVECs. After 6 hours, the
tubules were imaged at 10x magnification followed by quantification. Data were expressed as the number of tubules in the conditioned medium normalized to that of unconditioned medium.

**Quantification of VEGF-A expression levels** Cardiomyocytes isolated from 8-12 wk old male TG mice of their NTLs were treated with $\alpha_{1A}$-AR agonist, A61603 (25nM), and/or the $\alpha_1$-AR inhibitor, prazosin (1µM), and/or the MEK inhibitor, U0126 (0.5µM). After 48 hours, RNA was extracted and VEGF-A levels were quantified relative to an internal control, HPRT (Hypoxanthine-guanine phosphoribosyltransferase) by qPCR. Cardiomyocytes were harvested in lysis buffer treated with DNase I and resulting RNA was reverse transcribed according to manufacturer’s instructions using TaqMan gene expression Cells-to-Ct kit. (catalog# 4399002, Life Technologies). The mRNA was quantified using real-time qPCR (40 cycles at 15s step at 95ºC and a 1 min step at 60ºC) on a 7500 ABI-Prizm sequence detector (Applied Biosystems) using standard TaqMan probes (Applied Biosystems) for VEGF-A and HPRT. The conditioned medium from TG or NTL cardiomyocytes was collected and VEGF-A protein levels determined using ELISA (mouse VEGF Quantikine ELISA kit, catalog#DVE00, R&D systems).

**Statistical Analysis:** Data are expressed as mean ± SE. Statistical significance was determined using Student’s $t$-test or ANOVA plus Bonferroni post hoc test evaluations, where appropriate. Comparison of hemodynamics of post-MI rats treated with MEK inhibitor, U0126, or the VEGF (Flk-1/KDR) receptor antagonist, SU5416, were determined using two-way ANOVA. $p<0.05$ was taken as a minimal level of significance.


12. Xin Zhao, David Ho, Shumin Gao, Chull Hong, Dorothy E. Vatner, Vatner SF. Arterial pressure monitoring in mice. *Current protocols in mouse biology*. 2011;1:105-122


