Gene and MicroRNA Transcriptional Signatures of Angiotensin II in Endothelial Cells

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Abstract: Growth of atherosclerotic plaque requires neovascularization (angiogenesis). To elucidate the involvement of angiotensin II (Ang II) in angiogenesis, we performed gene microarray and microRNA (miRNA) polymerase chain reaction array analyses on human coronary artery endothelial cells exposed to moderate concentration of Ang II for 2 and 12 hours. At 12, but not 2, hours, cultures treated with Ang II exhibited shifts in transcriptional activity involving 267 genes (>1.5-fold difference; \( P < 0.05 \)). Resulting transcriptome was most significantly enriched for genes associated with blood vessel development, angiogenesis, and regulation of proliferation. Majority of upregulated genes implicated in angiogenesis shared a commonality of being either regulators (HES1, IL-18, and CXCR4) or targets (ADM, ANPEP, HES1, KIT, NOTCH4, PGF, and SOX18) of STAT3. In line with these findings, STAT3 inhibition attenuated Ang II-dependent stimulation of tube formation in Matrigel assay. Expression analysis of miRNAs transcripts revealed that the pattern of differential expression for miRNAs was largely consistent with proangiogenic response with a prominent theme of upregulation of miRs targeting PTEN (miR-19b-3p, miR-21-5p, 23b-3p, and 24-3p), many of which are directly or indirectly STAT3 dependent. We conclude that STAT3 signaling may be an intrinsic part of Ang II–mediated proangiogenic response in human endothelial cells.

Key Words: endothelium, angiotensin II, angiogenesis, microRNA, gene expression

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INTRODUCTION

Angiotensin II (Ang II) is the major regulatory derivative of angiotensin and its primary function concerns adaptive changes in blood pressure and vascular homeostasis via regulation of electrolyte/water balance and vascular tone. In some pathophysiological contexts, excessive secretion of Ang II is detrimental and promotes myocardial hypertrophy, fibrosis, and apoptosis. Formation of Ang II is also one of the major factors driving atherosclerosis via its stimulatory effects on proliferation and vascularization.1,2

Ang II has been shown to promote proliferation and angiogenesis via its type 1 receptor (AT1R). In breast cancer cells, introduction of AT1R inhibitor canceled Ang II–mediated stimulation of proliferation and xenografts in mice receiving candesartan exhibited reduced growth and vascularization.3 In hepatocellular carcinoma cells, addition of losartan suppressed Ang II–stimulated production of angiogenic factors including vascular endothelial growth factor (VEGF), angiopeitoin-2, and Tie-2.4 Similarly, the allografts of bone marrow mesenchymal stem cells responded to Ang II by increasing VEGF secretion and enhanced vascularization in ischemic hind limbs of mice, and these effects were attenuated by valsartan treatment.5

In the past decade, the transcriptional signature of Ang II has been studied in different organs and cell types, including hearts of acutely or chronically treated mice,6 smooth muscle cells,7 adrenocortical cells,8 and macrophages.9 The reported transcriptional patterns were very diverse, possibly highlighting extensive differences in cell type–specific responses. A recent study from our laboratory10 showed that small physiologic concentrations of Ang II (10–9 to 10–8 M) induced tube formation from human coronary artery endothelial cells (HCAECs).10 In the present study, we attempted to characterize Ang II–mediated transcriptional responses in HCAECs.

MATERIALS AND METHODS

Cells and Reagents

HCAECs and corresponding growth medium with VEGF endothelial cell growth kit were purchased from ATCC (Manassas, VA). Ang II was purchased from Sigma–Aldrich (Stoughton, MA) and dissolved in phosphate-buffered saline to make 1 mM stock solution. Inhibitor of STAT3 Stat specific was purchased from Santa Cruz (Santa Cruz, CA). Anto-mmu-miR21 miScript inhibitor and inhibitor negative control were purchased from Qiagen (Valencia, CA).
Details on gene microRNA (miRNA) expression analyses are presented in Supplemental Digital Content 1 (see Supplemental Material, http://links.lww.com/JCVP/A153).

Quantitative Polymerase Chain Reaction

Quantitative polymerase chain reaction (qPCR) was performed with predesigned primers selected from Primer-Bank and ordered from Integrated DNA Technologies (Coralville, IA). Real-time qPCR was performed using the Applied Biosystems 7900 real-time PCR system (Applied Biosystems, Foster City, CA). The comparative threshold cycles values were normalized for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) reference genes.

MicroRNA qPCR

Primers for real-time PCR reaction and qPCR analysis were purchased from Applied Biosystems, and the analysis was performed in triplicates for each data point according to the manufacturer’s instructions.

Transfection

Near confluent HCAECs were transfected with hsa-miR-21 inhibitor or negative control using HiPerFect transfection reagent (Qiagen) according to the manufacturer’s instructions. The cells were utilized for tube formation assay 72 hours after transfection.

Tube formation Assay

Fifty microliters of Matrigel basement membrane matrix (BD Biosciences, San Jose, CA) was pipetted into each well of
In our microarray study, STAT3 expression was approximately 50% higher in Ang II–treated group (P = 0.05). Taken together, these findings suggested that STAT3 might play a key role in Ang II–mediated proangiogenic signaling.

For analysis of the involvement of STAT3-mediated signaling in angiogenesis, we utilized a nonpeptidic small molecule STAT3 inhibitor Stattic. After determination of nontoxic concentration of inhibitor in endothelial cells using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay upregulation. To validate our microarray findings, we conducted qPCR analysis in a separate experiment (Fig. 2). With the exception of PGF, all tested genes exhibited same directional changes.

### Ang II–mediated miRNA Transcriptional Profile

Ang II–dependent miRNA transcriptome included 41 upregulated and 14 downregulated miRNAs (more than 4-fold; P < 0.05) (Fig. 3; see Table 2, Supplemental Digital Content 1, http://links.lww.com/JCVP/A153). Overall, differentially expressed miRNAs displayed a pattern consistent with the stimulation of angiogenesis (Table 1). Approximately 40% of upregulated miRNAs have been shown to act as positive regulators of angiogenesis and proliferation, including Let-7 family, miR-17-92 cluster, miR-10a-5p, miR-103a-3p, and miR-21-5p. On the other hand, several miRNAs with antiangiogenic action, such as miR-206, miR-320b, miR-34c-3p, miR-429, miR-509-3p, and miR-7-5p, were significantly downregulated.
(Fig. 4A), we measured tube formation on Matrigel in response to 10^{-8} M Ang II alone or in the presence of 100 nM Stattic. In these experiments, Ang II stimulated tube formation by approximately 20% (P < 0.05), and introduction of Stattic blocked this effect (Figs. 4A–C).

For further analysis of STAT3 involvement, we studied miR-21 that contains 2 STAT3 binding sites in its promoter. In separate experiments, exposure to Ang II resulted in 1.9-fold stimulation of miR-21 measured by real-time PCR (P < 0.05; Figs. 4A, B). As shown in Figures 4B and C compared with the cultures transfected with scrambled sequence, cells transfected with miR-21 inhibitor lost responsiveness to Ang II and did not exhibit any increase in tube formation.

FIGURE 3. Results of miRNA PCR array. Scatter plot for miRNAs’ expression and heat maps for upregulated and downregulated miRNAs in cultures exposed to 10 nmol/L Ang II for 12 hours. More comprehensive data on differentially expressed miRNA is provided in Supplemental Digital Content 1 (see Table 2, http://links.lww.com/JCVP/A153).

DISCUSSION

In the present study, we have shown that HCAECs react to Ang II in moderate concentrations by upregulating several genes implicated in blood vessel development and angiogenesis. Moreover, we have shown that proangiogenic effects of Ang II are, in part, linked to STAT3 and miR-21.

Our microarray data offer interesting clues regarding possible mechanisms implicating STAT3 as a key player in Ang II–mediated proangiogenic signaling. In a number of reports, Ang II has been shown to activate STAT3 signaling cascade in various cell types and utilizing both JAK2-dependent and JAK2-independent pathways. It is also interesting that STAT3 consensus sequences are found in
miR-21 promoter as well. Mir-21 seems to be central to angiogenic action of Ang II as its blockade using miR-21 inhibitor made endothelial cells unable to form tubular networks on Matrigel in response to Ang II exposure.

MiR-21 is well known for its oncogenic properties based, in part, on the stimulation of proliferation and angiogenesis. There is also a substantial body of evidence that miR-21 is intimately involved in vascular cell physiology. MiR-21 is induced by shear stress and after balloon injury where it stimulates proliferation and inhibits apoptosis. MiR-21 knockdown results in decrease in neointima formation after angioplasty in vivo and inhibition of proliferation in combination with increased apoptosis in vascular smooth muscle cell in vitro. MiR-21 has been also found to be upregulated during ischemic preconditioning and to be partially responsible for protective effect of ischemic preconditioning against cardiac ischemia/reperfusion injury.

The actions of miR21 are attributed to the central role of its primary target PTEN (phosphatase and tensin homolog deleted from chromosome 10) in Akt signaling. PTEN antagonizes PI3K by cleaving its major product, lipid PtdIns(3,4,5) 3, and thus preventing activation of downstream Akt signaling cascade. In recent studies, pharmacological inhibition of PI3K significantly reduced the formation of atherosclerotic lesions.

Judging from the pattern emerging from the PCR array analysis, Ang II triggers multilayered stimulation of angiogenesis and proliferation-related miRNAs while suppressing a number of miRNAs responsible for cell arrest and apoptosis. In our data set, expression of many members of Let-7 family (let-7a, c, d, e, f, and g) was stimulated by Ang II (see Table 2, Supplemental Digital Content 1, http://links.lww.com/JCVP/A153). Let-7g was shown to be necessary—via its effect on TIMP1/MMP

### Table 1. Differentially Expressed miRNAs Involved in Regulation of Angiogenesis

| miRNA         | Fold Change | P  | Target Genes     | References |
|---------------|-------------|----|------------------|------------|
| Stimulators   |             |    |                  |            |
| hsa-miR-103a-3p | 11.1        | 0.006 | DAPK, KLF4 | Chen et al  |
| hsa-miR-10a-5p | 38.4        | 0.003 | MAPK3K7, TAK1, β-TRC | Shen et al  |
| hsa-miR-10b-5p | 37.8        | 0.002 | HoxD10 | Shen et al  |
| hsa-miR-130a-3p | 9.1         | 0.04  | GAX, HOXA5 | Chen and Gorski |
| hsa-miR-155-5p | 7.5         | 0.02  | AT1R | Zheng et al |
| hsa-miR-19a-3p | 38.1        | 0.04  | TNF-α | Liu et al  |
| hsa-miR-19b-3p | 25.6        | 0.02  | PTEN | Olive et al |
| hsa-miR-21-5p | 6.7         | 0.01  | PTEN, Pdc4d, Trp1, TIMP3, HIF-1, VEGF | Liu et al |
| hsa-miR-218-5p | 11.2        | 0.01  | Robo1,2, GLCE | Small et al |
| hsa-miR-23b-3p | 12.1        | 0.02  | PTEN | Zaman et al |
| hsa-miR-24-3p | 6.6         | 0.03  | MXI1, PTEN | Xu et al  |
| hsa-miR-378a-5p | 4.2        | 0.003 | HMOX1, p53 | Skrzypek et al |
| Let-7c        | 7.99        | 0.003 | TIMP1 | Otsuka et al |
| Inhibitors    |             |    |                  |            |
| hsa-miR-100-5p | 15.2        | 0.001 | mTOR | Grundmann et al |
| hsa-miR-101-3p | 8.9         | 0.03  | EZH2 | Smits et al  |
| hsa-miR-125a/b-5p | 5.6/5.3 | 0.004 | Blocks Akt signaling, MMP11, VEGF | Wang et al, Scott et al |
| hsa-miR-128   | 4.2         | 0.04  | P70S6K1→HIF1, VEGF | Shih et al |
| hsa-miR-15a-5p | 10.9        | 0.006 | FGFr2, VEGF, BCL2 | Yin et al |
| hsa-miR-155-5p | 7.5         | 0.02  | AT1R, p61 | Zheng et al, Zhang et al |
| hsa-miR-16-5p | 55.3        | 0.03  | BCL2, VEGF | Tang et al |
| hsa-miR-195-5p | 60          | 0.03  | Bcl2 | Chen et al  |
| hsa-miR-206-5p | 8.4         | 0.04  | VEGF | Cuscio et al |
| hsa-miR-206   | -10.1       | 0.03  | VEGFa | Stahilut et al |
| hsa-miR-320b  | -4.2        | 0.04  | NRP-1 | Zhang et al |
| hsa-miR-34c-3p | -12         | 0.02  | Effector of p53, ATF1 | Liang et al |
| hsa-miR-378a-3p | -5.4      | 0.02  | MAPK1, IGF1R, GRB2, KSR1 | Ganesan et al |
| hsa-miR-429   | -7.3        | 0.02  | c-Myc | Wu et al  |
| hsa-miR-7-5p  | 8           | 0.03  | IRS-2→Akt | Giles et al |
| hsa-miR-720   | -9.1        | 0.01  | P63-Dicer | Botchkareva |

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FIGURE 4. A, MTT assay for Stattic, incubation 16 hours (upper graph and representative images), and tube formation assay (lower graph and representative images) on effects of 10^{-8} M Ang II in the presence or absence of 100 nM Stattic. B, Quantitative PCR validation of Ang II–dependent stimulation of miR-21 (upper graph and representative images) and effects of miR-21 inhibition on Ang II–mediated stimulation of tube formation on Matrigel (lower graph and representative images). Data from the experiment in quadruplicates. *P < 0.05.

activities—for the tube-forming ability of endothelial cells.\textsuperscript{22} Members of 17–92 cluster are regulated by MYC and have been directly implicated in various aspects of tumor angiogenesis due (presumably) to inhibition of their target genes thrombospondin-1 and connective tissue growth factor.\textsuperscript{53} However, recent studies showed that overexpression of miR-19a and several other cluster members had negative effect in 3-dimensional model of angiogenesis.\textsuperscript{54} Several miRNAs, including miR-19, miR-21, miR-23b-3p, and miR-24-3p, converge on the stimulation of prosurvival Akt pathway through inhibition of PTEN.\textsuperscript{17,19,20,48}

In agreement with experimentally validated proangiogenic action of Ang II, many antiangiogenic miRNAs were downregulated. On the other hand, a number of inhibiting miRNAs were overexpressed, probably reflecting the initiation of self-balancing program involving critical aspects of Ang II proangiogenic signaling. Several upregulated and downregulated miRNAs (miR-125, miR-128, miR-15a-5p, miR-16-5p and miR-20b-5p) target VEGF. It has also been shown that increase in VEGF production triggers the synthesis of miR-155\textsuperscript{55} (found to be upregulated in our study) that targets AT1R.\textsuperscript{15}

It should also be noted that many miRNAs that were found to be differentially expressed in Ang II–treated cultures are directly or indirectly implicated in STAT3 signaling and that observed shifts in their expression were consistent with STAT3 stimulation (see Figure 2, Supplemental Digital Content 2). For example, a number of miRNAs, including miR-21 and members of 17–92 cluster, are directly stimulated by STAT3.\textsuperscript{92} In line with the expectations, miR-21, miR-155, miR-23b-3p, and miR-24-3p, converge on the stimulation of prosurvival Akt pathway through inhibition of PTEN.\textsuperscript{17,19,20,48}

In summary, our studies show that one of the dominant themes of Ang II transcriptional signature in endothelial cells is stimulation of angiogenesis. Strong suppression of Ang II–mediated tube formation in response to inhibition of STAT3 and miR-21 suggests that the activation of STAT3–miR-21 signaling axis is one of the prerequisites of proangiogenic effect of Ang II.

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