Expression of CYR61, an Angiogenic Immediate Early Gene, in Arteriosclerosis and Its Regulation by Angiotensin II

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Background—The renin-angiotensin system is thought to be involved in development and progression of arteriosclerosis, thereby contributing to adverse cardiovascular events. To elucidate the role of angiotensin II (Ang II) at a cellular level, we analyzed the Ang II–induced gene expression profile.

Methods and Results—Genes induced on Ang II stimulation (10^{-7} mol/L, 45 minutes) in rat smooth muscle cells were analyzed by polymerase chain reaction selected subtraction. In addition to known genes, such as interleukin 6, leukemia inhibitory factor, and c-fos, we identified CYR61, an angiogenic immediate early gene. Northern blot analysis revealed a rapid 2.5-fold increase of CYR61 transcript levels by Ang II, peaking at 30 minutes, which was blunted by Ang II type 1 receptor blockade. Exposure of rat aortic rings to Ang II (30 minutes) revealed a 2-fold, and intraperitoneal injection of Ang II (30 minutes) in mice a 3-fold, increase of aortic CYR61 transcripts. In arteriosclerotic aortas of apolipoprotein E–deficient mice, CYR61 transcripts confirmed by in situ hybridization and proteins shown by immunohistochemistry were elevated, whereas they were hardly detectable in wild types. In human carotid atherectomies and arteriosclerotic coronary arteries, immunohistochemical analysis revealed expression of CYR61 within connective tissue in neointima, adventitia, and surrounding small capillaries and blood vessels, colocalized with ACE and Ang II. Normal human arteries showed no significant staining for CYR61.

Conclusions—CYR61, an angiogenic factor, is induced by Ang II in vascular cells and tissue. The expression of CYR61, colocalized with Ang II and ACE, in small vessels of human arteriosclerotic lesions is consistent with the notion that the activated renin-angiotensin system may contribute to plaque neovascularization by enhancing regulators of microvessel formation and cell proliferation. (Circulation. 2002;106:254-260.)

Key Words: angiotensin ■ arteriosclerosis ■ arteries ■ genes ■ stenosis

Clinical studies showed that an activated renin-angiotensin system (RAS) is associated with a higher incidence of myocardial infarction and stroke.1 Conversely, chronic inhibition of angiotensin II (Ang II) formation by ACE inhibitors reduces the risk of cardiovascular events in patients with arteriosclerosis and after myocardial infarction, as shown in large-scale trials such as the Heart Outcomes Prevention Evaluation study.2

We and others have recently demonstrated that the RAS is present in human arteriosclerotic lesions3,4 and is associated with inflammation processes, thereby potentially influencing plaque rupture.5 Ang II activates a multiplicity of signaling pathways, resulting in cell proliferation, matrix degradation, inflammation, and apoptosis.5,6 Its role for the pathogenesis of arteriosclerosis, however, is far from elucidated.

In this study, we analyzed the Ang II–induced gene expression pattern in rat aortic smooth muscle cells (RASMCs) to elucidate the role of Ang II in vascular remodeling and arteriosclerosis at the molecular level. Short-term stimulation with Ang II allows the detection of specific effects but limits the number of genes to be induced. In the present analysis, we found 16 genes, of which only CYR61 was not known to be Ang II inducible. Therefore, we investigated CYR61 in more detail.

CYR61 is a heparin-binding, secreted cysteine-rich protein that integrates into the extracellular matrix and binds directly to integrins (for review, see Perbal)7 and belongs to the novel CCN gene family (connective tissue growth factor, CTGF; cysteine-rich angiogenic protein 61, CYR61; nephroblastoma overexpressed).8 CYR61 was cloned as an immediate early gene expressed in fibroblasts on growth factor stimulation,9–11 and its expression has been associated with vascular restenosis,12 angiogenesis,13,14 and tumor growth,15,15–18

We analyzed the impact of Ang II on the expression of CYR61 in vitro and in vivo, as well as the expression of CYR61, Ang II, and ACE in arteriosclerotic lesions in...
apolipoprotein E (apoE)--deficient mice and human arteriosclerotic arteries. We found a striking colocalization for Ang II and CYR61, suggesting a possible regulation of CYR61 expression by Ang II in vivo.

Methods

Reagents
Losartan was from Dupont/Merck; Ang II and all other chemicals were purchased from Sigma.

Cell Culture
RASMCs were maintained in DMEM supplemented with 10% (vol/vol) FBS, 10 μg/mL streptomycin, and 100 U/mL penicillin. Cells were grown to 75% to 85% confluence and growth-arrested in serum-free DMEM for 24 hours before use, as published previously.19

PCR Select Subtraction
The Clontech PCR-Select Subtraction Kit (Clontech) was used according to the manufacturer’s protocol. A total of 2 μg Poly(A)^+ RNA isolated from RASMCs stimulated with Ang II (10^{-7} mol/L, 45 minutes) or unstimulated were used. Subtracted PCR products were subcloned into the pGEM-T vector (Promega). Individual clones were amplified by polymerase chain reaction (PCR) with nested primer 1 and nested primer 2R (Clontech) and tested for differential expression. Two identical membranes (Hybond NX; Amersham) were generated. Twin spots of PCR product of each candidate clone (2 μL) were dotted. Membranes were hybridized in ExpressHyb (Clontech) to either 32P-radiolabeled cDNAs from control or from Ang II-stimulated RASMCs. Duplicate blots were visualized by autoradiography and densitometrically analyzed. Induction of genes by Ang II was verified by Northern blot analysis in ≥3 independent experiments. cDNA clones from differentially expressed genes were sequenced, and the sequences were subjected to BLAST homology searches.

RNA Isolation and Northern Blot Analysis
Total RNA was isolated from cells or mouse tissue by TriFast (peqlab), separated by gel electrophoresis, and transferred to nylon membranes (Hybond XL; Amersham). Blots were visualized by autoradiography and densitometrically analyzed (GelDoc2000, BioRad).

Tissue Preparation

Human Tissue
Internal mammary artery (IMA) specimens were obtained from patients who had undergone coronary artery bypass graft surgery (n=4). Carotid arteries samples were collected from patients undergoing endarterectomy (n=7). Coronary artery specimens were isolated from explanted hearts after heart transplantation (n=3). Tissue samples were fixed in buffered 4% paraformaldehyde and embedded in paraffin.

Mouse Tissue
ApoE-deficient mice and controls of the same genetic background (C57BL6) 1 month and 6 months of age were killed after rapid excision of aortas. Aortic rings were fixed in buffered paraformaldehyde (4%) and embedded in paraffin. For RNA analysis in aortas, C57BL6 male mice were killed 30, 60, or 180 minutes after intraperitoneal injection of saline or Ang II (0.64 μg/g).

Rat Tissue
Aortas from male Sprague-Dawley rats were cut into rings and incubated in DMEM with or without Ang II (10^{-7} mol/L) for 30 minutes.

Reverse Transcription–PCR
For quantification of the CYR61 expression, reverse transcription (RT)-PCR was performed with G3PDH primers and mouse CYR61 primers (5'-CTGCGCGCTTGTGGTGT-3' and 5'-TGACCAAGGCACCATTCCATC-3'). RNA samples were tested for equal G3PDH content before CYR61 RT-PCR, which was performed under linear amplification conditions.

In Situ Hybridization
A PCR-generated CYR61 cDNA fragment 1292 bp long (primers as above) cloned into pGEM-T vector served as riboprobe. The DIG RNA in vitro labeling kit (Boehringer Mannheim) was used to label the antisense (Sp6) and the sense (T7) probes. Hybridization was performed on serial sections on polyaldehyde slides, which were dewaxed and rehydrated in RNASE-free water, with the in situ hybridization and detection system kit (Invitrogen) according to the manufacturer’s protocol.

Immunohistochemistry
Serial paraformaldehyde-fixed sections were mounted onto slides, dewaxed, rehydrated, and washed in PBS. Incubation with the primary antibodies was performed in a humidified chamber at room temperature for 3 hours with the following antibodies: anti-human CYR61 and anti-mouse CYR61 (2 μg/mL, Santa Cruz), anti–Ang II (1:100, generously provided by J. Nussberger), anti-ACE (1:80, generously provided by J. Nussberger), anti–smooth muscle α-actin (2 μg/mL, Sigma) and anti–von Willebrand factor (3 μg/mL, Dako). Sections were subsequently washed in PBS and incubated with a biotinylated secondary antibody (Vectastain ABC Elite secondary antibody, 1:50 dilution), followed by incubation with an avidin-biotin detection system and the peroxidase substrate diaminobenzidine according to the manufacturer’s instructions (Vector Laboratories). The sections were finally counterstained with hematoxylin, mounted with Glycergel (Dako), and visualized by light microscopy.

Statistical Analysis
All data are given as mean±SD. Differences were evaluated by t test and ANOVA. Statistical significance was defined as a value of P<0.05.

<table>
<thead>
<tr>
<th>Characteristics</th>
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<td>Secreted proteins</td>
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<td>Rat nuclear receptor 1 (RNR-1)</td>
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Results

PCR Select Subtraction Analysis Identifies CYR61 as a Gene Upregulated by Ang II in RASMCs

We analyzed the gene expression profile of Ang II (10^{-7} mol/L, 45 minutes)–stimulated RASMCs by use of a PCR select subtraction. Of some 400 clones tested, 40 were differentially upregulated by Ang II. BLAST homology searches revealed genes known to be inducible by Ang II (Table 1). In addition, 6 cDNA clones corresponding to the rat CYR61 gene were identified.

Ang II Induces CYR61 Expression in Smooth Muscle Cells Via AT1 Receptor Signaling

To confirm that CYR61 transcription is induced by Ang II stimulation, we performed time-course Northern blot analysis. A solid but transient increase of CYR61 transcripts peaking at 30 minutes and lasting up to 60 minutes was found after Ang II stimulation in RASMCs (Figure 1A) and in primary rat cardiac fibroblast cultures (data not shown). The selective nonpeptide Ang II type 1 (AT1) receptor antagonist losartan (10^{-5} mol/L) abolished Ang II–induced CYR61 mRNA expression (Figure 1B), indicating that Ang II induces CYR61 mRNA expression via the AT1 receptor. Losartan also abolished Ang II induction of c-fos, leukemia inhibitory factor, plasminogen activator inhibitor type 1, cyclooxygenase 2, and interleukin-6 (data not shown).

Ang II Promotes CYR61 Expression in Aortas Ex Vivo and In Vivo

To investigate whether Ang II induces CYR61 expression in arterial blood vessels, we incubated aortic rings for 30 minutes in DMEM with (n=3) or without (n=3) Ang II (10^{-7} mol/L). CYR61 transcript levels as determined by Northern blots were elevated 2-fold (P<0.05) in aortic rings exposed to Ang II compared with controls (data not shown).

Intraperitoneal injection of adult male mice with Ang II resulted in a transient increase of CYR61 mRNA, peaking 30 minutes after injection in aortic tissue, as determined by RT-PCR (Figure 2).

CYR61 Expression in Aortic Arteriosclerotic Plaques of ApoE-Deficient Mice

In 6-month-old but not in 4-week-old apoE-deficient mice, aortic CYR61 transcript levels were elevated 4 times compared with age-matched wild-type controls with the same genetic background (Figure 3A). CYR61 transcripts and protein were detected in aortas with plaques by in situ hybridization and immunohistochemical staining (Figure 3, B and C), whereas aortas of age-matched controls or young apoE-deficient mice without arteriosclerotic lesions were devoid of detectable CYR61 transcripts or protein (Figure 3, A–C).

CYR61 Protein Is Highly Expressed in Human Atherosclerotic Lesions

CYR61 protein expression in human arteriosclerotic lesions was examined (carotid artery specimens from 7 patients...
undergoing endarterectomy: mean age, 67 years; range, 60 to 80 years; and left anterior descending coronary artery samples from 3 patients with ischemic cardiomyopathy undergoing heart transplantation: mean age, 59 years; range, 57 to 63 years). Figure 4 shows solid expression of CYR61 protein (Figure 4A) of α-smooth muscle actin–positive and –negative cells (Figure 4C) within the neointima and the hyperplastic media within arteriosclerotic lesions of a carotid artery. For comparison, only marginal CYR61 staining was observed in normal arteries, IMA specimens, from patients undergoing coronary artery bypass operations (Figure 5) (n=4; mean age, 64 years; range, 51 to 71 years).

Colocalization of Ang II and CYR61 in Human and Mouse Arteriosclerotic Lesions

As previously shown,3 human arteriosclerotic plaques exhibit high levels of Ang II peptide accumulation. In carotid artery endarterectomy and in coronary artery specimens of patients with coronary heart disease, diffuse Ang II staining was observed in connective tissue within the hyperplastic media and the neointima (Figure 4B). Serial sections of an arteriosclerotic carotid artery show strong colocalization of CYR61 protein staining (Figure 4A) and Ang II peptide accumulation (Figure 4B) in connective tissue (Figure 4D) within the hyperplastic media and the neointima. Similarly, CYR61 protein and Ang II staining were colocalized within aortic plaques of apoE-deficient mice (Figure 3C).

Colocalization of Ang II and CYR61 in Microvessels of Human Atherosclerotic Lesions

Both the RAS and CYR61 have been implicated in angiogenesis.13,20 Arteriosclerotic lesions exhibit increased formation of microvessels within the adventitia and the hyperplastic media. Therefore, we examined protein expression of ACE, Ang II, and CYR61 within capillaries and small blood vessels in lesion areas. Serial sections of a carotid artery endarterectomy show enhanced CYR61, ACE, and Ang II protein expression in microvessels within the adventitia underlying the arteriosclerotic lesions (Figure 6). In small blood vessels, expression of CYR61, ACE, and Ang II was observed mainly in smooth muscle cells, whereas endothelial cells express predominantly ACE and Ang II but show only marginal CYR61 staining. In contrast, CYR61, ACE, and Ang II are expressed in endothelial cells of capillaries (Figure 6).
Discussion

In the present study, we screened for genes that are differentially expressed in response to Ang II stimulation in vascular smooth muscle cells. In addition to numerous genes known to be induced by Ang II, we identified the upregulation of CYR61, an angiogenic growth factor.13 Ang II induced the expression of CYR61 in vascular cells in vitro and in the vascular wall ex vivo and in vivo. The rapid onset of this expression after application of Ang II, even in the in vivo situation, suggests that Ang II can rapidly stimulate CYR61 transcription directly rather than indirectly by primary induction of growth factors and cytokines. Our observations, however, do not exclude the possibility that a delayed induction of CYR61 in response to Ang II (or, in general, during activation of the RAS) is in part attributable to indirect pathways, such as Ang II–induced activation of other growth factors and/or cytokines.

Notably, CYR61 expression was observed almost exclusively in arteriosclerotic arteries of both apoE-deficient mice and humans and colocalized with Ang II, supporting the notion that the expression of CYR61 is induced by Ang II in arteriosclerotic arteries.

CYR61 is a secreted heparin-binding protein that is associated with extracellular matrix and cell surface molecules.21 Purified CYR61 enhances basic fibroblast growth factor– and platelet-derived growth factor–induced DNA synthesis in fibroblasts,22 activation of intracellular signaling molecules including focal adhesion kinase, paxillin, and Rac, and sustained activation of p42/p44 mitogen-activated protein kinases (MAPKs).23 In addition, CYR61 enhances matrix metalloproteinase-1 (collagenase-1), and matrix metalloproteinase-3 (stromelysin-1) expression, which may cause matrix remodeling during angiogenesis and wound healing.23 Furthermore, CYR61 acts as a chemotactic factor for fibroblasts24,25 and promotes attachment of activated platelets26 and spreading of cultured endothelial cells.27 CYR61 stimulates the direct migration of human microvascular endothelial cells in culture and induces neovascularization in rat cornea.13 The fact that CYR61 binds to αβ1 integrins,27 αβ2 integrins,26 and αβ1 integrins24 raises the possibility that CYR61 acts through integrins as signaling receptors.27 A potential role of CYR61 in angiogenesis and vascularization has been suggested by 2 observations. First, CYR61 is able to induce neovascularization in rat cornea,13 and second, CYR61 is highly expressed in the neointimal lesions and in the tunica media of balloon-injured arteries in monkey.12

Figure 4. Representative immunohistochemistry of serial sections of an atheromatous plaque from a carotid artery specimen using anti-human CYR61 antibody (A), Ang II antibody (B), or α-smooth muscle actin antibody (C). Trichrome staining (Goldner-Weigert-elasticia) marks connective tissue (green) (D). Unspecific IgG (E). Adjacent sections show that CYR61 and Ang II staining are present in smooth muscle cells and in areas with connective tissue. NI indicates neointima; M, media. Magnification ×80.

Figure 5. Immunohistochemistry of a normal artery specimen (IMA) using anti-CYR61 (A), unspecific IgG (B), and trichrome staining (Goldner-Weigert-elasticia), where green indicates connective tissue; dark purple, elastic fibers; red, cytosol; and dark brown, nuclei (C). Similar staining was observed in IMA specimens of 3 additional patients. L indicates lumen; I, intima; and M, media. Magnification ×80.
CYR61 is colocalized with Ang II in arteriosclerotic lesions, it is reasonable to speculate that an activated RAS may modulate vascular CYR61 expression during the development and progression of arteriosclerosis. Stimulation of the AT1 receptor leads to a strong activation of the MAPK/extracellular signal–regulated kinase (ERK) signaling pathway. This pathway is crucial for CYR61 expression.28,29 The fact that Ang II–induced CYR61 expression was completely blocked by losartan, a selective AT1 antagonist, is consistent with the notion that Ang II induces CYR61 expression through activation of the MAPK/ERK pathway. In addition to its influence on important proarteriosclerotic processes, such as inflammation, smooth muscle cell growth, or cell adhesion, Ang II may contribute to arterial plaque formation by inducing expression of such genes as CYR61, ie, Ang II may act directly on smooth muscle cells and fibroblasts to stimulate CYR61 expression and secretion during the development of arteriosclerotic lesions, which in turn may contribute to extracellular matrix production, cell proliferation and migration, and neovascularization. The latter is thought to contribute to the progression of the arteriosclerotic process.30 Furthermore, in the event of plaque rupture, smooth muscle cells expressing high levels of CYR61 are exposed to activated platelets, which may enhance aggregation by the binding of CYR61 to αvβ3 integrins, with subsequent generation of thrombosis.

In contrast to CYR61, CTGF, another member of the CCN family that is expressed in arteriosclerotic lesions as well,31,32 shows no significant induction by Ang II stimulation in smooth muscle cells (data not shown). The presence in arteriosclerotic lesions of both of these proteins with almost identical properties7 may reflect a redundancy of the system. Whether these 2 members of the CCN family represent part of the mechanism for maintenance of the vessel function or are expressed in the vascular wall only during pathophysiological conditions, such as arteriosclerosis, remains to be elucidated.

In summary, the present study shows that in human arteriosclerotic lesions, ACE, Ang II, and CYR61 are colocalized and that Ang II stimulates the synthesis of CYR61 in smooth muscle cells and fibroblasts in vitro and in vivo. These observations may point to Ang II as a potential factor involved in the development of plaque neovascularization within arteriosclerotic lesions by enhancing regulators of microvessel formation and cell proliferation.

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References


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