Angiotensin II (Ang II), the main peptide of the renin-angiotensin system, is a true cytokine that regulates cell growth, inflammation, and fibrosis, contributing to the progression of vascular damage. 1–3 Extracellular matrix (ECM) accumulation in the cardiovascular system is involved in vascular and cardiac hypertrophy and heart failure. Hypertension causes structural changes in the arteries, including hypertrophy of vascular smooth muscle cells (VSMCs), collagen and fibronectin (FN) accumulation, and destruction of elastic fibers. 3 ECM overproduction has been attributed to hemodynamic changes associated with mechanical stress or factors such as Ang II and transforming growth factor-β (TGF-β). 3 The knowledge of molecular mechanisms involved in ECM accumulation may contribute to a better understanding of this pathological process and to improved therapeutic strategies.

Connective tissue growth factor (CTGF) is a novel and potent profibrotic factor, a member of the CCN family (cysteine-rich61, CTGF, and nephroblastoma overexpressed) of early immediate genes. CTGF is implicated in fibroblast proliferation, cellular adhesion, angiogenesis, and ECM synthesis. This protein of 38 kDa was originally identified in conditioned media from human umbilical vein endothelial cells and mice fibroblasts, primarily on the cell surface and ECM. 4 CTGF participates in fibrotic processes, including skin disorders, tumor development, and renal disease. 4 CTGF is overexpressed in human atherosclerotic lesions 5 and in the myocardium of infarcted rats and patients with cardiac ischemia. 6,7 In VSMCs, the cells primarily involved in ECM production, CTGF regulates cell proliferation/apoptosis, migration, and fibrosis. 4,7–9 For this reason, studies defining its role in vascular damage are necessary.

CTGF expression is regulated by several agents, including TGF-β, tumor necrosis factor-α, cAMP, high glucose, dexamethasone, factor VIIa, and mechanical stress, but not by growth factors, such as endothelial growth factor, platelet-derived growth factor, and fibroblast growth factor. 4 It has been postulated that CTGF is a downstream mediator of the effect of TGF-β on ECM regulation and apoptosis. 4,10,11 In different pathological settings and cell types, Ang II regulates TGF-β expression and could mediate some Ang II responses. 3,12 However, the relation between CTGF and Ang II has not been studied. Our aim was to investigate mediators and molecular mechanisms involved in Ang II–induced fibrosis associated with vascular damage, evaluating the hypothesis that CTGF could be a mediator of Ang II actions.
Methods

Ang II (50 ng \cdot kg^{-1} \cdot min^{-1}) by subcutaneous osmotic minipumps, n=8 rats each group) was infused systemically into female Wistar rats. One group was treated with the AT1 antagonist losartan (10 mg \cdot kg^{-1} \cdot d^{-1} in the drinking water) starting 24 hours before Ang II infusion and studied after 7 days. Animals were anesthetized with 5 mg/kg xylazine (Rompun, Bayer AG) and 35 mg/kg ketamine (Ketolar, Fisher) according to European guidelines. VSMCs from rat thoracic aorta were obtained and serum-starved for 48 hours before use.

Gene and Protein Studies

Gene expression was analyzed by reverse transcription–polymerase chain reaction (RT-PCR) and Northern blot. For in vivo studies, paraffin-embedded sections of rat aorta were studied by immunohistochemistry. Antibodies used were rabbit anti-CTGF (Torrey Pines Biolaboratories), anti-FN (Chemicon International), anti-collagen type I (Calbiochem), anti-laminin (Neomarkers), and peroxidase-conjugated secondary antibodies (Amersham). Negative controls without the primary antibody or using an unrelated antibody were included to check for nonspecific staining. For in vitro studies, cells were fixed in methanol/acetone at –20°C. In VSMCs, protein levels were also determined by Western blot.

Statistical Analysis

Results are expressed as n-fold increase over control in densitometric arbitrary units as mean±SEM of experiments made. Significance was established with GraphPAD Instat using Student’s t test (GraphPAD Software), Wilcoxon, and Student-Newman-Keuls test. Differences were considered significant at a value of P<0.05.

Results

Systemic Infusion of Ang II Increases CTGF Expression in Aorta

The aortic samples from control rats did not present staining for CTGF (Figure 1). In normal human arteries, CTGF mRNA or protein could not be detected. In rats infused with Ang II for 3 days, CTGF production was induced, showing positive immunostaining in VSMCs, which remained elevated after 7 days (Figure 1).

In human arteries with atherosclerotic lesions, high CTGF expression was found in intimal VSMCs of areas with ECM and fibrosis. Accumulation of several ECM proteins involved in vascular damage, such as FN, type I collagen, and laminin, were found in the aorta of Ang II–infused rats.
(Figure 1), as described previously. This effect was observed only after 7 days of Ang II infusion but not after 3 days (showed only in FN deposition), demonstrating that CTGF induction occurs earlier than ECM overproduction and suggesting that CTGF could be a mediator of vascular fibrosis caused by Ang II.

The AT1 antagonist losartan abolished blood pressure elevation observed in Ang II-infused rats (103 ± 11 vs 138 ± 7 mm Hg, n = 8, P < 0.05 versus Ang II at day 7) and aortic CTGF and ECM protein overexpression (Figure 1), suggesting that Ang II, through the AT1 receptor, induces CTGF and fibrosis in vivo.

**Angiotensin II Increases CTGF mRNA and Protein Levels in VSMCs**

Growth-arrested VSMCs expressed low CTGF mRNA levels, as shown by Northern blot as a band of 2.4 kb. Ang II stimulation rapidly increased CTGF mRNA (3.3-fold, 10^{-7} mol/L; 1 hour, Figure 2A), which was maximal after 3 hours and decreased thereafter, but showed another peak at longer times. Data were confirmed by RT-PCR (not shown).

CTGF protein production was investigated with an anti-CTGF antibody that recognizes the 247 to 260 CTGF amino acids (Figure 2B). In growth-arrested VSMCs, only the cell-associated fraction expressed a band of ≈ 38 kDa, the apparent CTGF molecular weight, which was undetectable in the supernatant. Ang II increased total cellular CTGF levels (cytosolic and cell-associated) and the release into the extracellular medium (soluble fraction) after 24 hours of treatment, remaining elevated up to 72 hours. Immunocytochemistry showed that growth-arrested VSMCs presented a slight CTGF staining, and stimulation for 48 hours with Ang II or 10% FCS (positive control)
clearly increased cytoplasmic staining (Figure 2C). These data suggest that in VSMCs, Ang II increases CTGF mRNA and protein production.

**Angiotensin II Increases CTGF via AT\(_1\) in VSMCs**

In VSMCs, Ang II acts through 2 specific receptors, AT\(_1\) and AT\(_2\).\(^{2,14}\) The AT\(_1\) antagonist losartan caused a significant diminution in Ang II–induced CTGF at both the mRNA and protein levels (Figure 3), whereas the AT\(_2\) antagonist PD123319 had no effect, suggesting that Ang II–induced CTGF upregulation is mediated through AT\(_1\) receptors.

**Molecular Mechanisms of Ang II–Induced CTGF Gene and Protein Production**

CTGF gene is regulated by Ang II in a 2-phase manner, a rapid induction after 1 hour and a maintained response until 24 hours (Figure 2). Pretreatment with actinomycin D, an inhibitor of RNA synthesis, markedly reduced CTGF mRNA induced by Ang II at different times studied, between 1 and 24 hours (Figure 4A). When ActD was added in the last 2 hours of incubation, Ang II–induced CTGF gene expression after 18 hours was also inhibited (not shown), suggesting that CTGF overexpression in Ang II–treated cells was a result of newly synthesized mRNA. Protein synthesis inhibition by cycloheximide strongly increased CTGF mRNA expression in basal and Ang II–treated cells at all times studied (Figure 4A). Our data demonstrate that early CTGF upregulation caused by Ang II is independent of the de novo protein synthesis of cytokines and transcription factors, showing that CTGF behaves as an early responsive gene as occurs in other cell types.\(^{17}\)

Ang II, via AT\(_1\), activates several intracellular mediators, including protein kinase C (PKC), phosphotyrosine kinases (PTK), and production of intracellular reactive oxygen species (ROS).\(^{2}\) In VSMCs, the PKC activator phorbol 12-myristate 13-acetate (PMA) increased CTGF expression and synthesis (Figure 2). Two PKC inhibitors, H-7 and bisindolylmaleimide, slightly but not signifi-
cantly diminished Ang II–mediated CTGF (Figure 4B). In VSMCs, exogenous H$_2$O$_2$ increases CTGF mRNA expression and protein production (Figure 5). The NADH/NADPH oxidase inhibitor diphenyleneiodonium and the O$_2^-$ scavenger Tiron markedly diminished Ang II–induced CTGF protein production (Figure 4B). CTGF gene upregulation after 18 hours was diminished by all of these inhibitors (not shown), indicating that CTGF is regulated at the transcription level. These data suggest that Ang II, via AT$_1$ through the activation of several protein kinases such as PKC and by a redox-sensitive mechanism, regulates CTGF protein production.

In VSMCs, TGF-$

\beta$ upregulates CTGF mRNA expression and protein synthesis (Figure 2). Compared with Ang II, TGF-$

\beta$ upregulates the CTGF gene with a similar kinetic response but causes a higher increase in CTGF gene expression at shorter times, whereas after 24 hours, the response was similar. TGF-$

\beta$ increased CTGF synthesis earlier than Ang II, showing augmented protein synthesis after 6 hours (Figure 2B). We blocked TGF-$

\beta$ actions by different methods: a neutralizing antibody against active TGF-$

\beta$ that blocks Ang II–induced ECM production$^{18}$ and decorin, a scavenger of its active form.$^{12}$ These TGF-$

\beta$ blockers inhibited Ang II–induced CTGF mRNA overexpression at 18 and 24 hours and diminished CTGF production from 24 to 72 hours (Figure 6), suggesting that endogenous TGF-$

\beta$ synthesis is involved, at least in part, in CTGF production caused by Ang II. In contrast, TGF-$

\beta$ blockade did not modify Ang II–induced CTGF mRNA expression at 1 hour (Figure 6A), showing that early CTGF upregulation is independent of TGF-$

\beta$. We have also observed that CTGF overproduction caused by exogenous H$_2$O$_2$ was not diminished by TGF-$

\beta$ blockade (Figure 5), showing a TGF-$

\beta$–independent CTGF regulation in VSMCs, as observed in other cells.$^{19}$

**Role of CTGF in Ang II–Induced Fibrosis**

In cultured cells, Ang II increased FN production in both the cell-associated and the soluble fractions.$^{18,20}$ We therefore investigated whether CTGF was involved in Ang II–induced fibrosis, evaluating the effect on soluble FN production by Western blot in VSMCs. To block CTGF actions, we used a CTGF antisense oligonucleotide, constructed with a 16-mer derived from the starting translation site, which contains the initial ATG whose sequence is 5'-TACTGGCGGCGGTCAT-3', which blocks TGF-$

\beta$ actions.$^{10}$ In VSMCs, incubation with the CTGF antisense oligonucleotide diminished Ang II–induced FN production (Figure 7). Recombinant CTGF may induce its own synthesis.$^{15}$ As a control, we have observed that the presence of CTGF antisense oligonucleotide abolished Ang II–induced CTF$

\gamma$ mRNA expression (not shown). These data suggest that CTGF is a downstream mediator of Ang II–induced FN upregulation and fibrosis.

**Discussion**

Hypertension is a risk factor for the development of coronary diseases and atherosclerosis. Fibrosis is one of the vascular changes caused by hypertension.$^{1-3}$ In a rat model of hypertension induced by cyclosporin A (CsA) and high-sodium diet, the enhanced expression of CTGF in epicardial arteries was closely correlated with blood pressure elevation.$^{21}$ Mechanical strain could be responsible for CTGF upregulation in hypertensive animals, because cyclic mechanical stretching induced CTGF, and increased immunoreactive Ang II in the culture medium and ECM synthesis.$^{15,22}$ However, in the CsA model, the AT$_1$ antagonist only partially diminished blood
The aortic FN upregulation was not a result of blood pressure. Losartan diminished aortic CTGF and ECM overexpression. In cultured VSMCs, an anti-sense CTGF oligonucleotide diminished Ang II overexpression was observed. In rats infused with Ang II, CTGF data show a similar behavior. In Ang II–treated cells, we used a CTGF antisense oligonucleotide (ODN; 20 μg/mL) added directly to medium, and CTGF sense ODN was its control. As positive control of FN production, 10% FCS was used. Values of mean ± SEM of 4 experiments are shown in lower panel. *P<0.05 vs control. #P<0.05 vs Ang II.

pressure and the development of left ventricular hypertrophy, whereas it normalized cardiac CTGF.21 We have observed that systemic infusion of Ang II into rats, which only slightly increased blood pressure, caused a marked aortic CTGF induction and vascular fibrosis. Moreover, in vitro, Ang II increases CTGF gene expression and production, showing that Ang II causes a direct, non–pressure-mediated CTGF upregulation. CTGF overexpression occurs in the active phase of matrix synthesis during wound repair.4 Our in vivo data show a similar behavior. In Ang II–infused rats, CTGF induction is prolonged for at least 7 days, when ECM overexpression was observed. In cultured VSMCs, an anti-sense CTGF oligonucleotide diminished Ang II–induced FN production, demonstrating that CTGF is involved in vascular fibrosis caused by Ang II.

Ang II acts through its binding to specific receptors.2 AT1 regulates cell proliferation and production of cytokines and ECM proteins and some pathological processes, including Ang II–induced hypertension, neointimal formation, and cardiac hypertrophy.1–3 However, other processes are controlled by AT2, such as cell growth inhibition and inflammatory cell recruitment.1–2 We have observed that in cultured VSMCs, Ang II increased CTGF expression and synthesis via AT1. Moreover, in Ang II–infused rats, the AT1 antagonist losartan diminished aortic CTGF and ECM overexpression. The aortic FN upregulation was not a result of blood pressure elevation but rather primarily of AT1.16 Our data clearly demonstrate that Ang II via AT1 could contribute to vascular damage increasing fibrosis through CTGF upregulation.

The molecular mechanisms linked to AT1 activation are common to classic cytokines and include activation of several kinases.2 Among the intracellular signaling systems involved in Ang II–induced matrix regulation, PKC and PTK activation play an important role in different cell types.18,20 In VSMCs, PKC mediated FN production caused by Ang II.20 Our data demonstrate that in VSMCs, Ang II regulates CTGF via AT1 and activation of PKC, whereas PTK activation made a minimal contribution. Opposite results have been described. The PI3-kinase-Akt pathway, independently of PKC, mediated vascular endothelial growth factor–induced CTGF.23 In rat kidney fibroblasts, only inhibitors of PKA but not of PKC or PTK blocked TGF-β–stimulated CTGF transcription.4 CTGF expression was inhibited by phosphorylation on serine/threonine and tyrosine by PKC and PTK.24 In VSMCs, we have observed that the PKC activator PMA increased CTGF gene and production, indicating that the PKC effect is cell specific. Ang II, via AT1, increased H2O2 generation through phospholipase D–dependent, NADH/NADPH oxidase–sensitive pathways.25 Diverse antioxidants blocked CTGF production elicited by Ang II, suggesting that ROS act as intermediates of AT1–mediated CTGF production. ROS also mediate other Ang II effects, including cell proliferation, protein synthesis and intracellular responses, such as activation of mitogen-activated protein kinase, nuclear factor-κB, and activator protein-1.1,14 Altogether, these data suggest that Ang II, via AT1, elicited several intracellular signals, such as PKC activation and ROS production, that contribute to CTGF and ECM regulation.

TGF-β is the most important regulator of ECM.12 ACE inhibitors and AT1 antagonists diminished tissue expression of TGF-β and fibrosis, and blockade of TGF-β diminished Ang II–induced ECM production.3,12,18 TGF-β and Ang II share intracellular mechanisms involved in regulation of ECM and, as shown here, of CTGF, including activation of PKC and PTK.12,17,18 The CTGF promoter contains a TGF-β response element.4 CTGF mediates several TGF-β actions,10,11 but other responses, such as changes in fibroblast morphology, are CTGF-independent.25 Here, we demonstrate that the blockade of TGF-β abolished Ang II–induced CTGF gene and protein production, suggesting that endogenous TGF-β synthesis is involved, at least in part, in CTGF production caused by Ang II. Intracellular ROS directly induced CTGF expression, via Janus kinase activation, independently of TGF-β in epithelial cells.19 In VSMCs, we have also observed that H2O2 upregulates CTGF by a TGF-β–independent process. Our data demonstrate that Ang II upregulates CTGF by a process that can be mediated by production of growth factors (TGF-β) and intracellular signals (protein kinases, ROS activation), showing the complexity of Ang II responses in vascular tissue.

The physiological functions of CTGF in vivo are not yet fully determined. CTGF stimulates proliferation in fibroblasts and endothelial cells,2,26 whereas it causes VSMC apoptosis.9 In human atherosclerotic plaques, elevated Ang II and CTGF1,3,5 and apoptosis of VSMCs27 were found. Ang II via AT1 increased but via AT2 inhibited cell proliferation.2 An in vivo study has shown that stimulation of AT1 or AT2 induces apoptosis in vessels.28 In Ang II–infused rats and in cultured VSMCs, we have not observed apoptosis (data not shown), indicating that in our experimental conditions and at the times...
studied, CTGF overexpression via AT1 activation is associated with fibrosis but not with apoptosis. In cardiac interstitial fibroblasts, Ang II via AT1 increases collagen. TGF-β induces CTGF in cardiac fibroblasts and cardiomyocytes. In both cell types, CTGF increases production of FN, collagen, and PAI-1, which shows its participation in cardiac fibrosis. CTGF overexpression has also been described in cardiac ischemia but its role in fibrosis or apoptosis is not clear. Future studies are necessary to determine the role of Ang II receptors and CTGF in heart failure.

In conclusion, our data show that Ang II in vivo and in vitro induces a novel profibrogenic factor: CTGF. In Ang II–infused rats, aortic CTGF expression preceded ECM overexpression, suggesting that CTGF could be a downstream mediator of Ang II–induced structural changes of the vascular wall. These data might contribute to increase our knowledge of the mechanisms underlying fibrosis in cardiovascular diseases.

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Mónica Rupérez, Óscar Lorenzo, Luis Miguel Blanco-Colio, Vanesa Esteban, Jesús Egido and Marta Ruiz-Ortega

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