Endothelin-1, via ET$_{A}$ Receptor and Independently of Transforming Growth Factor-$\beta$, Increases the Connective Tissue Growth Factor in Vascular Smooth Muscle Cells

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Abstract—Endothelin (ET)-1 is a potent vasoconstrictor that participates in cardiovascular diseases. Connective tissue growth factor (CTGF) is a novel fibrotic mediator that is overexpressed in human atherosclerotic lesions, myocardial infarction, and experimental models of hypertension. In vascular smooth muscle cells (VSMCs), CTGF regulates cell proliferation/apoptosis, migration, and extracellular matrix (ECM) accumulation. Our aim was to investigate whether ET-1 could regulate CTGF and to investigate the potential role of ET-1 in vascular fibrosis. In growth-arrested rat VSMCs, ET-1 upregulated CTGF mRNA expression, promoter activity, and protein production. The blockade of CTGF by a CTGF antisense oligonucleotide decreased FN and type I collagen expression in ET-1–treated cells, showing that CTGF participates in ET-1–induced ECM accumulation. The ET$_{A}$, but not ET$_{B}$, antagonist diminished ET-1–induced CTGF expression gene and production. Several intracellular signals elicited by ET-1, via ET$_{A}$ receptors, are involved in CTGF synthesis, including activation of RhoA/Rho-kinase and mitogen-activated protein kinase and production of reactive oxygen species. CTGF is a mediator of TGF-$\beta$- and angiotensin (Ang) II–induced fibrosis. In VSMCs, ET-1 did not upregulate TGF-$\beta$ gene or protein. The presence of neutralizing transforming growth factor (TGF)-$\beta$ antibody did not modify ET-1–induced CTGF production, showing a TGF-$\beta$–independent regulation. We have also found an interrelationship between Ang II and ET-1 because the ET$_{A}$ antagonist diminished CTGF upregulation caused by Ang II. Collectively, our results show that, in cultured VSMCs, ET-1, independently of TGF-$\beta$ and through the activation of several intracellular signals via ET$_{A}$ receptors, regulates CTGF. This novel finding suggests that CTGF could be a mediator of the profibrotic effects of ET-1 in vascular diseases. (Circ Res. 2005;97:125-134.)

Key Words: endothelin-1 ▪ connective tissue growth factor ▪ signal transduction ▪ vascular smooth muscle cells ▪ extracellular matrix

Several data suggest an important role for endothelin-1 (ET-1) in vascular diseases. Elevated plasma and tissue levels of ET-1 have been described in atherosclerosis, myocardial infarction, unstable angina, pulmonary hypertension, and heart failure. Chronic exposure to ET-1 results in vascular and myocardial fibrosis and hypertrophy. In experimental models of cardiovascular damage, the treatment with ET-1 antagonists presented marked beneficial effects.

ET-1 is a potent vasoconstrictor that can activate vascular smooth muscle cells (VSMCs), inducing proliferation, hypertrophy, and synthesis of extracellular matrix (ECM) proteins, such as fibronectin (FN) and type I collagen. ET-1 stimulates the production of cytokines, such as tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor-2, and strengthens the effects of transforming growth factor (TGF)-\(\beta\) and platelet-derived growth factor (PDGF). Mammals possess two main receptors, ET$_{A}$ and ET$_{B}$.

In blood vessels, ET$_{A}$ receptors are found in VSMCs, whereas ET$_{B}$ receptors are mainly localized on endothelial cells and, to some extent, in VSMCs and macrophages. ET-1, predominantly via ET$_{A}$ receptors, promotes vasoconstriction, cell growth, adhesion, fibrosis, and thrombosis. However, the role of ET$_{B}$ receptors is still controversial because those expressed on endothelial cells stimulate vasodilatation, release NO, and prevent apoptosis and may oppose the actions of ET$_{A}$ receptors. Moreover, in pathological conditions, upregulation of ET$_{B}$ on VSMCs has been described, with similar function to ET$_{A}$, which could amplify ET-1–induced responses.

Connective tissue growth factor (CTGF) is a cysteine-rich secreted protein that regulates cell proliferation/apoptosis, angiogenesis, migration, adhesion, and fibrosis. CTGF expression is strongly upregulated by mechanical stress or static pressure and by several factors involved in vascular damage, including elevated glucose concentrations, TGF-$\beta$, angioten-
sin (Ang) II, and VEGF, but not by other factors, such as epidermal growth factor and PDGF, and is downregulated by cAMP and TNF-α. However, there are no studies investigating whether ET-1 could regulate CTGF expression. Accumulation of ECM is one feature of cardiovascular diseases. The elucidation of the molecular mechanisms involved in this process would be very useful in the treatment of these disorders. The correlation of CTGF overexpression with fibrosis has been described in human atherosclerotic lesions, myocardial infarction, and in the aorta of Ang II–infused rats. The aim of this study was to investigate whether ET-1 could regulate CTGF in vascular cells and to investigate the potential role of ET-1 in ECM accumulation. Next, we have also evaluated the receptor subtype (ETA and ETB) and the molecular mechanisms involved in this process. ET-1 triggers several intracellular signaling systems, including free oxygen radical production, and activation of small G proteins and mitogen-activated protein kinase (MAPK), which are involved in vascular damage and fibrosis. For this reason, we studied whether these signaling pathways participate in ET-1–mediated CTGF regulation. Some data suggest that CTGF and TGF-β synergizes to promote chronic fibrosis and that CTGF acts as a mediator of TGF-β–induced apoptosis and fibrosis. In VSMCs, CTGF is also a mediator of the profibrotic effects of Ang II. Finally, we investigated the potential interrelationship of TGF-β, Ang II, and ET-1 on CTGF regulation.

**Materials and Methods**

**Materials**

Cell culture reagents (Life Technologies, Inc), Botulinum C3 exoenzyme (Calbiochem, La Jolla, Calif), ET-1 and antagonists (BACHEM, Germany); Y-27632 and fasudil (TOCRIS Cookson Ltd, Bristol, UK) were used. Antibodies were as follows: rabbit anti-CTGF antibody (Torrey Pines Biolabs, San Diego, Calif), phospho-ERK, ERK, and tubulin antibodies (Sigma) and secondary antibodies (Santa Cruz Biotechnology). The rest of the compounds were from Sigma-Aldrich.

**Cell Cultures**

VSMCs were obtained from thoracic aorta of Wistar–Kyoto rats by the collagenase method as described. Wistar Kyoto rats were obtained from the animal facilities of the Fundación Jiménez-Díaz and were treated following Institutional and European guidelines. Subcultured VSMCs from passages 2 to 7 were used in the experiments, showed >99% positive immunostaining against smooth muscle α-actin antibodies. For subsequent experiments, cells at 80% confluence in culture wells were growth-arrested by serum-starvation for 48 hours.

**Gene and Protein Studies**

Total RNA was isolated with Trizol. Northern blot was performed as described. Real-time polymerase chain reaction (PCR) was performed on a ABI Prism 7500 sequence detection PCR system (Applied Biosystems) according to the protocol of the manufacturer. TGF-β, type I procollagen, and GAPDH assay identification numbers are as follows: Rn00579697_m1, Rn00584426_m1 and Rn99999916_m1. Protein levels were determined by Western blot and ELISA (TGF-β1 immunoassay kit from R&D). Protein content was determined by the BCA method. For Western blot, Red Ponceau

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**Figure 1.** ET-1 increases CTGF mRNA expression in cultured VSMCs. A, Growth-arrested VSMCs were stimulated with 10⁻⁸ mol/L ET-1 or 10⁻⁇ mol/L Ang II from 1 to 24 hours. Dose response of ET-1 (10⁻⁸ mol/L to 10⁻¹⁰ mol/L) occurred at 24 hours. CTGF gene expression was determined by Northern blot, shown as a band of 2.4 kb. A, Representative Northern blot. B, mean±SEM values of 4 experiments. *P<0.05 vs control. The quantification of CTGF mRNA was determined densitometrically and expressed as ratio CTGF/G3PDH as n-fold over control. C, ET-1 increases CTGF promoter activity in VSMCs. Cells were transiently transfected with a CTGF promoter/SEAP reporter expression vector and CMV-β-galactosidase and were treated 24 hours later with 10⁻⁸ mol/L ET-1, 10⁻⁷ mol/L Ang II, or 10 ng/mL TGF-β for 24 hours. Values are mean±SEM of 5 experiments performed in triplicate. *P<0.05 vs control.
staining was used to show quality of proteins and efficacy of protein transfer. In experiments of cell-associated proteins, tubulin was used as loading control. The autoradiographs were scanned using the GS-800 Calibrated Densitometer (Quantity One, Bio-Rad, Spain), obtaining densitometric arbitrary units. Data were normalized against those of the corresponding tubulin. Results are expressed as n-fold increase over control in densitometric arbitrary units, expressed as mean ± SEM of the experiments performed.

Localization of RhoA was performed by indirect immunofluorescence with a rabbit polyclonal anti-RhoA antibody. Briefly, cells were fixed in merckofix (Merck) and treated with 0.1% Triton X-100. Nuclei were stained with propidium iodide (1 μg/mL). Controls were stained with nonimmune serum or with the secondary antibody alone (not shown). Coverslips were mounted in mowiol and examined by a laser scanning confocal microscope (Leika).

Transfection and Promoter Studies
VSMCs were seeded in 6-well plates, and 24 hours later, cells were transiently transfected with FUGENE (Roche Molecular Biochemicals), 1 μg CTGF promoter/SEAP reporter expression vector (kindly donated by Dr Noelynn Oliver, Fibrogen27) and 0.25 μg CMV-β-galactosidase (Clontech). After a 24-hour serum starvation step, cells were stimulated for 24 hours, and were assayed for SEAP/β-galactosidase activity.

Statistical Analysis
Significance was established with GraphPAD Instat using Student t test (GraphPAD Software) and Wilcoxon and Student–Newman–Keuls tests. Differences were considered significant when P<0.05.

Endothelin-1 Increases CTGF mRNA, Promoter Activity, and Protein Levels in VSMCs
Cultured VSMCs were treated with ET-1 for increasing times, and CTGF gene expression was determined by Northern blot. ET-1 upregulated CTGF mRNA expression as early as at 1 hour, peaking at 3 hours and remained elevated up to 24 hours. This CTGF upregulation was dose-dependent and maximal at 10⁻⁸ mol/L ET-1 (Figure 1).

Pretreatment with cycloheximide, a protein synthesis inhibitor, strongly increased CTGF mRNA expression in basal and ET-treated cells at all times studied (not shown), suggesting that this gene is regulated at the transcriptional level. We assessed whether ET-1 activates CTGF promoter. We found that ET-1 potently increased CTGF promoter activity in VSMCs (Figure 1C). These data indicate that ET-1–induced CTGF upregulation is controlled primarily at the level of transcription.

We determined whether ET-1 regulates CTGF protein production by Western blot analysis. ET-1 increased cell-associated and soluble CTGF protein synthesis after 24 hours. The maximal response of ET-1 was found at 10⁻⁸ mol/L, and maintained elevated up to 72 hours (Figure 2).
Role of CTGF in ET-1–Induced ECM Accumulation

We investigated whether CTGF was implicated in ET-1–induced ECM regulation. In VSMCs, CTGF increases ECM proteins, such as type I collagen and FN. We blocked CTGF actions with a CTGF antisense oligonucleotide. Incubation with a CTGF antisense oligonucleotide decreased type I collagen gene expression and FN production in ET-1–treated cells (Figure 3). These data suggest that CTGF is a downstream mediator of ET-1–induced ECM accumulation.

Endothelin-1 Increases CTGF via ETA in VSMCs

We have studied the receptor involved using specific ETA and ETB antagonists. The ETA antagonist BQ123 dose depen-
Role of MAPK Activation in ET-1–Induced CTGF Production

Pretreatment of VSMCs with the extracellular signal-regulated kinase (ERK) inhibitor, PD98059, markedly diminished ET-1–induced CTGF production, whereas the p38 MAPK inhibitor, SB203580, had no effect (Figure 7A), indicating the role of MAPK/ERK pathway in CTGF regulation. We next investigated the involvement of reactive oxygen species (ROS) production and Rho activation in ET-1–induced MAPK activation, evaluating ERK phosphorylation. VSMCs were treated with antioxidants and Rho-kinase inhibitors before stimulation with ET-1. Both treatments partially diminished ERK phosphorylation (Figure 7B). The involvement of ROS generation in mediating the ET-1 response on ERK has also recently described in A-10 VSMCs.31 These data suggest that regulation of CTGF by ET-1 involved first activation of Rho and production of ROS and then activation of MAPK/ERK pathway (Figure 7C).

ET-1 Regulates CTGF Independently of TGF-β but Both Factors Synergize in CTGF Production

In rat VSMCs, TGF-β is a potent activator of CTGF promoter and protein synthesis, showing a higher response than ET-1 (Figures 1 and 2). TGF-β is a mediator of CTGF upregulation and ECM accumulation caused by several factors involved in vascular fibrosis, such as Ang II and high glucose.17,18,32 In several models of cardiovascular diseases, the blockade of ET-1 receptors diminished tissue TGF-β expression1,2,7; however, there are no studies evaluating whether ET-1 directly regulates TGF-β in vascular cells. Incubation of rat VSMCs with ET-1 did not increase TGF-β gene expression, studied until 24 hours. In addition, TGF-β content in conditioned media from ET-1–treated cells was not increased compared with unstimulated cells. In the presence of a neutralizing antibody against TGF-β, ET-1–induced CTGF production was not modified (Figure 8). Interestingly, we observed that coincubation of ET-1 and TGF-β resulted in a synergistic effect on CTGF production (Figure 8). These data indicate that ET-1 regulates CTGF by a TGF-β–independent mechanism.

Interrelationship Between Ang II and ET-1

In rat VSMCs, Ang II and ET-1 increased CTGF gene expression with a similar kinetic response. Both peptides increased CTGF promoter activity and protein production in a comparable manner, although the effect of Ang II was slightly higher, showing that this peptide is a more potent profibrotic factor (Figure 1C and 2C). Several data suggest that some actions of Ang II are attributable to the endogenous production of ET-1.33,34 We found that the ETα antagonist BQ123 partially diminished Ang II–induced CTGF production (Figure 4), suggesting that ET-1 is involved in CTGF upregulation caused by Ang II.

Discussion

Many studies have demonstrated that ET-1 contributes to vascular structural changes in proliferative cardiovascular disease. Our results clearly show that in cultured VSMCs, ET-1 increases CTGF mRNA expression, promoter activity,
ET-1 is a vasoactive and mitogenic agent for VSMCs and contributes to the accumulation of ECM through the regulation of FN and type I collagen.\textsuperscript{1,2,9,10} CTGF induces the synthesis of these ECM proteins and plays a key role in the pathogenesis of fibrosis.\textsuperscript{17} In experimental models of vascular damage, including atherosclerosis and hypertension, tissue ET-1 and CTGF upregulation was correlated with fibrosis.\textsuperscript{18–21} We have observed that ET-1 causes a maintained CTGF protein production, up to 72 hours, and the blockade of endogenous CTGF, with a CTGF antisense oligonucleotide, diminishes ET-1–induced FN and type I collagen expression. These data suggest that CTGF could be a mediator of ECM accumulation caused by ET-1.

ET-1 acts through two receptors, ET\textsubscript{A} and ET\textsubscript{B}, both of them expressed in VSMCs.\textsuperscript{1,2} The ET\textsubscript{A} antagonist BQ123 significantly inhibited the in vitro growth-stimulating effects of ET-1.\textsuperscript{9,15} However, in pathophysiological states, such as hypercholesterolemia, the overall effect of ET\textsubscript{B} receptor activation may be vasoconstriction, amplifying ET-1–induced responses.\textsuperscript{16} In cultured fibroblasts, both receptors, ET\textsubscript{A} and ET\textsubscript{B}, mediate collagen synthesis.\textsuperscript{35} In VSMCs, with specific ET\textsubscript{A} and ET\textsubscript{B} antagonists and agonist, we have observed that ET-1 upregulates CTGF gene and protein via ET\textsubscript{A} receptors.

Much data indicate that ET-1 participates in atherosclerosis. In VSMCs of normal and diseased aorta, dense binding of ET-1 was observed by autoradiography. High expression levels of ET-1 have been found in human atherosclerotic plaques compared with normal vessels and after coronary angioplasty.\textsuperscript{36,37} ET-1 release is stimulated by vessel injury and by atherogenic oxidized low-density lipoproteins even when the endothelium remains intact.\textsuperscript{4} ET-1 plasma levels are \(\approx 1\) to 2 pg/mL,\textsuperscript{2} and tissue levels are increased during vascular damage.\textsuperscript{1,2} Our studies show that in cultured VSMCs, ET-1 at \(10^{-8}\) mol/L increases CTGF mRNA and protein expression, suggesting that local

![Figure 6](https://example.com/figure6.png)
ET-1 production in injured vessels could contribute to ECM accumulation, through CTGF production by VSMCs. ET₄₆ antagonism inhibits neointimal hyperplasia after both balloon and stent injury, by attenuating the proliferation of adventitial myofibroblasts and VSMCs as well as ECM formation.⁵ Moreover, ET₃ blockade decreases the development of atherosclerosis in experimental hypercholesterolemia⁶ and in apolipoprotein E–deficient mice.³ In diabetic rats, ET₄ antagonist diminished vascular hypertrophy and FN production.⁷ These data show that ET-1 via ET₄ regulates trophic and fibrotic responses in vascular diseases.

ET-1 seems to be involved in human and experimental hypertension.³⁸ The ET₄/₅ antagonist bosentan induced blood-pressure reductions in mildly hypertensive patients similar to those achieved with an angiotensin-converting enzyme inhibitor.³⁸ In different models of experimental hypertension vascular ET-1 overexpression and ECM accumulation have been described. In deoxycorticosterone acetate (DOCA)-salt–induced hypertension, ET₄ antagonists ameliorated interstitial and perivascular fibrosis, whereas the ET₅ protects against vascular and renal injuries.³⁹ In the early phase of this model, ET-1, via ET₄ receptor, activates TGF-β, and increases FN and collagen deposition in the heart.⁴⁰ The effects of ET-1 antagonists may be attributable to the blockade of direct ET-1 actions on VSMCs. In this sense, we have observed that ET-1 activates VSMCs to produce CTGF that mediates overexpression of FN and type I collagen. Our data reveal a novel mechanism that could explain the beneficial effects of ETA blockade in hypertension and other cardiovascular diseases.

Free radicals and redox stress participate in cellular signaling and regulate a number of important cellular events, including fibrosis and atherogenesis.⁴¹ ET-1 can induce ROS production in different cell types.¹,² In DOCA-salt hypertension, ROS generation was decreased by ETA blockade.⁴² In low-renin mineralocorticoid hypertension ET-1 augments vascular superoxide production, at least in part, via an ETA/NADPH oxidase pathway.⁴³ We examined the effect of DPI, a potent inhibitor of flavonoid-containing enzymes, such as NAD(P)H oxidase and the O₂⁻ scavenger Tiron. Both antioxidants inhibited ET-1 stimulation of CTGF production, which suggests the involvement of a redox mechanism in the regulation of CTGF.

ET-1 activates several intracellular mediators, including small G proteins.¹,² The Rho family of GTP-binding proteins contains many geranylgeranylated proteins, such as Rho, Rac, and Cdc42, that play an important role in cell adhesion, actin dynamics, and gene transcription regulation, including ET-1 and cytokines.⁴⁴ In VSMCs, we have confirmed that ET-1 activates RhoA. Inhibition of RhoA activity, by C3 exotransferase, or of the downstream ET-1 production in injured vessels could contribute to ECM accumulation, through CTGF production by VSMCs. ET₄₆ antagonism inhibits neointimal hyperplasia after both balloon and stent injury, by attenuating the proliferation of adventitial myofibroblasts and VSMCs as well as ECM formation.⁵ Moreover, ET₃ blockade decreases the development of atherosclerosis in experimental hypercholesterolemia⁶ and in apolipoprotein E–deficient mice.³ In diabetic rats, ET₄ antagonist diminished vascular hypertrophy and FN production.⁷ These data show that ET-1 via ET₄ regulates trophic and fibrotic responses in vascular diseases.

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Rho-kinase, by Y27632 or fasudil, prevented the induction of CTGF by ET-1. In *N*-nitro-L-arginine methyl ester-induced vascular damage, Y-27632 decreased vascular inflammation and arteriosclerosis progression. Activation of Rho-kinase system caused vasoconstriction, and it has been found in hypertensive animals. Our results showing that ET-1, via Rho/Rho-kinase activation, upregulates CTGF support the importance of this signaling pathway in hypertension-induced vascular changes.

ET-1 stimulates MAPK pathway including the ERK cascade, the stress-activated protein kinase/c-Jun N-terminal kinase cascade, and the p38 MAPK cascade. These pathways have been implicated in differentiation, hypertrophy, and fibrosis. We have observed that ERK, but not p38 activation, is necessary for ET-1–induced CTGF production. We have also found that ROS generation and Rho-kinase activation mediates ERK phosphorylation, indicating that CTGF production is induced by a ROS and Rho kinase-dependent ERK activation.

In cultured VSMCs, cyclic mechanical stretching and growth factors, such as TGF-β, Ang II and, as we have shown here, ET-1, upregulate CTGF. The regulation of CTGF can be mediated by the production of endogenous growth factors. In VSMCs, TGF-β mediates Ang II–induced CTGF production. Several data suggest an interrelation between TGF-β and ET-1. The blockade of ET-1 receptors diminished TGF-β production in cardiac, vascular, and renal tissues. TGF-β induces ET-1 synthesis. However, we have noted that in rat VSMCs ET-1 did not produce TGF-β. Moreover, the blockade of endogenous TGF-β did not decrease ET-1–induced CTGF upregulation. These data clearly indicate that ET-1 increases CTGF production independently of TGF-β. Current strategies designed to block fibrosis are focusing on CTGF, better than TGF-β, because of its specific role in ECM regulation, without affecting the inflammatory response, as occurs with TGF-β. Our data, showing that ET-1 regulates CTGF and fibrosis independent of TGF-β, support the searching for CTGF-related antifibrotic therapies.

Ang II and ET-1 shares some cellular responses, such as vasoconstriction, cell proliferation and ECM accumulation. However, our data show a different role for TGF-β in the regulation of CTGF caused by both peptides. Ang II regulates the production of ET-1 by a redox-sensitive ERK pathway. We have observed that the ET A antagonist BQ123 diminished Ang II–induced CTGF production, suggesting that ET-1 mediates, at least in part, CTGF production caused Ang II.

Our data, showing that ET-1 upregulated CTGF up to 72 hours, and coinunction of ET-1 and TGF-β causes a synergistic production of CTGF, support the idea that this growth factor contributes to the perpetuation of fibrosis. In a model of skin fibrosis, CTGF mRNA levels remained elevated in areas of persistent fibrosis. Injection of CTGF into the skin induces the formation of fibrous tissue and coinjection of CTGF and TGF-β results in sustained fibrosis.
Our results reveal that in cultured rat VSMCs, ET-1 via ET \(_{A}\) receptors increases CTGF and ECM production. The molecular mechanisms of CTGF regulation are complex, implicating the activation of several intracellular signals (redox processes, RhoA/Rho kinase, and MAPK/ERK) and the interrelationship with other growth factors systems (TGF-\(\beta\) and Ang II). Our findings suggest that CTGF could be a mediator of the profibrotic effects of ET-1 in vascular diseases and support the idea of the usage of CTGF blockers as a novel therapy for vascular diseases.

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References


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