Differential Regulation of Angiotensin II-induced Expression of Connective Tissue Growth Factor by Protein Kinase C Isoforms in the Myocardium*

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Protein kinase C (PKC) and angiotensin II (AngII) can regulate cardiac function in pathological conditions such as in diabetes or ischemic heart disease. We have reported that expression of connective tissue growth factor (CTGF) is increased in the myocardium of diabetic mice. Now we showed that the increase in CTGF expression in cardiac tissues of streptozotocin-induced diabetic rats was reversed by captopril and islet cell transplantation. Infusion of AngII in rats increased CTGF mRNA expression by 15-fold, which was completely inhibited by co-infusion with AT1 receptor antagonist, candesartan. Similarly, incubation of cultured cardiomyocytes with AngII increased CTGF mRNA expression by 2-fold, which was blocked by candesartan and a general PKC inhibitor, GF109203X. The role of PKC isoform-dependent action was further studied using adenoviral vector-mediated gene transfer of dominant negative (dn) PKC or wild type PKC isoforms. Expression of dnPKCα, -ε, and -ζ isoforms suppressed AngII-induced CTGF expression in cardiomyocytes. In contrast, expression of dominant negative PKCδ significantly increased AngII-induced CTGF expression, whereas expression of wild type PKCδ inhibited this induction. This inhibitory effect was further confirmed in the myocardium of transgenic mice with cardiomyocyte-specific overexpression of PKCδ (δTg mice). Thus, AngII can regulate CTGF expression in cardiomyocytes through a PKC activation-mediated pathway in an isoform-selective manner both in physiological and diabetic states and may contribute to the development of cardiac fibrosis in diabetic cardiomyopathy.

Myocardial pathologies in diabetic patients include diastolic dysfunction, microvascular disease, and interstitial fibrosis, which could be the result of active tissue remodeling that can contribute to the reduced cardiac contractility (1). The increase in myocardial fibrosis is part of a pro-fibrotic state associated with the pathogenesis of diabetic complications (2, 3) and may involve the up-regulation of fibrotic factors such as transforming growth factor β1 (TGFβ1)1 and plasminogen activator inhibitor-1. We have reported that the expression of connective tissue growth factor (CTGF) is increased in the myocardium of rats with streptozotocin (STZ)-induced diabetes and in mice engineered to overexpress PKCβ2 in the myocardium, preceding the increased expression of TGFβ1 (4). CTGF has been implicated to have diverse biological actions including the regulation of fibroblast proliferation, collagen synthesis, and cellular apoptosis (5). Its role in the regulation of fibrosis in tissues such as the retina, heart, aorta, lung, and kidney, in diabetes, and in other pathological conditions is beginning to be recognized (6). It has been suggested that CTGF is an essential mediator for the biological actions of TGFβ1 and bone morphogenetic protein (7), possibly via the modulation of the cytokine interaction with their cell surface receptors (7). Although a cell surface receptor mediating CTGF-induced biological responses has not been identified, tyrosine phosphorylation of low density lipoprotein receptor-related protein after its binding by CTGF with high affinity has been reported (8) and has been suggested to be associated with myofibroblast differentiation in the presence of TGFβ1 (9). Increased expression of CTGF has been reported in multiple fibrotic diseases such as renal (10) and lung (11) fibrosis. In diabetic states, up-regulation of the ctgf gene has been observed in tissues including kidney (12) and retina (13) from animals as well as in cells cultured in media containing high level of glucose (14). Multiple extracellular stimuli have been reported to regulate the expression of CTGF including mechanical stretch, growth factors, and cytokines such as TGFβ1 (6), vascular endothelial cell growth factor (15) and angiotensin II (AngII) (16).

Suppression of AngII action is beneficial in preserving cardiac or renal functions after myocardial ischemia and chronic hyperglycemia in diabetes (17). AngII, a vasotrophic hormone generated by angiotensin-converting enzyme (ACE) from angiotensinogen (18), may play an active role in tissue remodeling and the regulation of fibrosis. AngII binds to two types of G

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The abbreviations used are: TGF, transforming growth factor; CTL, control; GFP, green fluorescent protein; dnPKC, dominant negative isoform of protein kinase C; CTGF, connective tissue growth factor; STZ, streptozotocin; AngII, angiotensin II; PKC, protein kinase C; ACE, angiotensin-converting enzyme; PMA, phorbol 12-myristate 13-acetate; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; ICT, islet cell transplantation; NRCM, neonatal rat cardiomyocytes; GFX, GF109203X; ACEI, angiotensin-converting enzyme inhibitor; DM, diabetes mellitus.
protein-coupled cell membrane receptors, AT1R and AT2R (19) to elicit biological actions such as vasoconstriction, myocyte apoptosis and hypertrophy, sodium and water retention, and gene expression (20). Among the multiple signaling pathways that are known to mediate AngII action, protein kinase C (PKC) has been shown to be activated in cardiomyocytes, although the specificities of the various PKC isoforms to propagate AngII effects are yet to be determined (21–23). AngII has been shown to increase the expression of CTGF in vivo, potentially through a calcineurin-mediated pathway (16, 24). The regulation of CTGF expression by PKC activation has not been fully characterized except for studies showing that phorbol 12-myristate 13-acetate (PMA), an analogue of DAG and activator of conventional and novel PKC isoforms, can suppress CTGF expression in cultured fibroblasts (25). In contrast, we have reported that CTGF expression was increased in the myocardium of the cardiac PKC-β2 transgenic mice, preceding the increases of TGFβ2 expression (4). These findings suggest that PKC isoforms may have different effects on CTGF expression at basal states or after its activation by AngII. We have, therefore, investigated the role for a PKC isoform-dependent pathway in AngII-induced CTGF expression in cardiomyocytes. Furthermore, the regulation of CTGF expression specifically by PKCζ isoform activation after AngII stimulation was also examined in vitro and in vivo by using transgenic mice with targeted overexpression of PKCζ to the myocardium and adenoviral vectors overexpressing a wild type or a dominant negative isoform of PKCζ in cardiomyocytes.

**Experimental Procedures**

**Isolation and Culture of Neonatal Rat Cardiomyocytes**—Primary culture of neonatal rat cardiomyocytes were prepared as described previously (26). A total of 8 × 10⁶ cells was plated onto 100-mm culture dishes precoated with gelatin in DMEM-F-12 medium (Invitrogen) containing 5% horse serum and 100 μM/liter bromodeoxyuridine (Sigma). Cardiomyocytes were allowed to attach to the plate wall and were then serum-deprived for 48 h in DMEM-F-12 media containing 0.1% bovine serum albumin (BSA). Cells were then stimulated with 100 nM AngII (Sigma) dissolved in serum-free DMEM-F-12 media containing 0.1% BSA with or without pretreatment with 1 μM MGF109203X (Calbiochem) 30 min before the addition of AngII.

**Adenovirus Infection and AngII Stimulation**—Adenoviral vectors containing green fluorescent protein (GFP, Ad-GFP) and dominant negative PKCζ (dnPKCζ) cDNA fragment (dnPKCζ, ε (dnPKCε), and δ (dnPKCδ)) were constructed and used to infect cardiomyocytes as we have reported previously in various vascular cells and pancreatic β cells (27, 28). Briefly, cultured neonatal rat cardiomyocytes were infected with Ad-GFP, dnPKCα, dnPKCβ2, dnPKCζ, and dnPKCe at a multiplicity of infection of 2 in serum-free media for 2 h at a concentration of 200 and 100 nM, respectively for seeding the cells. The mixture was then applied to the cell monolayer. Cells were then incubated with 100 μM AngII dissolved in serum-free DMEM-F-12 media supplemented with 0.1% BSA for an additional 16 h in the incubator. Media containing adenovirus were removed and cells rinsed with ice-cold PBS twice and grown in serum-free DMEM-F-12 media supplemented with 0.1% BSA for an additional 48 h. Cells were then incubated with 100 μM AngII in serum-free DMEM-F-12 media containing 0.1% BSA for 2 h. Infectivity of these adenoviruses was evaluated by the percentage of green light-emitting cells under a fluorescent microscope (Nikon, Avon, MA). The presence of ~80% of GFP-positive cells was considered to be a successful infection and used for further experimentation.

**Generation of Transgenic Mice Overexpressing PKCζ in the Myocardium**—Mice were engineered to overexpress PKCζ using a method described previously (29). A 2031-bp BamH1-EcoRI (blunted with Klenow) human PKCζ cDNA fragment was inserted in the BamH1-ScaI site of the pBK-CMV vector (Stratagene, La Jolla, CA). The vector was linearized with SalI (blunted) and ligated downstream to a 3.3-kilobase Eagl-Sall fragment (blunted) containing the rat α-myosin heavy chain promoter (p-2936 + 1, provided by Dr. G. F. Fishman) (29). This in turn was located upstream of the SV40 poly(A) site. The recombinant plasmid pBK-CMV-sMHC-PKCζ was digested with Sål and MluI to generate a linear fragment for microinjection into pronuclei of fertilized FVB/Ncr mice eggs that were then implanted into pseudo-pregnant FVB foster mother mice (29). Successful gene transfer was determined by Southern blot analysis of tail genomic DNA using a full-length Nhel PKCζ cDNA fragment from pSVHNX-neo-PKCζ expression vector (provided by Dr. A. F. Reifel-Miller) labeled with [α-32P]dCTP (PerkinElmer Life Sciences) (29). Heterozygous transgenic mice and non-transgenic littermate control mice were used at ages indicated under “Results.”

**Histological Analysis**—Hearts were fixed in 10% neutral-buffered formalin, bisected transversely at the midventricular level, and embedded in paraffin. Sections (~5 μm thick) were made from the midventricle and stained with hematoxylin and eosin and with Masson’s trichrome stain at the Histology core of Joslin Diabetes Center. Ventricular fibrosis was measured using sections stained with Masson’s trichrome.

**AngII Infusion in Vivo**—A catheter was securely inserted into the left jugular vein of male Sprague-Dawley rats (pentobarbital 0.5 mg/g body weight intraperitoneally and mice ~0.1 mL/kg) and transplanted under the left kidney capsule of a recipient rat (30). Plasma glucose returned to normal (125 ± 29 mg/dl) in diabetic rats receiving ICT.

**RNA Extraction, Northern Blotting Analysis, and Taqman Reverse Transcription-PCR**—Total RNA was isolated using Tri-reagent (Molecular Research Center). Briefly, islet cell transplantation (ICT) was performed by Jennifer Beck in Dr. Gordon Weir’s laboratory at the Joslin Diabetes Center. Briefly, islet cells (3000) were isolated from healthy donor rats and transplanted under the left kidney capsule of a recipient rat (30). Plasma glucose returned to normal (125 ± 29 mg/dl) in diabetic rats receiving ICT.

**Induction of Diabetes**—Diabetes was induced in Sprague-Dawley rats by single intraperitoneal STZ injection (60 mg/kg) as described (4). After 4 weeks of diabetes, islet cell transplantation (ICT) was performed by Jennifer Beck in Dr. Gordon Weir’s laboratory at the Joslin Diabetes Center. Briefly, islet cells (3000) were isolated from healthy donor rats and transplanted under the left kidney capsule of a recipient rat (30). Plasma glucose returned to normal (125 ± 29 mg/dl) in diabetic rats receiving ICT.

**Protein Extraction and Fractionation**—Proteins were extracted from tissues and cells according to previously established methods (29). Briefly, tissues were crushed into frozen powder and homogenized in a pre-chilled buffer containing 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 1 mM phenylmethylsulfonfluoride, 1 mM diithiothreitol, 0.33 mM sucrose, 25 μg/ml leupeptin with a Polytron and further with a Dounce homogenizer. Tissue lysates were spun at 100,000 × g for 10 min, and the supernatant was further centrifuged at 100,000 × g for an additional 30 min at 4 °C. The resulting supernatant was retained as the cytosolic fraction, and the pellets were re-suspended in 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 1 mM phenylmethylsulfonfluoride, 1 mM diithiothreitol, 25 μg/ml leupeptin, and 1% Triton X-100 to extract membranous fraction. After rotating for 45 min at 4 °C, soluble membrane fractions were obtained by ultracentrifugation at
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Sprague-Dawley rats at 4 weeks of age were induced to have type 1 diabetes by STZ injection. Four weeks after the onset of hyperglycemia, diabetic rats were divided into three groups including no treatment (DM) or treated by ICT or oral application of 1 mg/ml captopril in drinking water (ACEI). These rats were followed for an additional 4 weeks. Animals were then sacrificed, and total RNA was isolated from their ventricles. mRNA expression of CTGF was assessed by quantitative reverse transcription-PCR and normalized to the expression of non-diabetic control rats (CTL). Results were expressed as the mean ± S.D.

Western Blotting Analysis—Fifty μg of denatured proteins were electrophoresed on pre-cast 10% Tris-glycine gel (Invitrogen) for 2 h by 200 volts and then transferred to nitrocellulose membranes (Invitrogen). The membranes were then blocked with 5% BSA at 4 °C overnight and then hybridized with polyclonal antibodies against different isoforms of PKC including α, β1, β2, ε, and γ (Santa Cruz Biotechnology) for at least 1 h at room temperature. After three consecutive washings of 10 min using PBS-T buffer, the membranes were then washed three times with PBS for 10 min each, and a hybridization signal was visualized using an ECL kit (Amersham Biosciences).

Statistical Analysis—All results were expressed as mean ± S.D. Differences between groups were compared using one-way analysis of variance with a post-hoc test using Student-Newman-Keul analysis. Comparison of the mean values between two groups was performed using unpaired Student t test. The level of significance was set at p < 0.05.

RESULTS

Effect of ACEI on CTGF Gene Expression in the Myocardium of Diabetic Rats—The effect of ACEI inhibition on CTGF mRNA expression was assessed in myocardium from STZ-induced diabetic rats since we have previously reported that CTGF expression in the myocardium of diabetic rodents was increased (4). After 4 weeks of diabetes, subsets of diabetic rats were randomly assigned to an untreated group, ACEI (captopril 1 mg/ml in drinking water)-treated group, or ICT group for an additional 4 weeks. At the end of this study, a 2.8-fold increase in CTGF expression was observed in the myocardium from rats with 8 weeks duration of diabetes as compared with the control rats receiving injections of vehicle solutions (p < 0.01 versus CTL, n = 6, Fig. 1). ICT, which maintained euglycemic control in diabetic rats, reversed the increased CTGF mRNA expression (p < 0.05 versus DM; p > 0.05 versus CTL, n = 6, Fig. 1). Treatment with ACEI for 4 weeks significantly reduced cardiac CTGF expression to a level comparable with

100,000 x g for 30 min. Both the cytosolic and membranous fractions were further examined for protein expression of different isoforms of PKC and PKC kinase activity.

PKC Kinase Assay—PKC activity was measured in proteins isolated from both the membranous and cytosolic fractions of the myocardium as previously described (29, 31). Proteins were concentrated using a DEAE-Sephacel column (Amersham Biosciences) and then eluted with a buffer containing 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 25 μg/ml leupeptin, and 1% Triton X-100. PKC kinase activity was measured and defined as the transfer of 32P from [γ-32P]ATP into the Thr residue of a synthetic peptide (Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu), mimicking the substrate sequence specific to PKC stimulated by Cu2+, phosphatidylserine, and diacylglycerol (31).

FIG. 1. Evaluation of CTGF mRNA expression in the myocardium of control and diabetic rats treated with ICT and ACEI. Sprague-Dawley rats at 4 weeks of age were induced to have type 1 diabetes by STZ injection. Four weeks after the onset of hyperglycemia, diabetic rats were divided into three groups including no treatment (DM) or treated by ICT or oral application of 1 mg/ml captopril in drinking water (ACEI). These rats were followed for an additional 4 weeks. Animals were then sacrificed, and total RNA was isolated from their ventricles. mRNA expression of CTGF was assessed by quantitative reverse transcription-PCR and normalized to the expression of non-diabetic control rats (CTL). Results were expressed as the mean ± S.D.

Statistical Analysis—All results were expressed as mean ± S.D. Differences between groups were compared using one-way analysis of variance with a post-hoc test using Student-Newman-Keul analysis. Comparison of the mean values between two groups was performed using unpaired Student t test. The level of significance was set at p < 0.05.

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Regulation of CTGF Expression by AngII through a AT1 Receptor-dependent Pathway in Vivo—The direct effect of AngII on the induction of CTGF mRNA expression in the myocardium was studied in vivo in both mice and rats without diabetes. AngII, which was infused into mice intrajugularly at 38.7 μg/ml leupeptin, 1% Triton X-100. PKC kinase activity was measured and defined as the transfer of 32P from [γ-32P]ATP into the Thr residue of a synthetic peptide (Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu), mimicking the substrate sequence specific to PKC stimulated by Cu2+, phosphatidylserine, and diacylglycerol (31).

FIG. 2. Characterization of CTGF mRNA expression by AngII stimulation in the ventricle in vivo. A, non-diabetic mice at 8 weeks of age were infused with AngII at different concentrations for 2 h. Cardiac CTGF mRNA expression was then assessed by Northern blot analysis (top panel) and was normalized to the expression of 36B4 (lower panel). CTGF mRNA level was expressed as a -fold change relative to that of the untreated mouse hearts. Infusion of AngII at 25 ng/kg·min−1 significantly increased CTGF mRNA expression compared with controls received saline infusion (n = 4, p < 0.05). B, infusion of AngII into non-diabetic Sprague-Dawley rats through intrajugular veins increased the mRNA expression of CTGF (n = 4, p < 0.001). Co-infusion of candesartan prevented the induction of CTGF expression. Results are presented as mean ± S.D.

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infusion of AngII to rats at a rate of 100 ng·kg⁻¹·min⁻¹ for 2 h also increased the expression of CTGF by ~15-fold (p < 0.001 versus vehicle-infused control, n = 4, Fig. 2B). Cell membrane receptors mediated the AngII effect on CTGF expression were examined further using candesartan, an AT1 receptor-selective inhibitor. Co-infusion of candesartan at a rate of 25 mg·kg⁻¹·min⁻¹ abolished AngII-induced CTGF gene expression (Fig. 2B), whereas infusion of candesartan alone did not alter basal CTGF expression in the myocardium (p > 0.05 versus saline-infused controls, Fig. 2B).

Effect of AngII on CTGF Expression in Cultured Cardiomyocytes—To determine whether AngII can directly regulate CTGF expression independent of its effect on blood pressure regulation, we characterized the AngII effect on cultured cardiomyocytes. Neonatal rat cardiomyocytes (NRCM) were isolated and stimulated with AngII (100 nM) after serum starvation. As shown in Fig. 3, CTGF mRNA expression increased by 2-fold (p < 0.05, n = 4) after AngII stimulation. The induction of ctgf gene expression was prevented by preincubating cells with 1 µM candesartan (p < 0.05 versus AngII, n = 4, Fig. 3), similar to that observed in vivo. Candesartan alone did not affect basal CTGF mRNA expression in cardiomyocytes (Fig. 3).

Effect of PKC Activation on AngII-induced CTGF Expression in Cardiomyocytes—Because we have previously reported that targeted overexpression of PKCβ2 in the myocardium induced CTGF expression and severe fibrosis (4, 29), we examined the effects of PKC and its isoforms activation on CTGF expression in this study. General activation of PKC by using 100 nM PMA, an activator of conventional and novel PKC isoforms, for 3 h suppressed CTGF expression in cardiomyocytes by 60% (p < 0.05, n = 3, Fig. 4A). In contrast, the addition of a general PKC inhibitor, GF109203X (GFX, 5 µM) increased expression of CTGF by 1.6-fold (Fig. 4B). Surprisingly, the addition of the GFX completely inhibited AngII-induced CTGF expression (Fig. 4B).

Effect of PKC Isoforms on AngII-induced Expression of CTGF in Cardiomyocytes—To assess the involvement of PKC isoforms in the regulation of CTGF expression by AngII, cultured NRCM were infected with adenoviral vectors containing different dominant negative PKC isoforms α, β2, δ, ε, and ζ. In NRCM infected with GFP control virus, AngII induced a 2-fold increase (n = 6, p < 0.05) in CTGF expression (Fig. 4C), similar to that in non-infected control cells. However, CTGF mRNA expression was suppressed by 78% in dnPKCα-infected cells incubated with AngII as compared with those infected cells without AngII (p < 0.05, n = 3). Similarly, expression of dnPKCζ isoforms also lowered CTGF mRNA expression by 86% in the presence of AngII as compared with infected cells without AngII (p < 0.05, n = 3). Inhibition of PKCζ isoform by infecting cardiomyocytes with dnPKCζ blocked AngII-induced CTGF mRNA expression to a level similar to those infected cells without AngII. Although CTGF mRNA expression has been previously reported to be increased in the myocardium of cardiac PKCβ2 transgenic mice (4), expression of dnPKCβ2 did not reduce CTGF expression induced by AngII as compared with Ad-GFP-infected control cells stimulated with AngII. Interestingly, overexpression of dnPKCδ alone in cardiomyocytes increased AngII-induced CTGF mRNA expression by ~10-fold, which was ~5-fold higher than AngII-induced CTGF expression in NRCM infected with adenoviral vector containing GFP (n = 3, p < 0.05 versus GFP-AngII).

Effect of Overexpression of PKC Isoforms on CTGF Expression in Cardiomyocytes—To probe the role of PKC isoform action on CTGF expression in cardiomyocytes, we also overexpressed wild type PKC (wtPKC) isoforms α, β2, and δ in NRCM using adenoviral vectors and studied CTGF mRNA expression. Northern blotting analysis showed that overexpression of...
PKCα increased CTGF expression by 1.9-fold (Fig. 5A). However, overexpression of PKCβ2 did not alter CTGF mRNA expression as compared with un-infected control cells or cells infected with Ad-GFP (Fig. 5B). Interestingly, although overexpression of wtPKCδ did not alter basal CTGF mRNA expression in cardiomyocytes (Fig. 5C), it suppressed AngII-induced CTGF mRNA expression (p > 0.05, n = 3, Fig. 5C). Basal CTGF expression in cardiomyocytes was not changed by the infection of dnPKCδ (Fig. 5C). Contrary to that of wtPKCδ, the addition of AngII dramatically increased CTGF expression by ~4-fold in cardiomyocytes overexpressing dnPKCδ (Fig. 5C), detected by Northern blotting analysis.

**Effects of in Vivo PKCδ Overexpression in Cardiomyocyte on CTGF Expression**—To evaluate the inhibitory effects of PKCδ activation on AngII-induced CTGF expression in vivo, we generated mice with targeted overexpression of PKCδ in cardiomyocytes using rat myosin heavy chain promoter (Fig. 6A). Successful gene transfer was confirmed by Southern blotting analysis of tail genomic DNA using a PKCδ-specific probe (Fig. 6B). Approximately 9 copies of the transgene were present in this line. PKCδ protein expression as determined by Western blotting revealed a 2.5-fold induction compared with littermate controls, whereas the expression of PKCa, β1, β2, and ε isoforms was not changed (Fig. 6C). In addition, a 1.4-fold increase in membranous PKC activity was observed in ventricular tissues (Fig. 6D). This corresponds to a 14-fold increase of membranous PKCδ protein expression from δTg mice (Fig. 6E). Membranous translocation was not changed for PKC isoforms α, β1, β2, ε, and ζ in the myocardium of the δTg mice (Fig. 6E). Heterozygous mice bearing the PKCδ transgene were viable and reproduced normally. Heart/body weight ratios did not differ between the δTg and the wt controls mice at 8 weeks of age (Table I). H&E staining revealed no evidence of necrosis, inflammation, fibrosis, or calcification in δTg mice, although mild cardiomyocyte hypertrophy was apparent (Fig. 7A and B). Mason’s trichrome staining revealed no difference in collagen content between δTg and wt mice (Fig. 7, C and D). No differences in myocardial mRNA expression for CTGF, TGFβ1 (Fig. 7E), and collagen IV (data not shown) were observed between 8-week-old wt and δTg mice.

**Effects of AngII on CTGF mRNA Expression in the Myocardium from δTg Mice**—Both wt and δTg mice were subjected to intrajugular Ang II infusion (25 ng/kg−min−1), and CTGF mRNA expression was determined by quantitative real time PCR. AngII infusion increased CTGF expression by 2.5-fold in the myocardium of wt mice (p < 0.05, n = 4, Fig. 8). In contrast, AngII increased CTGF expression by only 1.3-fold in ventricles from δTg mice (Fig. 8), which was not significantly different from δTg mice receiving saline infusion (Fig. 8).

**DISCUSSION**

This study has demonstrated that AngII and PKC are potent regulators of CTGF expression in the myocardium and cardiomyocytes both in physiological and diabetic conditions. The effect of AngII is mediated through the activation of AT1R, since candesartan, a selective AT1R blocker, inhibited CTGF induction by AngII both in vivo and in vitro. Previously, Finckenberg et al. (24) has reported that hypertensive transgenic rats carrying the renin-angiotensinogen transgene have elevated CTGF expression in the myocardium, presumably via a calcineurin-mediated pathway. However, the above study was not able to distinguish whether this is due to AngII action through hypertension or a direct action on cardiomyocytes. Our data provide direct evidence that AngII can increase CTGF mRNA expression in vitro and in vivo, independent of its action on blood pressure regulation. Furthermore, we have shown that the AngII effect on CTGF expression can be derived from cardiomyocytes in addition to that previously observed in myofibroblasts (32, 33). Infusion of AngII in vivo induced an ~15-fold increase of CTGF mRNA expression in the ventricular tissues. This is much higher than that achieved in cultured cardiomyocytes. This could be due to the fact that increased blood pressure by AngII infusion might have an additive effect on CTGF expression. Furthermore, ventricular tissue consists of multiple cell types including cardiomyocytes, fibroblast, and endothelial cells. Some of these cell types may respond with higher CTGF expression to AngII than pure cardiomyocytes in culture.

In cardiomyocytes, AngII has been previously shown to activate intracellular signaling pathways including the phospholipase C-PKC cascade through AT1R (34). Although PKC has been shown to be activated by AngII and contributes to multiple biological consequences (21, 35), the biological effects of individual PKC isoforms has not been established. Our results have demonstrated that activation of PKC, depending on the isoforms involved, could either increase or suppress CTGF expression in response to AngII. Previously, Fan et al. (25) have shown that CTGF expression is inhibited in fibroblasts by PMA, a potent general PKC activator of conventional and novel PKC isoforms, suggesting that PKC activation might inhibit...
PKC activity in the cytosolic and membranous fraction of the ventricular tissues from wt and ΔTg mice. The expression of the PKCδ in the Western blot (lower panel) was quantitated and expressed in the bar graph (top panel). D, PKC activity in the cytosolic and membranous fraction of the ventricular tissues from wt and ΔTg mice. E, protein expression of PKCα, -β1, -β2, and -ε in the ventricular tissue of wt and ΔTg mice. The expression of the PKCδ in the Western blot (lower panel) was quantitated and expressed in the bar graph (top panel).

TABLE I
Cardiac characterization of wt control and ΔTg mice

|                  | wt (n = 10) | ΔTg (n = 12) | p     |
|------------------|-------------|--------------|-------|
| Heart weight (mg)| 122.1 ± 18.9| 110.3 ± 16.3| 0.14  |
| Body weight (g)  | 25.7 ± 2.9  | 25.0 ± 1.5   | 0.5   |
| Ratio            | 4.9 ± 0.6   | 4.7 ± 0.3    | 0.64  |

CTGF expression. However, this observation did not provide information regarding the specific action of individual PKC isoforms in cardiomyocytes. Our results also showed that the addition of PMA inhibited CTGF expression, whereas GFX, a general PKC inhibitor, increased the expression of CTGF. Paradoxically, GFX inhibited the effects of AngII in cardiomyocytes, suggesting that the activation of various PKC isoforms may have different effects on CTGF expression. We have further clarified these paradoxical findings by showing that inhibition of PKCα, -β1, and -ε PKC isoforms decreased AngII-induced CTGF expression, whereas inhibition of PKCδ resulted in a robust induction of CTGF expression after AngII treatment. Because GFX did not inhibit PKCε activation, these results suggested that PKCα and -ε could be the main isoforms involved in AngII-induced CTGF expression.

At basal state, the expression of PKCα isoform increased CTGF expression, yet PKCβ2 and -δ isoforms had no effect. In diabetes, we have previously reported that CTGF expression is increased in the myocardium of rodents with STZ-induced diabetes (4). Because PKCβ isoforms are preferentially activated in the myocardium in diabetes (36), we have initially hypothesized that this activation could be responsible for the induction of CTGF expression in diabetic states. Furthermore, the overexpression of PKCβ2 isoform selectively in the myocardium of transgenic mice increased cardiac CTGF expression and induced severe fibrosis (4). However, inhibition of PKCβ2 activation directly using ruboxistaurin, a PKCβ isoform-selective inhibitor, did not decrease diabetes-induced CTGF expression in rats (4), suggesting that PKCβ2 activation may not be responsible for the direct induction of CTGF expression in diabetes. In this study, we have clarified this issue by showing that overexpression of either wild type or dominant negative PKCβ2 failed to induce CTGF expression in cultured cardiomyocytes and, thus, concluded that PKCβ2 activation did not directly induce gene expression for CTGF. The increased CTGF expression observed in cardiac PKCβ2 transgenic mice is, therefore, likely the result of myocardial injury induced by PKCβ2 activation through a mechanism yet to be determined. Thus, overexpression of PKCβ2 apparently causes myocardial inflammation and cardiomyocyte death in cardiac PKCβ2 transgenic mice, leading to CTGF overexpression and fibrosis (4, 29). Consistent with this finding, induction of CTGF expression has previously been shown to be induced in multiple tissues that have been injured (37). In addition, the increase in CTGF expression appears to be related to the enhanced action of AngII, potentially through the activation of the PKCα and -ε isoforms.

The surprising finding that the inhibition of PKCδ resulted in a significant increase in AngII-induced CTGF expression suggests a potential explanation for the ability of PMA to inhibit CTGF expression since PKCδ can be activated by PMA. This finding is unusual since activation of PKCδ generally has inhibitory effects on cellular proliferation and gene expression...
PKC isoform activation has been reported to affect the integrity of cytoskeletal structure in the myocardium (39). Inhibition of PKCδ translocation to the membranous fraction in the heart, a putative marker of its activation, has been shown to minimize ischemia-reperfusion-induced damage (40), possibly through the inhibition of cardiomyocyte death (41). Our findings have provided the first mechanistic evidence that PKCδ activation is likely to induce hypertrophy since PKCδ null mice has severe restenosis after vein graft surgery, suggesting that inhibiting PKCδ activation might be protective against fibrosis through the inhibition of CTGF expression. Leitges et al. (38) have recently reported that PKCδ null mice have severe restenosis after vein graft surgery, suggesting that inhibiting PKCδ activation is in favor of arterial smooth muscle cell proliferation and extracellular matrix production. Because AngII activation has also been suggested to participate in the pathogenesis of atherosclerosis with extensive fibrosis in its advanced stage, it is possible that PKC activation, especially PKCδ isoform, may also regulate AngII-induced CTGF expression in the arterial system. This is also supported by Ruperez et al. (16), who showed that AngII can stimulate CTGF expression in aortic tissues (16). Our results have demonstrated the inhibitory role of PKCδ activation in AngII-induced CTGF expression in vivo by studying the myocardium of transgenic mice overexpressing PKCδ selectively in cardiomyocytes. These δTg mice developed only mild cardiomyocyte hypertrophy without overt cardiac hypertrophy. Gene expression for TGFβ1, CTGF, and collagen IV in the myocardium were similar in both wt mice and δTg mice. The lack of clear cardiomyocyte loss or pathologies are surprising since it has been previously suggested that activation of the PKCδ isoform in cardiomyocytes could result in cell death and amplification of ischemia-reperfusion-induced cardiomyocyte damage (42). It is possible that overexpression of PKCδ alone is not sufficient to induce these changes, and additional insults such as ischemia-reperfusion are required. A previous study by Chen et al. (42) has shown that transgenic mice overexpressing ψ6RACK in the myocardium develop cardiac hypertrophy, providing indirect evidence showing that PKCδ activation is likely to induce hypertrophy since ψ6RACK is a peptide that facilitate the translocation of PKCδ to the membranous fraction of the cell (42). Our study provided direct evidence showing that the overexpression of PKCδ alone does not induce significant cardiac hypertrophy or myocardial pathologies. However, the activation of PKCδ isoform can regulate the actions of AngII in the myocardium, such as on the induction of ctgf gene expression. Furthermore, the inhibition of other PKC isoforms such as α, ε, and ζ can suppress CTGF expression induced by AngII. Thus, the effect of PKC on CTGF expression depends on the isoforms that are being activated by cytokines and the expression level of those PKC isoforms. In summary, we have characterized PKC isoform-dependent action mediating the AngII effect on CTGF expression in cardiomyocytes and, therefore, provide potential molecular explanation on the increased gene expression of CTGF in pathological conditions such as in diabetic cardiomyopathy. Our study suggested that the activation of PKC could elicit distinctive or even opposite actions regardless of whether they belong to the same class. It is, therefore, of great importance to develop isoform specific inhibitors of PKC for clinical applications to avoid any adverse effects. Because inhibition of PKCδ might exacerbate AngII-induced CTGF expression and potentially fibrosis, caution should be taken in considering using general PKC inhibition as a strategy in the treatment of pathological conditions, especially in diabetes where activation of specific PKC isoforms is associated with the pathologies of microvascular and cardiovascular complications (35).

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