Adapter molecule DOC-2 is differentially expressed in pressure and volume overload hypertrophy and inhibits collagen synthesis in cardiac fibroblasts

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Kumbar DH, VanBergen A, Ocampo C, Muangmingsuk S, Griffin AJ, Gupta M. Adapter molecule DOC-2 is differentially expressed in pressure and volume overload hypertrophy and inhibits collagen synthesis in cardiac fibroblasts. J Appl Physiol 102: 2024–2032, 2007. First published January 25, 2007; doi:10.1152/japplphysiol.00924.2006.—DOC-2 (differentially expressed in ovarian carcinoma) is involved in Ras-, β-integrin-, PKC-, and transforming growth factor-β-mediated cell signaling. These pathways are implicated in the accumulation of extracellular matrix proteins during progression of hypertrophy to heart failure; however, the role of DOC-2 in cardiac pathophysiology has never been examined. This study was undertaken to 1) analyze DOC-2 expression in primary cultures of cardiac fibroblasts and cardiac myocytes and in the heart following different types of hemodynamic overloads and 2) examine its role in growth factor-mediated ERK activation and collagen production. Pressure overload and volume overload were induced for 10 wk in Sprague-Dawley rats by aortic constriction and by aortocaval shunt, respectively. ANG II (0.3 mg·kg⁻¹·day⁻¹) was infused for 2 wk. Results showed that, compared with myocytes, DOC-2 was found abundantly expressed in cardiac fibroblasts. Treatment of cardiac fibroblasts with ANG II and TPA resulted in increased expression of DOC-2. Overexpression of DOC-2 in cardiac fibroblasts led to inhibition of hypertrophy agonist-stimulated ERK activation and collagen expression. An inverse correlation between collagen and DOC-2 was observed in in vivo models of cardiac hypertrophy; in pressure overload and after ANG II infusion, increased collagen mRNA correlated with reduced DOC-2 levels, whereas in volume overload increased DOC-2 levels were accompanied by unchanged collagen mRNA. These data for the first time describe expression of DOC-2 in the heart and demonstrate its modulation by growth-promoting agents in cultured cardiac fibroblasts and in vivo models of heart hypertrophy. Results suggest a role of DOC-2 in cardiac remodeling involving collagen expression during chronic hemodynamic overload.

angiotensin II; extracellular signal-regulated kinase; type 1 collagen; cardiac remodeling

INCREASED HEMODYNAMIC OVERLOAD results in cardiac hypertrophy. Although this response is initially beneficial, its continued activation through local and systemic factors eventually contributes to contractile dysfunction via adverse cardiac remodeling, a process characterized by myocyte hypertrophy, inflammation, pathological accumulation of extracellular matrix (ECM), and necrosis. In this process, whereas myocytes participate by increasing cell size, fibroblasts proliferate, differentiate, and secrete ECM proteins such as fibrillar collagen and other ECM constituents (29).

Recent studies have demonstrated that a critical balance between matrix synthesis and degradation is essential for maintaining myocardial integrity. Disruption of this balance leads to cardiac remodeling and dysfunction (35). Excessive collagen deposition (mainly type I) and fibrosis are integral features of failing heart from chronic pressure overload (21, 5), whereas enhanced collagen degradation has been suggested as a prerequisite for the cardiac dilatation from chronic volume overload-induced heart failure (9). The mechanisms involved in the regulation of cardiac collagen content during these two different overloads are not yet fully understood.

Several studies have suggested that ECM protein production is regulated by neurohumoral factors and by locally produced growth factors, such as basic fibroblast growth factor, transforming growth factor-β (TGF-β), and ANG II, in a paracrine and autocrine manner (6, 8). These factors regulate fibroblast cell proliferation and differentiation by interacting with cell surface receptors involving specific signaling mechanisms inducing G-protein-coupled receptors (ANG II), receptor tyrosine kinases (PDGF, EGF receptor), nontyrosine receptor kinases (c-Src, FAK), TGF-β, ras, and PKC pathways. With significant degree of cross talk, these pathways elicit their biological response by activating downstream kinase cascade. For example, ras activation is accompanied by the induction of the Raf-1/MEK/ERK cascade in many cell types, including cardiac fibroblasts (36). Several adapter proteins control ras activation in which Grb-2 plays a central role (25). Grb-2 is recruited either directly by nontyrosine receptor kinases or indirectly by G-protein-coupled receptor agonist-linked tyrosine receptor kinases to the cell membrane (28) where it promotes the activation of ras via its interaction with SOS (Son of Sevenless) (10). The importance of Grb-2-SOS complex in growth factor-mediated ras activation has been demonstrated in many cell types, including cardiac myocytes. The constitutively active form of ras has been shown to produce hypertrophic changes, in terms of both increased cell size and induction of fetal gene program in cardiac myocytes (26, 31). More importantly, in vivo, Grb-2 function is required for pressure overload-induced cardiac fibrosis (39).

Besides forming complex with SOS, Grb-2 has been shown to interact with DOC-2 (differentially expressed in ovarian carcinoma) (38). DOC-2, also known as Dab2, is considered a tumor suppressor (22, 32). Through its interaction with many...
cellular proteins, DOC-2 is known to influence several signaling pathways involved in cell growth. Binding of the COOH-terminal domain of DOC-2 with Grb-2 has been shown to disrupt the Grb-2-SOS complex, resulting in inhibition of growth factor-mediated activation of Ras/Raf-1/MEK/ERK cascade (38, 40). The NH2-terminal domain of DOC-2 interacts with TGF-β-signaling intermediates, Smad2 and Smad3, and influences TGF-β-mediated cell signaling (16). DOC-2 inhibits cell cycle progression by regulating the activity of cyclin-dependent kinase (14). Studies have also suggested that DOC-2 inhibits PKC-mediated signal pathways involving ERK and AP1 activity (28) and attenuates Wnt signaling via stabilization of β-catenin degradation (17). More recently, DOC-2 has been shown to interact with β-integrin and to negatively regulate fibrinogen adhesion and cell signaling (18). Therefore, DOC-2 represents a potent signaling factor that modulates many exogenous stimulus-mediated signal pathways that are implicated in cardiac remodeling from hemodynamic overload. However, no reports are available describing its role in cardiac pathophysiology.

This study was undertaken to characterize expression of DOC-2 in the normal heart and after different types of hemodynamic overload and analyze its role in growth factor-mediated ERK activation and collagen production by cardiac fibroblasts. We report that DOC-2 is predominantly expressed in cardiac fibroblasts. In vivo, DOC-2 expression is altered in hemodynamic overload in a stimulus-specific manner and was found inversely related to collagen expression in pressure and volume overload hypertrophy. We further demonstrate that DOC-2 negatively regulates hypertrophy agonist-mediated ERK activation, leading to inhibition of collagen synthesis. These findings provide a distinction between pressure and volume overload hypertrophy and contribute to our understanding of DOC-2 function in cardiac remodeling involving collagen expression.

MATERIALS AND METHODS

Induction of hemodynamic overload. This investigation conforms to the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health (NIH publication no. 85-23, revised 1996) and was approved by the animal care committee at UIC. Adult Sprague-Dawley rats (330 ± 20 g) were anesthetized with pentobarbital sodium (35 mg/kg ip). Pressure overload was induced by constricting of abdominal aorta. Briefly, a midline abdominal incision was made, and the suprarenal portion of the abdominal aorta was constricted with a 2.5-mm diameter wire. Volume overload was induced by creating aortocaval shunt according to the method of Garcia and Diebold (12) with our modifications (23). Briefly, a superficial purse-string suture was applied to the adventitia of abdominal aorta (distal to renal arteries and proximal to iliac arteries). A connection between aorta and inferior vena cava (IVC) was created by inserting a 22-gauge angiocatheter into the purse-string suture and advancing it into the IVC. After successful creation of the shunt, the catheter was withdrawn, and purse-string sutures were placed to seal the puncture site. Communication between the aorta and IVC was verified by the presence of turbulent blood flow in the IVC. The abdominal incision was sutured, and rats were allowed to recover. After 10 wk of surgical procedure, rats belonging to both the volume and pressure overload groups were killed. ANG II infusion (0.3 μg/kg·h·day−1) was given via Alzet osmotic pumps (model 2001). For this, rats were anesthetized as above, and pumps were implanted subcutaneously in the posterior region of neck. These pumps were primed with either ANG II or normal saline 4 h before implantation. After 2 wk, rats were killed, and hearts were removed, quickly frozen in liquid nitrogen, and stored at −80°C for later use.

Cell culture, transfections, and treatments. Rat neonatal ventricular cardiac cultures were prepared from the hearts of 1- to 2-day-old Sprague-Dawley rats, as described previously (13). Ventricles were separated from atrial tissue and washed in digestion solution (in mM: 116 NaCl, 20 HEPES, 1 NaH2PO4, 5.5 glucose, 5.4 KCl, and 0.8 MgSO4·7H2O, with collagenase (95 U/ml) and pancreatin (0.6 mg/ml)). Cardiac cells were dissociated in fresh digestion buffer. These cells, which were a mixture of myocytes and nonmyocyte fibroblasts, were suspended in plating medium (DMEM and M-199 at a ratio of 4:1) and purified by plating onto 150-mm-diameter noncoated culture dishes. After a 1-h incubation, unattached cells were collected and grown in DMEM with high glucose, supplemented with 10% FBS. More than 95% of these cells were myocytes as determined by cell morphology and myosin staining. The attached cells were grown to confluency in DMEM containing 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mmol/l glutamine, and 10% FBS and were split (passage 1) as required. These cells were mainly composed of fibroblasts as confirmed by positive staining for vimentin and desmin and negative for antifactor VIII, excluding the presence of vascular muscle cells or endothelial cells in our culture preparation. To avoid possible phenotype variations between culture preparations, all experiments utilized fibroblasts of passage 1. The cells were plated at 1.5 × 106 cells/9.6 cm2; after 48 h of plating, cells were infected with adenovirus containing 3.7 kb of DOC-2 cDNA (a gift from Dr. Hsieh, University of Texas) at indicated multiplicity units of infection (MOI). Adenovirus encoding green fluorescent protein (GFP) served as control. After 3 days, the serum medium was replaced with serum-free medium for overnight, and cells were treated with either phenylephrine (PE; 50 or 500 μM), ANG II (50 or 250 nM), or TPA (1 or 10 μM) for indicated time intervals.

Northern analysis. For RNA extraction from cardiac tissues, frozen heart samples were homogenized in Trizol reagent (Invitrogen) and processed according to the manufacturer’s instructions. For cell cultures, cells were harvested in Trizol reagent and RNA was isolated. Total RNA (20 μg) was resolved on 2% formaldehyde agarose gel by electrophoresis and transferred to nitrocellulose membranes. Probes used were α(II) procollagen probe H65774 (American Type Culture Collection) and 3.7 kb of DOC-2 cDNA (a gift from Dr. Hsieh). Full-length cDNA probe encoding GAPDH was used for normalization of RNA loading. Probes were labeled by random priming using α-32PdCTP, and a minimum of 2 × 106 cpm/ml were used in hybridizing membranes. Signal was detected by autoradiography and quantitated by Scion software.

Preparation of protein lysates. For Western analysis, frozen heart tissue (100 mg) was homogenized in 1.5 ml of lysis buffer [20 mM HEPES (pH 7.4), 2 mM EGTA, 1 mM DTT, 1 mM sodium orthovanadate, 1% Triton X-100, 10% glycerol, 2 μM leupeptin, 1 mM PMSF, 45 μg/ml aprotinin, and 1 mM PMSF] at 8,000 rpm. The homogenate was centrifuged at 0°C at 14,000 g, the supernatant was collected, and protein concentration was determined by Bradford assay. Aliquots of tissue lysates were stored at −80°C. For cultured cells, medium was aspirated, and cells were washed with ice-cold PBS, lysed in RIPA buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 45 μg/ml aprotinin, and 1 μg/ml sodium orthovanadate), and centrifuged at 10,000 rpm at 4°C for 10 min. The supernatant was collected and stored at −85°C.

Western analysis. Proteins (30–100 μg) were resolved on 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane. Membranes were blocked with 10% milk and hybridized overnight at 4°C with anti-DOC-2 (1:10,000; a gift from Dr. Hsieh), anti-phospho-ERK1/2 (1:1,000; Cell Signaling), anti-ERK2 (1:1,000; Santa Cruz), anti-type 1 collagen (1:250; Southern Biotech) antibodies. Membranes were washed with 1× PBS-Tween 20 and hybridized with appropriate secondary antibody (1:2,000) in 5% milk. After samples were washed

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with 1× PBS-Tween 20, the signal was developed using chemiluminescence reagent (Santa Cruz) and detected by autoradiography.

**Statistical analysis.** Statistical analyses were performed by paired Student’s t-test.

**RESULTS**

Altered expression of DOC-2 in chronic hemodynamic overload. Cardiac hypertrophy is known to modulate expression of several cardiac genes. In our preliminary gene-chip microarray expression analysis of 10-wk volume overload rat hearts, we observed a twofold increase in DOC-2 expression compared with sham controls (7). DOC-2 is originally cloned as a differentially expressed gene in ovarian carcinoma with two transcripts of ~3.7 and 2.9 kb in size and spliced isoforms of predicted molecular masses of ~96 and 87 kDa in different tissues (1, 22, 32, 37). Its expression in the heart has never been examined.

To characterize the expression pattern of DOC-2 in rat heart and verify microarray findings, we performed Northern blotting using a 3.7-kb rat DOC-2 cDNA probe and Western blotting using monoclonal anti-DOC-2 antibody. As shown in Fig. 1A, three transcripts of 6.5, 3.7, and 2.9 kb were detected in both sham controls and volume overload hearts. Although 3.7- and 2.9-kb transcripts have been reported earlier in other tissues (32, 37), the 6.5-kb transcript was found unique to the cardiac tissues. Furthermore, compared with controls, we found increased levels of all three transcripts in volume overload hearts: 75% increase in 6.5 kb ($P < 0.05$), 2.2-fold increase in 3.7 kb ($P < 0.001$), and 2.4-fold increase in 2.9 kb ($P < 0.001$). The expression of GAPDH, which served as a negative control, was not changed in control and volume overload hearts. By Western analysis, we observed two spliced isoforms of ~96 and 87 kDa in the heart (Fig. 1B), implying that the 6-5 kb transcript observed in cardiac tissue is either not translatable or gets processed to 96- or 87-kDa isoforms. Future cloning of the 6.5-kb transcript will resolve this issue.

Comparison of 96- and 87-kDa isoforms in sham and volume overload hearts revealed that, in addition to increases in DOC-2 mRNA, a significant increase in the DOC-2 protein level was also observed in volume overload. The increase was much more in the p87 (3-fold) than in the p96 (80%) isoform.

To examine whether other types of hemodynamic overload influence DOC-2 expression, pressure overload was induced by constricting abdominal aorta for 10 wk. Similar to volume overload hearts, these hearts demonstrated increased heart weight-to-body weight ratios and increased expression of the
Doc-2 is mainly expressed in fibroblasts. These data suggest that, in rat heart, in relation to myocytes, Doc-2 is mainly expressed in fibroblasts.

Data presented in Fig. 1 show that Doc-2 expression is altered in chronic hemodynamic overload. We next examined whether agents that influence cardiac growth have an effect on Doc-2 expression in primary cultures of cardiac fibroblasts.

For this, under serum-free conditions, cardiac fibroblasts were treated with α-adrenergic receptor agonist PE, PKC agonist, TPA, and the profibrotic and hypertrophy-inducing agent ANG II. After 24 h, cell lysates were analyzed for Doc-2 expression by Western blotting. Results showed that Doc-2 expression is increased after treatment with 250 nM ANG II and 1.0 and 10 μM TPA but not with either dose of PE (Fig. 2B). Lack of PE effect on Doc-2 expression is not surprising because cardiac fibroblasts are known to be devoid of α-adrenergic receptors (27). We also observed that Doc-2 is phosphorylated in vivo because, when cell lysate was subjected to alkaline phosphatase treatment, a lower shift in the mobility of both p96 and p87 isoforms of Doc-2 was observed (Fig. 2B). These data collectively suggest that, in cardiac fibroblasts, Doc-2 is a phosphoprotein and that both TPA and ANG II promote Doc-2 expression.

**Inhibition of ERK activity after Doc-2 overexpression in cardiac fibroblasts.** TPA and ANG II are both known to activate Ras/Raf/MEK/ERK cascade (reviewed in Ref. 28). Because Doc-2 is known to interfere with Grb-2-SOS complex, which plays a central role in ras activation, we asked whether Doc-2 overexpression could influence ERK1/2 activation induced by ANG II and TPA. For this, cardiac fibroblasts were infected with adenovirus containing full-length Doc-2 cDNA or adenovirus containing GFP (control) for 72 h. This resulted in an approximately eightfold increase in the expression of p96 and a threefold increase in p87 isoform of Doc-2 (Fig. 3A). In relation to nontransfected cells, transfection of GFP adenovirus had no effect on Doc-2 expression.

![Fig. 2](http://jap.physiology.org/Downloadedfrom) **Fig. 2.** Effect of growth factors on Doc-2 in cardiac fibroblasts. A. *left:* cardiac fibroblasts and cardiac myocytes were purified from newborn rat hearts and grown in serum (+S) or serum-free (−S) medium before they were harvested. Cellular proteins (30 μg) were resolved by SDS-PAGE and transferred to membranes. Sequential immunoblotting was performed by hybridizing the membranes first with anti-Doc-2 antibody and then with anti-β-actin antibody. Arrows indicate molecular weights. A. *right:* quantification of Doc-2 isoforms in fibroblasts and myocytes. Compared with myocytes, both isoforms of Doc-2 were more abundant in cardiac fibroblasts (*P < 0.001). B: cardiac fibroblasts were grown in serum medium for 48 h (+S) and then serum-free medium (−S) overnight and treated for 24 h with phenylephrine (PE), ANG II (AII), or phorbol ester (TPA) at indicated concentrations. Cell lysates treated with TPA were also subjected to alkaline phosphatase treatment (phosphatase, 1 U for 15 min at room temperature, last lane). Cell lysates were analyzed by Western blot as above using anti-Doc-2 antibody followed by anti-β-actin antibody. Typical results of 3 independent experiments are shown. Data on additional experiments are shown in supplemental Fig. 2. (The online version of this article contains supplemental data.)
After 72 h of DOC-2 infection, cells were treated with either TPA (Fig. 3B) or ANG II (200 nM) (C) for the indicated durations. Membranes were sequentially probed with anti-phospho-ERK or total ERK2 antibodies. Experiments were repeated a minimum of 3 times, and typical results are shown. Data on additional experiments are shown in supplemental Fig. 3. (The online version of this article contains supplemental data.)

Reduced collagen expression in DOC-2-expressing cardiac fibroblasts. In Fig. 2, we observed significant expression of DOC-2 in cardiac fibroblasts. Because one of the functions of cardiac fibroblasts is to synthesize ECM proteins, we next examined whether DOC-2 influences hypertrophy agonist-mediated increases in collagen expression. For this, cells were first infected with either DOC-2 adenovirus or with control (GFP) adenovirus. After 48 h, cells were serum starved overnight and treated with ANG II for 24 h. Vehicle-treated cells served as controls. As shown in Fig. 4A, treatment of cardiac fibroblasts with ANG II significantly increased procollagen α1(I) mRNA, which was inhibited by infection of cardiac fibroblasts with DOC-2 adenovirus. GAPDH mRNA was not changed by DOC-2 overexpression. When we used an adenovirus construct encoding GFP as a control, procollagen α1(I) mRNA levels remained unaffected, documenting the specificity of DOC-2 on procollagen α1(I) mRNA. DOC-2 also inhibited basal levels of type 1 collagen protein in a dose-dependent manner, decreasing it at 25 MOI and almost completely repressing at 75 MOI, with no affect on β-actin expression (Fig. 4B). These data collectively suggest a repressive effect of DOC-2 on the basal level of collagen expression as well as after ANG II treatment.

Role of ERK in collagen expression. Because DOC-2 inhibited ERK activation and collagen expression, we next asked whether DOC-2 influences hypertrophy agonist-mediated increases in collagen expression. For this, cells were first infected with either DOC-2 adenovirus or with control (GFP) adenovirus. After 48 h, cells were serum starved overnight and treated with ANG II for 24 h. Vehicle-treated cells served as controls. As shown in Fig. 4A, treatment of cardiac fibroblasts with ANG II significantly increased procollagen α1(I) mRNA, which was inhibited by infection of cardiac fibroblasts with DOC-2 adenovirus. GAPDH mRNA was not changed by DOC-2 overexpression. When we used an adenovirus construct encoding GFP as a control, procollagen α1(I) mRNA levels remained unaffected, documenting the specificity of DOC-2 on procollagen α1(I) mRNA. DOC-2 also inhibited basal levels of type 1 collagen protein in a dose-dependent manner, decreasing it at 25 MOI and almost completely repressing at 75 MOI, with no affect on β-actin expression (Fig. 4B). These data collectively suggest a repressive effect of DOC-2 on the basal level of collagen expression as well as after ANG II treatment.
whether ERK inhibition by DOC-2 contributes to collagen repression. For this, we pretreated cardiac fibroblasts with a specific inhibitor of ERK (PD-98059) for 6 h before treatment with ANG II for 24 h. Similar to its effects on procollagen α(I) mRNA (Figs. 4A and 5A), ANG II treatment also induced type 1 collagen protein \((P < 0.001)\) in cardiac fibroblasts (Fig. 5B). Pretreatment with PD-98059 inhibited ANG II-induced collagen expression in a dose-dependent manner for both procollagen α(I) mRNA (Fig. 5A) and type 1 collagen protein (Fig. 5B; \(P < 0.001)\) without affecting GAPDH mRNA or β-actin protein levels. We also observed that PD-98059 inhibited basal levels of collagen expression \((P < 0.05;\) Fig. 5B). Together, these data indicate that one of the mechanisms by which DOC-2 represses collagen expression by cardiac fibroblasts involves ERK inhibition.

**Inverse relationship between DOC-2 levels and collagen expression in chronic hemodynamic overload.** In chronic pressure overload, an increase in type 1 collagen has been observed, whereas collagen degradation is reported during chronic volume overload \((5, 9)\). Consistent with these reports, we also observed increased procollagen α(I) mRNA after chronic pressure overload but no changes during volume overload in rat heart (Fig. 6A). In Fig. 4, we observed that overexpression of DOC-2 inhibits ANG II-induced procollagen α(I) mRNA and type 1 collagen protein; however, paradoxically, in Fig. 2 we observed that treatment with high doses of ANG II induces DOC-2 expression in cultured cardiac fibroblasts. One possible explanation could be that DOC-2 upregulation by ANG II in cultured fibroblasts may be a transient response of a negative feedback mechanism to inhibit collagen synthesis. To clarify this, we utilized a chronic model of ANG II infusion in rats and examined DOC-2 expression. Data presented in Fig. 6B show that, in contrast to acute effects of ANG II in cultured cardiac fibroblasts, chronic infusion with ANG II in vivo repressed DOC-2 expression. We next asked whether any correlation exists between endogenous levels of DOC-2 and type 1 collagen in pressure overload and ANG II infusion. For this, we performed a scatterplot analysis of procollagen α(I) mRNA content in relation to DOC-2 levels of individual hearts. As shown in Fig. 6, hearts containing lower levels of DOC-2 always demonstrated high collagen expression and those containing higher DOC-2 showed low collagen mRNA, documenting an inverse correlation between DOC-2 levels and collagen expression with \(R^2 = 0.79\) for pressure overload and \(R^2 = 0.93\) for ANG II-treated hearts (Fig. 6C).

**DISCUSSION**

DOC-2 represents a potent adapter protein that modulates many pathways involved in cell growth and cell differentiation, including Ras-, PKC-, TGF-β-, and β-integrin-mediated cell signaling. Although each of these pathways is intricately involved in cardiac hypertrophy, the present study is the first to characterize the expression of DOC-2 in the heart and demonstrates a new role for DOC-2 in collagen expression in cardiac fibroblasts. Our results show that DOC-2 is predominantly expressed in cardiac fibroblasts, and its expression and phosphorylation are induced by growth-promoting agents (ANG II and TPA). Furthermore, in vivo models of cardiac hypertrophy, the expression of DOC-2 is differentially regulated by distinct stimuli; it is reduced during pressure overload and after chronic infusion with ANG II, whereas it is increased during chronic volume overload. In addition, endogenous levels of...
DOC-2 were found to be inversely related to procollagen \( \alpha_1(I) \) mRNA in different hemodynamic overloads. The cause-and-effect relationship of this association was demonstrated by in vitro experiments that utilized cultured cardiac fibroblasts, where overexpression of DOC-2 resulted in inhibition of hypertrophy agonist-mediated ERK phosphorylation and repression of collagen synthesis, as reflected by drastic reductions in procollagen \( \alpha_1(I) \) mRNA and type 1 collagen protein.

The major collagen types found in the heart are type 1 (>80%), type III (10–15%), and type V (<5%). The collagen network not only participates in maintaining ventricular size and shape but has also been documented to influence diastolic and systolic functions of the heart (3, 4). Specific changes in both type 1 and type III collagens have been reported in response to the persistent increase in ventricular pressure or volume overload. Both in animal model and in patients with aortic stenosis, chronic pressure overload has consistently been shown to result in increased expression of type 1 collagen (11, 15), in line with the findings reported in our study. Chronic volume overload on the other hand shows a time-related change in collagen expression, initially increasing during the first week of volume overload followed by return to basal levels by 6 wk of volume overload (9). Our data obtained with hearts subjected to a further prolonged period of volume overload (10 wk) showed that procollagen \( \alpha_1(I) \) mRNA levels remained unchanged in volume overload. The changes in collagen expression during volume overload have earlier been shown to involve collagenase activation (matrix metalloproteinase-1) (9) and reduced expression of inhibitor of matrix metalloproteinase-1 (tissue inhibitor of metalloproteinase-1) (34). A decreased level of collagen have been found to be necessary for reduced ventricular stiffness, increased ventricular compliance, and ventricular dimensions seen during heart failure from volume overload (9, 20).

We have shown here that DOC-2 is differentially expressed in pressure and volume overload hypertrophy; chronic volume overload...
overload involves increased DOC-2 expression, whereas pressure overload and ANG II result in reduced expression of DOC-2. We postulate that reduced DOC-2 levels in pressure overload and after ANG II infusion contribute to increased collagen expression, and increased DOC-2 expression in volume overload is likely to maintain collagen levels within normal range. Results obtained from cultures of cardiac fibroblasts support this hypothesis in the following ways. 1) In cardiac hypertrophy, fibroblasts contribute to cardiac remodeling by proliferating and secreting ECM proteins; our results showed that DOC-2 is abundantly expressed in cardiac fibroblasts where its expression is induced by hypertrophy agonists. 2) Overexpression of DOC-2 in cardiac fibroblasts repressed basal and ANG II-stimulated increases in type I collagen protein without affecting β-actin. This must be a direct effect and not mediated by collagen degradation, as it was also observed at the procollagen α1(I) mRNA level. Thus DOC-2 appears to be an inhibitor of collagen synthesis, and accordingly its decreased expression observed in our pressure overload and after ANG II infusion could contribute to increased collagen expression seen in these models of hemodynamic overload.

Both TPA and ANG II are known to induce Ras activation mediated by Grb-2-SOS complex; the former involves RTK, whereas the latter involves G-protein-coupled receptor signaling leading to ERK activation (2, 28). In our study, overexpression of DOC-2 in cardiac fibroblasts significantly repressed TPA and ANG II-induced ERK phosphorylation. The possible mechanism of this effect could be derived from the ability of DOC-2 to sequester Grb-2 away from recruiting downstream signaling effectors such as SOS (38, 40) and that may in turn inhibit the activation of the Ras/Raf-1/MEK/ERK signaling cascade. Because an earlier study reported activation of ERK in pressure overload hypertrophy (30), data presented in our study will argue that reduction in endogenous levels of DOC-2 observed in pressure overload may allow more Grb-2 to bind to SOS, leading to stimulation of the Ras/Raf/MEK kinase cascade and resulting in ERK activation.

In our study, data obtained with the specific inhibitor of ERK on collagen expression suggested that ERK plays an important role in collagen expression. Treatment of fibroblasts with PD-98059 not only repressed ANG II-stimulated collagen expression but also inhibited basal levels of type I collagen protein. This finding is consistent with a recent report in which ERK activation was found to be important in cyclic mechanical load-induced increases in procollagen α1(I) mRNA in cultured cardiac fibroblasts (24). Because DOC-2 overexpression inhibited ERK activation, we believe that the ability of DOC-2 to inhibit ERK activation may also be contributing to collagen repression by DOC-2. Future experiments involving small interfering RNA-mediated DOC-2 knockout in cultured fibroblasts and cardiac specific overexpression and knockout of DOC-2 in transgenic mice will establish a role of DOC-2 in overload-induced cardiac remodeling. Data presented here lay out a foundation for conducting such studies. Previous studies have also suggested a role of DOC-2 in β-integrin-mediated and in TGF-β-mediated cell signaling (16, 18). Because both of these pathways are intricately involved in cardiac remodeling involving collagen expression (reviewed in Ref. 19), it is likely that collagen repression by DOC-2 reported here may also utilize these additional pathways and warrant further investigation.

Thus our study revealed that hypertrophy, induced by pressure overload and ANG II infusion, differs from volume overload hypertrophy, in the expression pattern of DOC-2 and that the DOC-2 levels in these in vivo models of hypertrophy are inversely related to collagen expression. The study also provided a direct role of DOC-2 in collagen repression in cardiac fibroblasts that involved ERK inhibition. Collectively, these data suggest that DOC-2 may have a regulatory role in cardiac remodeling during chronic cardiac overload involving collagen expression.

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