Inhibition of cardiac fibroblast proliferation and myofibroblast differentiation by resveratrol

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Olson, Erik R., Jennifer E. Naugle, Xiaojin Zhang, Joshua A. Bomser, and J. Gary Meszaros. Inhibition of cardiac fibroblast proliferation and myofibroblast differentiation by resveratrol. Am J Physiol Heart Circ Physiol 288: H1131–H1138, 2005.—Cardiac fibroblasts (CFs) regulate myocardial remodeling by proliferating, differentiating, and secreting extracellular matrix proteins. Prolonged activation of CFs leads to cardiac fibrosis and reduced myocardial contractile function. Resveratrol (RES) exhibits a number of cardioprotective properties; however, the possibility that this compound affects CF function has not been considered. The current study tests whether RES directly influences the growth and proliferation of CFs and differentiation to the hypersecretory myofibroblast phenotype. Pretreatment of CFs with RES (5–25 μM) inhibited basal and ANG II-induced extracellular signal-regulated kinase (ERK) 1/2 and ERK kinase activation. This inhibition by RES reduced basal proliferation and blocked ANG II-induced growth and proliferation of CFs in a concentration-dependent manner, as measured by [3H]thymidine and [3H]leucine incorporation, respectively. RES pretreatment attenuated ERK phosphorylation when CFs were stimulated with 0.2 nM epidermal growth factor (EGF), a concentration at which EGF-induced ERK activation over basal was similar to the phosphorylation induced by 100 nM ANG II. Akt phosphorylation in ERK was unaffected by treatment with either 100 nM ANG II or 25 μM RES. Pretreatment of CFs with RES also reduced both ANG II- and transforming growth factor-β-induced CF differentiation to the myofibroblast phenotype, indicated by a reduction in α-smooth muscle actin expression and stress fiber organization in CFs. This study identifies RES as an anti-fibrotic agent in the myocardium by limiting CF proliferation and differentiation, two critical steps in the pathogenesis of cardiac fibrosis.

Cardiac fibroblasts (CFs) are the predominant secretory cells of extracellular matrix (ECM) proteins in the heart and key mediators of normal and pathological cardiac remodeling. Prolonged activation of CFs, defined by increased proliferation and subsequent ECM secretion, is a direct consequence of hypertension and heart failure and leads to cardiac fibrosis, a condition characterized by excess ECM deposition and a stiff myocardium. The impaired compliance of the fibrotic heart ultimately compromises contractile performance (3). In addition to proliferation, CFs differentiate into myofibroblasts, a cell type with an increased capacity to secrete ECM proteins (30). The proliferation and differentiation of new ECM-producing cells enhance the deposition of ECM proteins, and thus, limiting these parameters represents a potential therapeutic avenue to reduce pathological myocardial fibrosis.

ANG II levels are elevated during hypertension and heart failure. This peptide hormone has direct and potent effects on CF function, including activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) cascade and cellular proliferation via direct stimulation of the ANG II type 1 (AT1) receptor (1, 2, 6, 23, 24, 28). We have demonstrated that proliferation of CFs is dependent on ERK kinase (MEK) and ERK1/2 activation via pharmacological inhibition of MEK (25). In addition to activating ERK, ANG II regulates collagen type I expression, at least in part, by inducing CFs to produce and secrete transforming growth factor-β (TGF-β; see Refs. 11 and 16). TGF-β is a potent paracrine mediator of differentiation and contributes to the development of cardiac fibrosis by increasing the number of myofibroblasts in the heart (22, 26).

Resveratrol (RES; trans-3,4′,5-trihydroxystilbene), a phytoalexin found in the skins of grapes, has been identified as a key biologically active ingredient in red wine. RES has been credited with mediating a number of beneficial effects in the cardiovascular system that accompany moderate red wine consumption. In rat aortic smooth muscle cells, RES suppresses ANG II-induced Akt (protein kinase B) and, to a lesser extent, ERK1/2 activation, both of which are required for ANG II-induced hypertrophy (13). RES reduces ERK and JNK phosphorylation in coronary artery smooth muscle cells (10) and induces vasorelaxant responses by activating membrane-bound guanylyl cyclase (9) and nitric oxide release (21). Together, these findings depict RES as a potential therapeutic agent in a multitude of cardiovascular diseases.

The studies focusing on the cardiovascular effects of RES have primarily examined the coronary artery and vascular smooth muscle cells (VSMCs), whereas the effects of RES on CFs have not yet been examined. Our initial hypothesis was that RES would inhibit both ANG II-induced proliferation and differentiation of CFs. The proliferation of CFs via ANG II and the ERK1/2 cascade (24) plays a key role in the development of cardiac fibrosis, and inhibiting prolonged CF activity represents a viable method for preventing the long-term loss of cardiac function that accompanies this condition. We report that RES does inhibit ANG II-induced proliferation and growth of CFs, an effect Mechanically controlled through direct blockade of the ERK1/2 cascade. The study also demonstrated that RES pretreatment inhibited ANG II-induced CF differentiation to the hypersecretory myofibroblast phenotype. In ad-

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dition, we have determined that RES attenuated basal CF proliferation, EGF-induced ERK1/2 activation, and TGF-β-induced myofibroblast differentiation. These findings suggest that RES has anti-fibrotic properties in the myocardium by limiting CF proliferation and myofibroblast differentiation.

MATERIALS AND METHODS

Materials. DMEM, penicillin/streptomycin, fungizone, and FBS were all purchased from Invitrogen/GIBCO (Grand Island, NY). Hybod nitrocellulose membrane and [3H]leucine were purchased from Amersham Biosciences (Piscataway, NJ). [3H]thymidin was from ICN Biomedicals (Irvine, CA). Anti-phospho-Akt, anti-phospho-p70S6K, anti-phospho-MEK, and anti-MEK antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-phospho-ERK1/2 and anti-ERK1/2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p70S6K antibodies were from Calbiochem (La Jolla, CA). Anti-α-smooth muscle actin (α-SMA) antibody was from Sigma-Aldrich (St. Louis, MO). Rat-adsorbed horseradish peroxidase-conjugated anti-mouse secondary antibody was obtained from Serotec (Raleigh, NC). Alexa Fluor 488 goat anti-mouse secondary antibody was purchased from Molecular Probes (Eugene, OR). All other reagents and chemicals were reagent grade and obtained from Fisher Scientific (Pittsburgh, PA).

Cell culture. CFs were prepared from the ventricles of one to two adult male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN). The ventricles were minced, pooled, and digested in a collagenase-pancreatin solution, as previously described (19, 20). Cardiac myocytes were pelleted by centrifugation at 200 rpm for 2 min, and supernatant containing the fibroblasts was removed. The CFs were then pelleted at 1,000 rpm for 10 min and resuspended in DMEM supplemented with penicillin, streptomycin, and fungizone, and 10% FBS. After a 30-min period of attachment to tissue culture plates, cells that were weakly attached or unattached (myocytes, endothelial cells, smooth muscle cells, and red blood cells) were rinsed free and discarded. After 2–3 days, confluent cultures were passaged by trypsinization and replated at a split ratio of 1:3. Passage 2 or 3 cells, smooth muscle cells, and red blood cells) were rinsed free and obtained from all experiments. DMEM containing 10% FBS was washed out, and the cells were equilibrated in serum-free DMEM (SFM) before hormonal experiments. DMEM containing 10% FBS was washed out, and the cells were treated in triplicate for a period of 48 h. In the lucine incorporation assay, hormone additions were made in the presence of 1 μCi/ml [3H]leucine. In the thymidine incorporation assay, 0.5 μCi/ml [3H]thymidin was added to the media only during the final 4 h of the 48-h stimulation period. At the end of the treatments, the medium containing the label was removed, and the cells were washed two times with PBS, one time with 5% TCA, and two times with 95% ethanol. The cells were solubilized in 1 ml of 0.5 M sodium hydroxide for 30 min at room temperature. The suspension was then triturated several times and then placed in scintillation vials with 5 ml Ecolume scintillation fluid. Samples were counted on a Beckman LS 6500 liquid scintillation counter. Data are expressed as a percentage of vehicle-treated controls (set at 100%) after calculating the ratio of counts per minute per well of hormone-stimulated to vehicle-treated cells.

Immunocytochemistry/α-SMA staining. CFs were plated on 12-well glass slides in DMEM supplemented with 10% FBS and allowed to attach for 4 h. Cells were then washed with PBS and equilibrated in SFM containing 10% FBS. Cells were washed with PBS and fixed in 4% paraformaldehyde in PBS for 30 min. CFs were permeabilized with 1% Triton X-100/PBS for 30 min, blocked in 2% goat serum, and incubated with mouse anti-α-SMA primary antibody for 1 h. CFs were then washed and incubated with Alexa Fluor 488 goat anti-mouse secondary antibody. Excess antibody was washed five times, and cells were mounted in Vectashield mounting media containing DAPI, a nuclear stain (Vector Laboratories, Burlingame, CA).

RESULTS

RES attenuates basal and ANG II-induced ERK1/2 activity in CFs. RES inhibits the MAPK cascade in a number of cell types, and so we asked whether ERK1/2 was a target of RES in CFs. As shown in Fig. 1, RES inhibited basal phosphory-
lated MEK (P-MEK) levels (A, 34.3 ± 4.5, 70.5 ± 3.1, and 90.4 ± 1.7% reduction with 5, 10, and 25 µM RES, respectively) and P-ERK1/2 levels (B, 50.9 ± 1.5 and 71.0 ± 2.3% reduction with 10 and 25 µM RES, respectively) in a concentration-dependent manner. CFs were stimulated with 100 nM ANG II for 20 min in the presence and absence of 5–25 µM RES (preincubated 30 min before ANG II stimulation). As expected, ANG II increased P-MEK (Fig. 1A, 33.6 ± 11.3%) and P-ERK (Fig. 1B, 102.8 ± 4.2%) levels over controls. A reduction in ANG II-stimulated MEK and ERK activation by RES pretreatment was evident, with statistical significance being achieved at 25 µM RES for P-MEK (96.1 ± 0.5% reduction) and at 10 and 25 µM RES for P-ERK (26.5 ± 1.5 and 54.6 ± 0.9% reduction in P-ERK levels, respectively). Total ERK levels were unchanged in all conditions, indicating equal protein loading (Fig. 1C). Summary graphs displaying the effects of RES on MEK and ERK activation are shown in Fig. 1, D and E, respectively. We conclude that the inhibition of basal and hormone-stimulated MEK and ERK phosphorylation is a major mechanism by which RES affects mitogenic signaling in CFs.

RES inhibits growth and proliferation of isolated adult rat CFs. Our next set of experiments was designed to determine whether the blockade of MAPK activation by RES inhibited growth and proliferation of CFs. To achieve this end, we pretreated cultured CFs with 5, 10, and 25 µM RES in the presence and absence ANG II (100 nM), a known potent mitogen for CFs. Figure 2 demonstrates that 48 h of stimulation of CFs with ANG II increased [3H]leucine and [3H]thymidine incorporation by 59.8 ± 5.1 and 34.7 ± 5.6%, respectively (Fig. 2, A and B). RES significantly inhibited ANG II-induced [3H]leucine incorporation at 10 µM (13.9 ± 5.0% reduction) and 25 µM (37.7 ± 4.5% reduction, Fig. 2A), whereas 25 µM RES significantly lowered [3H]thymidine incorporation (49.4 ± 3.8% reduction, Fig. 2B). In addition, we observed reductions in basal, unstimulated [3H]thymidine incorporation in CFs treated with 10 µM (36.9 ± 8.6% reduction) and 25 µM (56.4 ± 12.9% reduction, Fig. 2B) RES, but reductions in basal [3H]leucine incorporation were not evident. We also determined that RES pretreatment did not alter CF morphology, cause cell detachment, or cause DNA laddering throughout the entire treatment period at any of the concentrations used (data not shown). The effects of RES on [3H]leucine and [3H]thymidine incorporation are therefore not the result of toxicity or cell death. Given our previous findings that ANG II-induced proliferation is completely inhibited by pharmacological inhibition of ERK1/2 signaling (25) and the data presented in Figs. 1 and 2, we conclude that the blockade of the ERK1/2 signaling cascade is a major target for RES to achieve its inhibitory effect on CF proliferation.

RES attenuates epidermal growth factor-induced ERK1/2 activation in CFs. We next sought to test whether the inhibitory actions of RES on ERK1/2 signaling were the result of a specific blockade of the AT1 receptor or inhibition of downstream intracellular signaling. Epidermal growth factor (EGF) is a more robust stimulus for proliferation and activation of ERK1/2 in isolated CFs, because of direct receptor tyrosine kinase-mediated activation of the MAPK cascade. Initially, we found that 25 µM RES was ineffective in reducing ERK1/2 phosphorylation induced by 10 nM EGF (data not shown). Treatment with either 100 nM ANG II or 10 nM EGF was sufficient to induce a maximal ERK phosphorylation for the given hormones, but 10 nM EGF induced nearly a 20-fold greater phosphorylation of ERK1/2 over basal compared with
concentration-dependent manner, reaching statistical significance at 25 

isolated from separate animals, and are expressed as % vehicle-treated controls 

Evident. Data are pooled from 3 experiments, each done in triplicate in cells 

A 2-fold increase induced by 100 nM ANG II (Fig. 3, A and B).

As previously demonstrated in Fig. 1B, pretreatment with 25 

RES did not induce Akt or p70S6K phosphorylation over basal levels in CFs. Figure 4B confirms that 100 nM ANG II is sufficient to activate Akt in VSMCs, and, compared with Fig. 4A, it is clear that this pathway is not activated by 100 nM ANG II in CFs. Thus we conclude that 100 nM ANG II and 10 or 25 

M RES do not affect Akt signaling in CFs, indicating that this pathway is not playing a major role in proliferation of CFs at the concentrations of ANG and RES used in the experiments. These results indicate cell type-specific differences in the effects of both ANG II and RES, specifically highlighting an important contrast between VSMCs and CFs.

RES prevents ANG II- and TGF-β-induced cardiac myofibroblast differentiation. Because of the recent appreciation that myofibroblasts contribute to the development of cardiac fibrosis, we next investigated the effects of RES on cardiac myofibroblast differentiation. The expression and organization of α-SMA are hallmarks of myofibroblast differentiation, and we have assessed the expression levels of α-SMA after 48- to 72-h treatments with either 200 pM TGF-β or 100 nM ANG II in the presence and absence of 25 

M RES. Figure 5A displays immunocytochemical α-SMA staining in CFs treated with TGF-β with and without RES pretreatment for 30 min. We selected TGF-β as an agonist, since it has been shown repeatedly to be a potent inducer of in vitro cardiac myofibroblast differentiation (17, 22). Pretreatment of CFs with RES was effective in limiting the staining intensity and organization of α-SMA induced by TGF-β. Differences in cell number are apparent between treatment conditions, which would be expected, since the agonists used affect both proliferation and differentiation. Figure 5B contains the representative Western blot demonstrating the expected increase in α-SMA levels induced by TGF-β and ANG II over vehicle-treated controls.

Fig. 2. RES attenuates ANG II-stimulated [3H]leucine and [3H]thymidine uptake in cultured rat CFs in a concentration-dependent manner. A: [3H]leucine uptake in response to 48 h treatment with 5, 10, or 25 

M RES in combination with 100 nM ANG II normalized to unstimulated controls (basal = 100%). RES reduced ANG II-stimulated [3H]leucine uptake in a concentration-dependent manner, but treatment with RES alone had no effect on basal [3H]leucine uptake. B: [3H]thymidine uptake in response to 48 h treatment with RES and 100 nM ANG II. RES reduced ANG II-stimulated [3H]thymidine uptake in a concentration-dependent manner, reaching statistical significance at 25 

M. A concentration-dependent reduction in basal [3H]thymidine uptake was also evident. Data are pooled from 3 experiments, each done in triplicate in cells isolated from separate animals, and are expressed as % vehicle-treated controls (calculated by pooling cpm/well). *P < 0.05 and ***P < 0.001, statistically significant vs. basal levels. †P < 0.05 and ‡‡‡P < 0.001, significant reduction vs. 100 nM ANG II alone. Significant differences between conditions were determined by Tukey’s multiple-comparison test.

ERK1/2 activation, we conclude that the effects of RES are not specific for ANG II signaling and that RES acts as a nonspecific, general inhibitor of ERK1/2 signaling in CFs.

DISCUSSION

The current study was designed to determine whether RES has direct effects on CF activation and function. We have examined key early events that occur during CF activation, proliferation, and myofibroblast differentiation. CFs have been

- Figures and tables
- Integrated discussion with specific experiments and data
- Clarification on the role of RES in cardiac fibroblast function

This detailed analysis provides a comprehensive understanding of RES's effects on cardiac fibroblast function and differentiation, highlighting its potential therapeutic implications in cardiac fibrosis.
Fig. 3. RES inhibits epidermal growth factor (EGF)-induced ERK activation in CFs. A: representative Western blot of P-ERK and total ERK levels in CFs after stimulation with 0.2–10 nM EGF for 10 min. B: densitometric analysis of Western blots comparing ANG II- and EGF-induced ERK phosphorylation. Densitometric data for ANG II were obtained previously and carried over from Fig. 1 for comparison. The amount of ERK phosphorylation in CFs treated with 10, 5, 2, and 1 nM EGF was significantly elevated over 100 nM ANG II. ERK activation induced by 0.5 and 0.2 nM EGF was not significantly different from 100 nM ANG II. Data are pooled from 3 experiments in cells isolated from separate animals (n = 3) and are expressed as %basal phosphorylation. †P < 0.05, ††P < 0.01, and †††P < 0.001, statistical significance vs. 100 nM ANG II determined by Tukey’s multiple-comparison test. C: representative Western blot of P-ERK and total ERK levels in CFs after 30 min pretreatment with 25 μM RES and stimulation with 0.5 and 0.2 nM EGF (the concentrations that did not show a significant elevation in ERK phosphorylation over 100 nM ANG II) for 10 min. The Western blot in C was taken from A and is representative of 3 experiments examining the effects of RES pretreatment on 0.5 and 0.2 nM EGF-induced ERK activation. D: densitometric analysis of experiments represented in C. Data are pooled from 3 experiments in cells isolated from separate animals (n = 3) and are expressed as %basal phosphorylation. †P < 0.05, statistical significance between means determined by a t-test.

Fig. 4. RES does not affect protein kinase B (Akt) or p70S6K phosphorylation in CFs. A: representative Western blot demonstrating the effects of 10 or 25 μM RES followed by stimulation with 100 nM ANG II for 5 min on phosphorylated Akt (P-Akt) in CFs at Ser473 and Thr308 and phosphorylated p70S6K (P-p70S6K). Total p70S6K levels are unchanged, indicating equal protein loading. RES had no effect on P-Akt at either Ser473 or Thr308 or P-p70S6K. B: representative Western blot demonstrating phosphorylation of Akt at Ser473 and Thr308 by 100 nM ANG II in vascular smooth muscle cells (VSMCs). Western blots are representative of 3 experiments in cells isolated from separate animals (n = 3). The appearance of multiple bands is the result of some nonspecific binding of the primary antibody.
reported to respond to a variety of hormonal stimuli via proliferation, which predisposes the myocardium to excessive ECM production and ultimately cardiac fibrosis. Differentiation of these cells to a myofibroblast phenotype can contribute to hypersecretion of ECM proteins as well, and we hypothesize that the myofibroblasts secrete the majority of the myocardial collagen and other ECM proteins. Therefore, interventions that target CF proliferation and differentiation to myofibroblasts represent a viable therapeutic avenue in the prevention of cardiac fibrosis.

We report here that RES, when administered to cultured CFs, inhibits proliferation by attenuation of ERK1/2 signaling. The MAPK family has been shown to be involved in a variety of chronic disease states and to play a major role in cardiac hypertrophy. Agents that are effective in attenuating MAPK signaling cascades, particularly those that are naturally occurring compounds, may prove to be viable therapeutic tools in the prevention of many hyperplastic and/or hypertrophic diseases. The anti-proliferative effects of RES have been examined intensively, and several studies in various cancer cell lines and smooth muscle cells have determined that the ERK1/2 cascade is a key pathway targeted by RES (10, 13, 31). Results from the present study provide further support that RES targets the ERK1/2 pathway in CFs by directly inhibiting MEK activation. However, the precise mechanism by which RES acts is poorly understood and likely involves multiple intracellular targets.

CF proliferation is vital for ventricular remodeling, and several studies have identified the hormones and growth factors that mediate this process. Growth factor-induced proliferation of CFs is mediated by the classic MAPK signaling pathway through phosphorylation of Raf, MEK, and ERK. G protein-coupled receptors, including the AT1 receptor, have been postulated to activate MAPKs by transactivation of the EGF receptor (EGFR) in both CFs and VSMCs, and this transactivation is necessary for ERK activation, DNA synthesis, and protein production (8, 14, 29). Data obtained in the current study indicate that RES is effective in inhibiting ERK phosphorylation regardless whether the initial stimulus is via the AT1 receptor or the EGFR. However, the mitogenic response resulting from ANG II stimulation may only be partially attributed to transactivation of the EGFR, since ANG II acti-
vates multiple signaling pathways to induce mitogenesis, and there likely exist other targets of RES upstream of MEK and distinct from the classic MAPK cascade that still remain to be identified.

One target of RES and the mitogenic pathway activated by ANG II in VSMCs is the phosphatidylinositol 3-kinase (PI3-kinase)/Akt pathway. Akt has a multitude of effector proteins and is involved in a number of cellular processes, including proliferation and survival via inhibition of proapoptotic proteins. Akt induces hypertrophy of VSMCs by activating p70S6K. Treatment of VSMCs with RES was found to be an effective method in preventing ANG II-induced hypertrophy by inhibiting phosphorylation of PI3-kinase, the kinase directly upstream of Akt/PKB (13). These data suggest that RES is likely exerting beneficial effects on the cardiovascular system by interacting with multiple signaling pathways and is not limited strictly to the MAPK cascade. Our findings indicate that levels of phosphorylated Akt are unaffected by 100 nM ANG II in CFs, which agrees with a previous study in which the investigators had determined that the concentration of ANG II that would activate Akt over basal in CFs was in the micromolar range, far above the concentration of interest in the current study (27). We found that neither 10 nor 25 μM RES had any effect on Akt/p70S6K signaling in CFs, results that highlight important cell-specific differences in the inhibitory actions of RES.

We and other investigators postulate that myofibroblast differentiation is stimulated by a number of hormonal and nonhormonal factors, and, even in the normal myocardium, these hypersecretory cells are important players in the wound-healing and remodeling processes. CFs and myofibroblasts produce and secrete TGF-β, a potent inducer of differentiation (4). TGF-β acts in a paracrine fashion to stimulate myofibroblast differentiation and a concurrent production of collagen (18). Under normal circumstances, the myofibroblasts are removed from the wound site by apoptosis (5). However, a lack of apoptosis of myofibroblasts leads to an overproduction of ECM proteins because of their prolonged presence (12). In a cardiac pressure-overload hypertrophy model, increased fibrosis, myofibroblast number, and TGF-β mRNA were observed from 3 to 28 days after suprarenal aortic constriction (15). Inhibition of TGF-β function by the injection of anti-TGF-β antibodies resulted in a reduction in both fibrosis and myofibroblast number as well as a reduction in type I and III collagen mRNA. Prevention of cardiac myofibroblast differentiation may therefore represent a potential target for therapies aimed at limiting fibrosis in the heart. In the present study, we found that pretreatment of cultured CFs with RES was effective in preventing myofibroblast differentiation induced by ANG II and TGF-β.

In conclusion, we have determined that RES directly inhibits two critical stages of CF activation, proliferation, and differentiation to myofibroblasts. Both of these physiological parameters are key determinants of cardiac fibrosis, and limiting these activation steps can greatly reduce the overall production of ECM components within the myocardium. This study therefore contributes additional evidence identifying RES as a cardioprotective agent: in addition to its cholesterol-lowering, anti-atherosclerotic properties and its inhibition of VSMC hypertrophy, RES provides protection against excessive CF activity, which has implications for limiting aberrant remodeling and fibrosis in the myocardium.

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