Several studies have demonstrated that the proliferation of its content is a major determinant of myocardial stiffness. Therefore, in the cardiovascular system, collagen-1 is usually present in two-thirds of the total collagens in most tissues (10). Types, of which type 1 (collagen-1) and type 3 (collagen-3) chains and comprise a family of at least 19 genetically distinct proteins in the extracellular space, is closely associated with heart failure. Among the extracellular matrix proteins, up to 85% are collagens, which consist of triple helix polypeptide, which was inhibited by pravastatin and pioglitazone in a dose-dependent fashion. Pretreatment of fibroblasts with low therapeutic concentrations of either pravastatin (0.1 μM) or pioglitazone (5 μM) only slightly decreased ANG II-induced NADPH oxidase expression, superoxide anion production, and procollagen-1 expression; however, the combination of pravastatin and pioglitazone markedly modulated these effects of ANG II. The combination also blocked ANG II-mediated p38 MAPK and p44/42 MAPK activation. Electrophoretic mobility shift assay showed that ANG II activated transcription factors NF-κB and activator protein-1 (AP-1). Although pravastatin and pioglitazone alone had a variable effect on NF-κB and AP-1 activation, their combination exerted a potent inhibitory effect on the activation of both NF-κB and AP-1. The effects of pravastatin and pioglitazone in combination on superoxide generation and procollagen-1 expression mimicked those of α-tocopherol and γ-tocopherol, two potent antioxidants. Thus it appears that there is a positive interaction between pravastatin and pioglitazone in modulating ANG II-mediated oxidative stress, inhibiting MAPK activation, and procollagen-1 expression.

The activation of the renin-angiotensin system (RAS) with oxidative stress and procollagen-1 expression in cardiac fibroblasts: blockade by pravastatin and pioglitazone

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Chen, Jiawei, and Jawahar L. Mehta. Angiotensin II-mediated oxidative stress and procollagen-1 expression in cardiac fibroblasts: blockade by pravastatin and pioglitazone. Am J Physiol Heart Circ Physiol 291: H1738–H1745, 2006. First published May 19, 2006; doi:10.1152/ajpheart.00341.2006.—Angiotensin II (ANG II), a product of renin-angiotensin system activation, enhances collagen synthesis, which is a key event in cardiac remodeling after myocardial infarction. Inhibition of cardiac remodeling is now a target of multiple therapies, including 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, commonly known as statins, and peroxisome proliferator-activated receptor-γ (PPAR-γ) ligands. We examined the potential antifibrotic effect of the combination of a statin (pravastatin) and a PPAR-γ ligand (pioglitazone) in ANG II–treated mouse cardiac fibroblasts. ANG II treatment induced procollagen-1 expression, which was inhibited by pravastatin and pioglitazone in a dose-dependent fashion. Pretreatment of fibroblasts with low therapeutic concentrations of either pravastatin (0.1 μM) or pioglitazone (5 μM) only slightly decreased ANG II-induced NADPH oxidase expression, superoxide anion production, and procollagen-1 expression; however, the combination of pravastatin and pioglitazone markedly modulated these effects of ANG II. The combination also blocked ANG II-mediated p38 MAPK and p44/42 MAPK activation. Electrophoretic mobility shift assay showed that ANG II activated transcription factors NF-κB and activator protein-1 (AP-1). Although pravastatin and pioglitazone alone had a variable effect on NF-κB and AP-1 activation, their combination exerted a potent inhibitory effect on the activation of both NF-κB and AP-1. The effects of pravastatin and pioglitazone in combination on superoxide generation and procollagen-1 expression mimicked those of α-tocopherol and γ-tocopherol, two potent antioxidants. Thus it appears that there is a positive interaction between pravastatin and pioglitazone in modulating ANG II-mediated oxidative stress, inhibiting MAPK activation, and procollagen-1 expression.

Fibrosis, characterized by abundant accumulation of matrix proteins in the extracellular space, is closely associated with heart failure. Among the extracellular matrix proteins, up to 85% are collagens, which consist of triple helix polypeptide chains and comprise a family of at least 19 genetically distinct types, of which type 1 (collagen-1) and type 3 (collagen-3) constitute two-thirds of the total collagens in most tissues (10). In the cardiovascular system, collagen-1 is usually present in the form of thick fibers with a high tensile strength. Therefore, its content is a major determinant of myocardial stiffness. Several studies have demonstrated that the proliferation of cardiac fibroblasts and the deposition of collagen-1 are directly associated with heart failure (26). Collagen-1 is derived from its precursor procollagen-1, which is synthesized by cardiac fibroblasts. The amount of collagen in the heart depends not only on its production but also on its degradation, which is carried out by a family of proteinases called matrix metalloproteinases (MMPs).

The activation of the renin-angiotensin system (RAS) with oxidative stress and procollagen-1 expression in cardiac fibroblasts: blockade by pravastatin and pioglitazone

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The combination of pravastatin and pioglitazone markedly modulated ANG II-mediated oxidative stress, induces collagen-1 synthesis in rat cardiac fibroblasts and that the peroxisome proliferator-activated receptor-γ (PPAR-γ) ligand pioglitazone attenuates the profibrotic effect of ANG II (3).

3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, also known as statins, have also been shown to exert antioxidant effects. Recent studies (1, 16) also support the notion that statins might improve cardiac remodeling and prevent the development of heart failure.

We postulated that the combination of a PPAR-γ ligand and a statin may have an additive effect against ANG II-mediated superoxide generation. Furthermore, we thought that their combination would block intracellular signals leading to the formation of collagen in cardiac fibroblasts. For clinical relevance, we used therapeutic concentrations rather than the high pharmacological concentrations used in previous studies (4, 5, 15, 21).

MATERIALS AND METHODS

Cell culture and treatment. Mouse cardiac fibroblasts were isolated and cultured as described earlier (3). In brief, hearts were removed from C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME). After being washed with phosphate-buffered saline (PBS), heart tissues were minced and digested in 0.25% collagenase solution (37°C, 1 h). After digestion, cells were pelleted by centrifugation at 1,500 rpm for 10 min and suspended in DMEM supplemented with 1% penicillin and streptomycin and 10% fetal bovine serum. The suspension was...
transferred to a culture dish. After 1 h of incubation at 37°C, cells that were weakly attached or unattached were removed, and the attached cells were cultured in the dish with DMEM. The purity of these cultured cardiac fibroblasts was >95% on the basis of positive staining for vimentin and negative staining for smooth muscle cell α-actin and von Willebrand factor. Mouse cardiac fibroblasts cultured to the fifth passage were used in all experiments. In the first set of experiments, cells were treated with ANG II (1 μM for 24 h) alone or pretreated with pravastatin (0.1–1 μM) or pioglitazone (1–10 μM) for 3 h before treatment with ANG II. In other experiments, cells were pretreated with pravastatin (0.1 μM) or pioglitazone (5 μM) or their combination for 3 h before exposure to ANG II for 24 h. In parallel experiments, cells were pretreated with α-tocopherol (25 μM) or γ-tocopherol (25 μM), followed by ANG II treatment (15). The studies were approved by the Animal Use Committee of the University of Arkansas for Medical Sciences (Little Rock).

Measurement of superoxide anion generation in cardiac fibroblasts. Intracellular superoxide anion generation was measured with the superoxide anion-sensitive chemiluminescent probe coelenterazine (14). In brief, cells were harvested and suspended in Krebs-Ringer buffer (pH 7.4) containing 10 μM coelenterazine. The chemiluminescence of coelenterazine was detected with a scintillation counter (LS 7000; Beckman) in out-of-coincidence mode.

In some experiments, intracellular ROS generation was measured with the use of the fluorescent signal carboxy-H2-2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA; Invitrogen), a cell-permeable indicator for ROS (5). Carboxy-H2DCF-DA is nonfluorescent until the acetate groups are removed by intracellular ROS. The ROS-mediated fluorescence was observed under a fluorescent microscope with excitation set at 485 nm and emission set at 530 nm.

Western blot analysis of procollagen-1, NADPH oxidases, MMPs, and MAPKs. Whole cell protein extracts were prepared as described previously (14). After incubation in blocking solution (5% nonfat milk; Sigma), membranes were incubated with primary antibodies overnight at 4°C. Antibodies used were 1:500 dilution goat polyclonal antibody to procollagen-1 (Santa Cruz), 1:500 dilution mouse monoclonal antibody to MMP-2 (Santa Cruz), 1:1,000 dilution goat polyclonal antibody to MMP-3 (Santa Cruz), 1:1,000 dilution goat polyclonal antibody to MMP-9 (Santa Cruz), 1:200 dilution goat polyclonal antibody to p22phox (Santa Cruz), 1:200 dilution goat polyclonal antibody to p67phox (Santa Cruz), 1:1,000 dilution rabbit polyclonal antibody to p38 MAPK (Santa Cruz), 1:500 dilution mouse monoclonal antibody to phosphorylated p38 MAPK (Santa Cruz), 1:1,000 dilution rabbit polyclonal antibody to p44/42 MAPK (Santa Cruz), 1:500 dilution mouse monoclonal antibody to phosphorylated p44/42 MAPK (Santa Cruz), and 1:5,000 dilution mouse monoclonal antibody to β-actin (Sigma).

Membranes were then washed with 1× Tris-buffered saline with Tween 20 solution and incubated with 1:5,000 dilution secondary antibody (Amersham Life Sciences) at room temperature for 1 h. The proteins were detected with the ECL system (Amersham). The relative intensities of the protein bands were analyzed by Scan-gel-it software (14), and the intensity of each protein band was normalized with that of β-actin.

MMP activity measurement. MMP activity was measured by zymography. Briefly, after treatment of cells as described above, the conditioned culture medium was collected, and 10 μl of the medium were subjected to electrophoresis in SDS polyacrylamide gel containing 0.1% gelatin under nonreducing conditions. The gels were soaked in 2.5% Triton X-100 for 1 h and washed with water for 1 h. The gels were then incubated in a developing buffer containing 50 mM Tris, pH 7.4, 5 mM CaCl2, and 0.02% sodium azide overnight at 37°C. The gels were then stained with Coomassie blue for 1 h and photographed.

Electrophoretic mobility shift assay. Cell nuclear extracts were prepared as previously described (2). Complementary oligonucleotides containing putative NF-κB binding site (5′-AGT TGA GGG

Fig. 1. ANG II-mediated procollagen-1 expression and modulation by pravastatin and pioglitazone. ANG II treatment (1 μM for 24 h) markedly upregulated expression of procollagen-1, which was attenuated by pretreatment of cells with pravastatin or pioglitazone in a dose-dependent manner. Although low concentrations of pravastatin (0.1 μM) and pioglitazone (5 μM) had minor inhibitory effect, their combination completely blocked ANG II-induced procollagen-1 expression. Each panel shows a representative experiment (top) and summary of densitometric data from 3 separate experiments (bottom). Values are means (SD). AU, arbitrary units.
plexes were separated by electrophoresis in a 6% nondenaturing polyacrylamide gel. The gels were dried and exposed to radiographic films for 24 h at –80°C.

Measurement of cardiac fibroblast growth. Cell growth was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay using Cell Growth Determination Kit (Sigma) (3).

Data analysis. All data represented the mean of samples from at least three separately performed experiments. Data were subjected to analysis of variance and are presented as means (SD). A value of P < 0.05 was considered significant.

RESULTS

ANG II-mediated procollagen-1 expression and inhibitory effects of pravastatin and pioglitazone. In accordance with previous findings in rat cardiac fibroblasts (3), ANG II induced the expression of procollagen-1 in mouse cardiac fibroblasts. Whereas the low concentrations of pioglitazone and pravastatin had minimal effect on ANG II-mediated collagen expression, the higher concentrations markedly inhibited it (Fig. 1). Importantly, pretreatment of cells with the combination of low concentrations of pravastatin (0.1 μM) or pioglitazone (5 μM) completely blocked ANG II-mediated upregulation of procollagen-1 expression (Fig. 1).

Because collagen accumulation depends not only on its production but also on its MMP-mediated degradation, we examined the expression of MMPs. Western blot analysis showed that the expression of MMP-3 and -9 significantly, and the expression of MMP-2 only modestly, was enhanced by ANG II. MMP activity was also enhanced by ANG II. Interestingly, the low concentration of pravastatin (0.1 μM), pioglitazone (5 μM), or their combination had only a minimal effect on the expression of MMPs; however, the combination of drugs normalized MMP activity (Fig. 2).

ANG II-induced oxidative stress and fibroblast proliferation and modulation by pravastatin and pioglitazone. Superoxide anion generation increased markedly after ANG II treatment of cardiac fibroblasts (Fig. 3). Pretreatment of cells with low concentration of pravastatin (0.1 μM) had no inhibitory effect, and pioglitazone (5 μM) only slightly inhibited it. However, the combination of pravastatin and pioglitazone reduced superoxide anion production to a level lower than with either agent alone (Fig. 3).

We examined the underlying mechanism of superoxide anion generation by measuring different subunits of NADPH oxidase, p22phox and p67phox, and observed that ANG II induced the expression of both subtypes. We also observed that the combination of pravastatin and pioglitazone completely blocked ANG II-mediated expression of NADPH oxidase (Fig. 3).

Although a large body of evidence has suggested that oxidative stress may induce apoptosis and cell death in endothelial cells, a recent study reported that oxidative stress is associated with cardiac fibroblast growth (4). In the present study, we examined fibroblast growth in response to ANG II and its modulation by pravastatin and pioglitazone. We found that, whereas ANG II did induce fibroblast growth, the low concentration of pravastatin (0.1 μM), pioglitazone (5 μM), or their combination had no inhibitory effect on cell growth over 24 h of incubation (Fig. 4).

ANG II-mediated activation of MAPKs and transcription factors and attenuation by pravastatin and pioglitazone. It has been shown that ANG II stimulates MAPK activity in rat cardiac fibroblasts (5, 21), but the role of different MAPK subtypes is unclear. Accordingly, we determined whether the p38 or p44/42 isoform of MAPK or both are activated by ANG II in mouse cardiac fibroblasts. The data in Fig. 5 show that the activity of both p38 and p44/42 MAPK isoforms was enhanced by ANG II without any change in the protein expression of either isoform. Low concentrations of pravastatin (0.1 μM) and pioglitazone (5 μM) alone modestly inhibited p38 MAPK activation, but the combination completely blocked p38

Fig. 2. ANG II-mediated matrix metalloproteinases (MMP) expression and activity and modulation by pravastatin (Prava) and pioglitazone (Pio). ANG II treatment markedly increased MMP-3 and MMP-9 expression, which was minimally affected by pravastatin or pioglitazone or their combination. ANG II also stimulated MMP activity, which was normalized by pravastatin-pioglitazone combination. Each panel shows a representative experiment (top) and summary of densitometric data from 3 separate experiments (bottom). Values are means (SD).
MAPK activation (Fig. 5). In contrast, the activation of p44/42 MAPK was not affected by pravastatin (0.1 μM) but was blocked by pioglitazone (5 μM) alone or combined with pravastatin (0.1 μM; Fig. 5).

Next we examined whether ANG II would activate transcription factors NF-κB and AP-1. We confirmed previous observations (17) that ANG II activates both NF-κB and AP-1 as in rat cardiac fibroblasts (Fig. 6). Interestingly, we found that the low concentration of pravastatin (0.1 μM) attenuated
AP-1 activation, whereas low concentration of pioglitazone (5 μM) reduced NF-κB activation (Fig. 5). The combination of pravastatin and pioglitazone reduced the activation of both NF-κB and AP-1 (Fig. 6).

**Antifibrotic effect of α-tocopherol and γ-tocopherol in ANG II-treated cardiac fibroblasts.** To provide another line of evidence that antioxidant effect is likely to be the basis of the interaction between pravastatin and pioglitazone in modulating collagen formation, we examined the effects of two well-known antioxidants, α-tocopherol and γ-tocopherol, in ANG II-treated cardiac fibroblasts. As expected, ANG II-induced oxidative stress was markedly attenuated by both α-tocopherol and γ-tocopherol (Fig. 7, top). We also observed that ANG II-induced procollagen-1 upregulation was markedly inhibited by both α-tocopherol and γ-tocopherol (Fig. 7, bottom). More importantly, these effects of α-tocopherol and γ-tocopherol were qualitatively similar to those of the combination of pravastatin and pioglitazone.

**DISCUSSION**

In the present study, we show that ANG II induces intracellular oxidative stress, activates MAPKs and the transcription factors NF-κB and AP-1, and stimulates cell growth and procollagen-1 synthesis. We also show that the therapeutically achieved concentrations of pravastatin and pioglitazone exert only minor inhibitory effect on oxidative stress and subsequent oxidative stress-mediated signaling pathway, as well as procollagen-1 synthesis. Importantly, the combination of pravastatin and pioglitazone, each in low concentration, exerts a potent inhibitory effect on oxidative stress and related signaling pathways, leading to reduced procollagen-1 expression.

**ANG II-induced oxidative stress and collagen expression.** The activation of RAS is a central mediator in progressive cardiac remodeling that follows ischemic and nonischemic stress. The remodeling process denotes alterations in the structural components of the myocardium, involving both myocytes and nonmyocyte compartments (24). ANG II, the main effector hormone of RAS activation, is a powerful mediator of myocardial fibrosis, characterized by fibroblast growth and collagen formation (24). We now confirm that ANG II induces oxidative stress, activates redox-sensitive signals, and leads to the expression of procollagen-1. Because MMP-1 is rarely detected in mouse fibroblasts, we focused on the expression of MMP-2, -3, and -9, which are present in abundance in these cells. We observed that the expression of these MMPs increased in response to ANG II. MMP activity was also enhanced by ANG II. It is possible that the increased expression and activity of MMPs reflects an autoregulatory step to combat enhanced collagen-1 synthesis. It is of note that MMP expression also increases in rat cardiac fibroblasts exposed to anoxia-reoxygenation (4).

**ANG II** has been reported to induce the proliferation of smooth muscle cells (25) and cardiomyocytes (6) and apoptosis of endothelial cells (18, 12). In the present study, we show that ANG II slightly but significantly increased the growth of
mouse cardiac fibroblasts over 24 h. In previous studies, Chen et al. (3) showed that exposure to ANG II causes growth of rat cardiac fibroblasts when the cells were exposed to ANG II for a longer period (3). These observations collectively suggest that this peptide hormone stimulates the growth of cardiac fibroblasts but only when fibroblasts are exposed to ANG II for a prolonged time.

We also examined intracellular mechanisms responsible for ANG II-mediated collagen synthesis and found the activation of both p38 and p44/42 MAPK isoforms (Fig. 4). Xie et al. (24) showed that, in adult rat cardiac fibroblasts, p44/42 MAPK, but not p38 MAPK, is activated by ANG II. Tharaux et al. (21) also suggested that p44/42 MAPK is involved in ANG II-induced expression of collagen-1 gene, whereas p38 MAPK is not. In contrast, Li et al. (13) suggested that it is the activation of p38 MAPK that plays a critical role in ANG II-mediated collagen-1 synthesis in rat fibroblasts. As such, the role of different isoforms of MAPK in ANG II-mediated collagen synthesis remains debatable. Our data show that ANG II activates both p38 and p44/42 isoforms of MAPK, at least in mouse cardiac fibroblasts.

We also examined the activation of transcription factors by ANG II. Consistent with other studies (17), we observed the activation of both NF-κB and AP-1 in cardiac fibroblasts after exposure to ANG II. Tharaux et al. (21) have suggested that NF-κB does not play a role in ANG II-induced collagen gene expression. Rippe et al. (17) have even suggested that NF-κB activation may inhibit the expression of collagen-1. Yet, others (7) have implicated both AP-1 and NF-κB in ANG II-mediated collagen synthesis and cardiac remodeling (22). It is possible that the variability in the results on the involvement of NF-κB and AP-1 is related to animal species and/or experimental conditions. In a discussion of the role of ANG II in collagen expression, the studies of Siwik et al. (19) are noteworthy. These investigators treated rat cardiac fibroblasts with H2O2 or xanthine/xanthine oxidase as a source of oxidant stress and showed a reduction in collagen synthesis. It is of note that there were major differences in their and our studies, such as prolonged ANG II exposure versus a short-term exposure to ROS-generating systems and use of fibroblasts from different species.

Antioxidant and antifibrotic effects of pravastatin, pioglitazone, and their combination. Besides their lipid-lowering effects, potent statins, such as simvastatin and atorvastatin, have been shown to inhibit oxidative stress, NF-κB activation, and the expression of adhesion molecules in endothelial cells (16). Nonetheless, these effects of statins are concentration dependent, and the low concentrations have only slight inhibitory

Fig. 7. Antioxidant and antipрocollagen-1 expression effects of α-tocopherol and γ-tocopherol. ANG II-mediated oxidative stress was blocked by α-tocopherol and γ-tocopherol. Top: representation of 4 separate experiments. ANG II-mediated procollagen-1 upregulation was also inhibited by α-tocopherol or γ-tocopherol in parallel experiments. β-Actin was used as loading control. Bottom: representative experiment (top) and summary of data from 4 separate experiments (bottom). Values are means (SD).

ANTIOXIDANT AND ANTIFIBROSIS EFFECT OF PRAVASTATIN AND PIOGLITAZONE

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effects on oxidative stress and related signaling pathways. When compared with other statins, pravastatin is the most hydrophilic and has less of an antioxidant or antiproliferative effect (22).

Indeed, this was observed in the present study, wherein 0.1 μM pravastatin minimally modulated the effects of ANG II in cardiac fibroblasts. Similarly, the PPAR-γ ligand pioglitazone in 5 μM concentration, which is achieved after therapeutic use, only slightly inhibited oxidative stress and activation of MAPK and AP-1, with no effect on NF-κB.

There is immense interest in the use of PPAR-γ ligands and statins in the management of cardiovascular diseases; hence we postulated that the combination of low concentration of pravastatin and pioglitazone may have complementary effects. Indeed, the combination of the two agents, each in low concentration, dramatically reduced superoxide anion generation and the activation of both isoforms of MAPK as well as NF-κB and AP-1 transcription factors. We also show that the combination of these agents “normalized” the expression of p22phox and p67phox NADPH oxidases. Other NADPH oxidases were not examined. This alteration in redox signaling was associated with dramatic reduction in procollagen-1 synthesis. It is of note that pioglitazone and pravastatin or their combination had no effect on fibroblast growth. Probably, an effect on fibroblast growth requires a prolonged contact. Furthermore, pioglitazone and statin or their combination had no effect on the expression of MMPs but reduced ANG II-enhanced MMP activity. This observation suggests that the upregulation of MMP expression and activity in fibroblasts may be an autoregulatory response to ANG II-mediated expression of procollagen-1.

We believe that the inhibitory effects on collagen-1 synthesis are related to the antioxidant effects of pioglitazone and pravastatin. To examine this relationship further, we treated fibroblasts with two well-known antioxidants, α-tocopherol and γ-tocopherol. Both tocopherol preparations eliminated superoxide generation and subsequent procollagen-1 expression. Although the experiments with tocopherols do not provide convincing evidence of the link between oxidant stress and collagen-1 synthesis in response to ANG II, the modulation of the effects of ANG II by pioglitazone and pravastatin is qualitatively similar to the effects of tocopherols and indicates that there may be a link between oxidant stress and collagen formation. Nevertheless, there may well be other mechanisms by which pioglitazone and pravastatin reduce collagen formation. For example, both statins and PPAR-γ ligands have been shown to reduce the expression of ANG II type 1 receptor (23, 20), the activation of which is associated with cardiac fibrosis.

In conclusion, we show in this study that ANG II induces oxidative stress and procollagen-1 expression in mouse cardiac fibroblasts. The combination of pravastatin and pioglitazone, each in therapeutic concentration, blocks the effects of ANG II. In addition, we show that, whereas pravastatin and pioglitazone individually affect somewhat different signaling pathways, their combination results in a positive interaction with regard to blockade of oxidative stress and related signaling pathways with a major inhibitory effect on procollagen-1 formation.

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