Oxidative stress regulates collagen synthesis and matrix metalloproteinase activity in cardiac fibroblasts

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Siwik, Deborah A., Patrick J. Pagano, and Wilson S. Colucci. Oxidative stress regulates collagen synthesis and matrix metalloproteinase activity in cardiac fibroblasts. Am J Physiol Cell Physiol 280: C53–C60, 2001.—Oxidative stress has been implicated in the pathophysiology of myocardial failure. We tested the hypothesis that oxidative stress can regulate extracellular matrix in cardiac fibroblasts. Neonatal and adult rat cardiac fibroblasts in vitro were exposed to H2O2 (0.05–5 μM) or the superoxide-generating system xanthine (500 μM) plus xanthine oxidase (0.001–0.1 mU/ml) (XXO) for 24 h. In-gel zymography demonstrated that H2O2 and XXO each increased gelatinase activity corresponding to matrix metalloproteinases (MMP) MMP-13, MMP-2, and MMP-9. H2O2 and XXO decreased collagen synthesis (collagenase-sensitive [3H]proline incorporation) without affecting total protein synthesis ([3H]leucine incorporation). H2O2 increased mRNA, suggesting a selective transcriptional effect on collagen synthesis. H2O2, but not XXO, also decreased the expression of nonfibrillar procollagen α1(IV) and α2(IV) mRNA. To determine the role of endogenous antioxidant systems, cells were treated with the superoxide dismutase (SOD) inhibitor diethyldithiocarbamic acid (DDC, 100 μM) to increase intracellular superoxide or with the glucose-6-phosphate dehydrogenase inhibitor dehydroisoandrosterone 3-acetate (DHEA; cellular superoxide or with the glucose-6-phosphate dehydrogenase (G6PD), which is essential for regenerating cells. H2O2 and XXO each increased gelatinase activity corresponding to matrix metalloproteinases (MMP) MMP-13, MMP-2, and MMP-9. H2O2 increased mRNA, suggesting a selective transcriptional effect on collagen synthesis. H2O2, but not XXO, also decreased the expression of nonfibrillar procollagen α1(IV) and α2(IV) mRNA. To determine the role of endogenous antioxidant systems, cells were treated with the superoxide dismutase (SOD) inhibitor diethyldithiocarbamic acid (DDC, 100 μM) to increase intracellular superoxide or with the glucose-6-phosphate dehydrogenase inhibitor dehydroisoandrosterone 3-acetate (DHEA; 10 μM) to increase intracellular H2O2. DDC and DHEA decreased collagen synthesis and increased MMP activity, and both effects were inhibited by an SOD/catalase mimetic. Thus increased oxidative stress activates MMPs and decreases fibrillar collagen synthesis in cardiac fibroblasts. Oxidative stress may play a role in the pathogenesis of myocardial remodeling by regulating the quantity and quality of extracellular matrix.

Fibillar collagen plays an important role in determining the structural integrity of the myocardium (22). The quantity and quality of the extracellular collagen is determined by the balance between synthesis and degradation (27). Collagen synthesis is regulated transcriptionally and posttranslationally. Degradation is mediated by matrix metalloproteinases (MMPs) that are regulated transcriptionally, posttranslationally through activation of latent proenzymes (pMMPs), and by endogenous tissue inhibitors (TIMPs). Collagen synthesis, MMPs, and TIMPs are localized to the cardiac fibroblasts (8, 11, 12).

Recently it has been shown that MMP activity is increased in the failing myocardium of patients (9, 35) and animal models of myocardial remodeling and failure (32). It was further shown that inhibition of MMPs can decrease the severity of remodeling early postmyocardial infarction (28) and in chronic pacing-induced failure (31). The mechanisms responsible for these changes in collagen metabolism are not known. It has been shown that there is increased oxidative stress in the myocardium of patients with heart failure (21) and animal models of heart failure (10) and that antioxidants attenuate the development of myocardial failure (17). Reactive oxygen species (ROS) are known to regulate collagen metabolism in a variety of noncardiac cell types, including rat lung, dermal fibroblasts, and human venous endothelial cells (2, 19, 25). However, it is not known whether ROS can regulate collagen metabolism in cardiac fibroblasts, which are the major cell type responsible for collagen synthesis and degradation in the myocardium.

Accordingly, this study had two goals. We first examined the ability of ROS to regulate collagen metabolism in cardiac fibroblasts by measuring the effect of two sources of ROS (H2O2 and the superoxide-generating system of xanthine plus xanthine oxidase) on collagen synthesis and MMP activity. Second, we tested the role of endogenous antioxidant systems in regulating collagen metabolism by 1) inhibiting cytosolic Cu,Zn-superoxide dismutase (Cu,Zn-SOD) and extracellular SOD with diethyldithiocarbamic acid (DDC) (16) to increase intracellular superoxide levels or 2) inhibiting glucose-6-phosphate dehydrogenase (G6PD), which is essential for regeneration of reduced glutathione and catalase activity, with dehydroisoandrosterone 3-acetate (DHEA) (36, 37) to increase intracellular H2O2 levels.

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methods

Rat cardiac fibroblast cultures and treatments. Neonatal rat cardiac fibroblasts were prepared as previously described (5). Adult rat cardiac fibroblasts were prepared by plating the nonmyocyte fraction of adult rat hearts digested as described previously (33). First and second passage fibroblasts plated on 6- or 24-well plates, or 35- or 100-mm dishes (Falcon) were grown to confluence in DMEM (GIBCO) containing 7% (vol/vol) heat-inactivated fetal bovine serum (GIBCO) and 1% (vol/vol) penicillin-streptomycin (PS; GIBCO) and changed to serum-free DMEM containing PS for 48–72 h before exposure to experimental treatments.

Fibroblasts were treated in DMEM containing PS for 24 h with H2O2 (0.05–5 μM; stabilized; Sigma) or xanthine (500 μM; Sigma) plus xanthine oxidase (0.001–0.1 mU/ml; Boehringer Mannheim) (XXO). Control cells were treated with DMEM containing PS alone. To inhibit antioxidant systems, fibroblasts in DMEM containing PS were treated for 24 h with DDC (100 μM; Sigma) or DHEA (10 μM; Sigma) alone or in combination with the superoxide dismutase/catalase mimetic EUK-134 (50 μM; 30 min pretreatment; Eukarion) (1). Control cells were treated with DMEM containing PS alone for DDC groups or DMEM containing PS and 0.1% DMSO (vehicle) for DHEA groups.

G6PD activity. G6PD activity was measured in fibroblasts plated on 100-mm dishes treated for 24 h with 10 μM DHEA as described by Tian et al. (37). Briefly, cells are scraped into ice-cold homogenization buffer (in mM: 320 sucrose, 20 HEPES, and 0.5 EDTA, pH 7.2). Samples were homogenized and centrifuged at 2,000 × g for 10 min. Protein concentration in the supernatant was determined by the Bradford assay (Bio-Rad Protein Dye Reagent; Bio-Rad) against a BSA standard. Equal amounts of protein were added to the total dehydrogenase assay buffer (in mM: 50 Tris base, 1 MgCl2, 0.2 glucose 6-phosphate, 0.2 6-phosphogluconate, and 0.1 NADP+, pH 8.1; all reagents from Sigma) and the 6-phosphogluconate dehydrogenase (6PGD) assay buffer (in mM: 50 Tris base, 1 MgCl2, 0.2 6-phosphogluconate, and 0.1 NADP+; pH 8.1). NADP+ reduction to NADPH was measured as the rate of change of the absorbance at 340 nm over 6 min. G6PD activity was calculated as the total dehydrogenase activity minus the 6PGD activity.

Dichlorofluorescein fluorescence. The ability of DHEA treatment to increase H2O2 in cardiac fibroblasts was measured as the EUK-134-inhibitable 2,7-dichlorofluorescein (DCF) fluorescence. Fibroblasts were plated on 24-well plates and were treated with 10 μM DHEA alone or in combination with 50 μM EUK-134 for 24 h. Cells were washed three times with phenol-free DMEM and were incubated for 1 h at 37°C with 10 μM DCF diacetate (Molecular Probes) in phenol-free DMEM. Cells were again washed three times with phenol-free DMEM, and fresh phenol-free DMEM was added. DCF fluorescence (485 nm excitation; 538 nm emission) was measured as the average of nine 100-ms readings at room temperature.

Superoxide dismutase activity. Superoxide dismutase (SOD) activity in fibroblasts plated on 100-mm dishes and treated for 24 h with 100 μM DDC was measured as inhibition of pyrogallol auto-oxidation as previously described (30).

Cytochrome c reduction. The ability of DCC treatment to increase superoxide in cardiac fibroblasts was measured as SOD-inhibitable cytochrome c reduction. Fibroblasts were plated on six-well plates and were treated with 100 μM DDC for 24 h. Cells were washed three times with phenol-free DMEM and were incubated for 1 h at 37°C with 15 μM acetylated cytochrome c (Sigma) and 1 mM diethylentri-aminopentaacetic acid with or without 600 μM bovine erythrocyte SOD (Sigma) in phenol-free DMEM. Reduction of cytochrome c was measured as the absorbance of the media at 550 nm.

Collagen synthesis. Collagenase-sensitive [3H]proline incorporation was determined by a modification of the technique described by Botstein et al. (3). Briefly, confluent fibroblasts in 35-mm dishes were treated with H2O2, XXO, DHEA, or DDC alone or in combination with EUK-134 for 24 h, and 10 μCi/ml [3H]proline (NEN) and 50 μg/ml ascorbate (Sigma) were added for the final 4 h of treatment. Cells and media were collected by scraping and proteins were precipitated overnight in 20% (wt/vol) trichloroacetic acid at 4°C. Precipitated proteins were washed and digested with chromatographically purified collagenase (0.5 mg/ml; Worthington Biochemical) as described by Guarda et al. (14). The percent of total protein synthesis sensitive to collagenase was calculated as described by Guarda et al. (15).

Total protein synthesis. Fibroblasts were plated on 24-well plates and treated with H2O2, XXO, DHEA, or DDC alone or in combination with EUK-134 for 24 h in 100-mm dishes. The media were collected, centrifuged for 5 min at 500 g to remove cells and debris, lyophilized to dryness, resuspended in 1/20 volume of water, and protein was determined by the Bradford assay. MMP activity per 500 ng protein was measured by in-gel zymography with gelatin (Type A from porcine skin; Sigma) as the substrate. Samples were loaded under nonreducing conditions onto 4% stacking/10% separating SDS-polyacrylamide gels with 1 mg/ml gelatin polymerized in the separating gel and were electrophoresed at 15 mA while stacking and 20 mA while separating. After separation, gels were washed in 2.5% Triton X-100 for 30 min with gentle shaking and then were rinsed with water for an additional 30 min. MMP identity was confirmed by an additional 30-min incubation of selected gels with the serine protease inhibitor phenylmethylsulfonfluryl fluoride (PMSEF; 5 mM), or the metal chelators EDTA (10 mM), or 1,10-phenanthroline (1 mM). All gels were incubated overnight at 37°C in substrate buffer (50 mM Tris-Cl, pH 8.5, 5 mM CaCl2, and 0.02% Na2EDTA), stained in Coomassie blue R-250 in 7% acetic acid and 40% methanol, and then destained in 7% acetic acid and 40% methanol. Clear, digested regions representing MMP activity were quantified using an imaging densitometer (GS700; Bio-Rad), and molecular weights were estimated using prestained molecular weight markers.

Assessment of mRNA levels. Fibroblasts plated on 100-mm dishes were treated with H2O2 or XXO for 24 h. Total RNA was collected as previously described (34). Northern blots and hybridizations were performed as previously described (34) except for the hybridization buffer (100 μg/ml herring sperm DNA, 20% (vol/vol) dextran sulfate, 1% (wt/vol) SDS, 50% (vol/vol) formamide, and 15 mM NaCl). cDNAs for pro-collagen α1(1), α1(II), α1(III), α2(IV), α2(V), and fibronecin (American Type Culture Collection) were labeled with [32P]dCTP (NEN) as previously described (34). Blots were exposed to storage phosphor screens (Molecular Imaging Screen B; Bio-Rad) for 2–3 h and quantified with a storage phosphor imager (GS-563; Bio-Rad), or exposed to XO-.
oxIdative stress regulates fibrillar collagen

Effect of oxidative stress on collagen synthesis. Exposure of neonatal rat cardiac fibroblasts to H$_2$O$_2$ or XXO for 24 h had concentration-dependent effects on cell number. At the highest concentrations used, H$_2$O$_2$ (5 μM) and XXO (xanthine, 500 μM; xanthine oxidase, 0.1 mU/ml) decreased cell number by 18 ± 10% (n = 4; P = not significant (NS)) and 38 ± 7% (n = 12, P < 0.001), respectively. Lower concentrations of H$_2$O$_2$ (0.05 and 0.5 μM) or XXO (xanthine oxidase, 0.001–0.1 mU/ml) had no effect on cell number (data not shown). After correction for cell loss, neither H$_2$O$_2$ nor XXO had an effect on total protein synthesis as reflected by $^3$H]leucine incorporation (Fig. 1A). However, both H$_2$O$_2$ and XXO caused a concentration-dependent decrease in collagen synthesis measured as collagenase-sensitive $^3$H]proline incorporation normalized to total protein synthesis (Fig. 1A).

H$_2$O$_2$ and XXO had similar effects in adult rat cardiac fibroblasts. H$_2$O$_2$ (5 μM) and XXO (500 μM + 0.1 mU/ml) decreased cell number by 26 ± 2% and 43 ± 17% (n = 3; P < 0.05), respectively. Neither XXO nor H$_2$O$_2$ had an effect on total protein synthesis (n = 3; P = NS; Fig. 1B). However, XXO and H$_2$O$_2$ decreased collagen synthesis (normalized to total protein synthesis) by 24 ± 7% and 19 ± 6%, respectively (n = 3; P < 0.05; Fig. 1B).

Effect of oxidative stress on collagen mRNA expression. The effects of H$_2$O$_2$ (5 μM) and XXO (500 μM + 0.1 mU/ml) on collagen synthesis were further examined by Northern analysis. Treatment of neonatal fibroblasts with H$_2$O$_2$ or XXO for 24 h decreased the expression of mRNA for procollagens α$_1$(I), α$_2$(I), and α$_1$(III), the major fibrillar collagen forms in the rat heart (Fig. 2). In contrast, H$_2$O$_2$ and XXO tended to increase the expression of fibronectin (Fig. 2). H$_2$O$_2$ also decreased the expression of mRNA for the nonfibrillar procollagens α$_1$(IV) and α$_2$(IV), whereas XXO had no effect on the mRNA levels of α$_1$(IV) and α$_2$(IV) (Fig. 2).

Effect of oxidative stress on MMP activities. MMP activity in the media of cultures treated with H$_2$O$_2$ or XXO was determined by in-gel zymography using gelatin as the substrate. H$_2$O$_2$ and XXO each increased total MMP activity in neonatal and adult cardiac fibroblasts (Fig. 3). These effects were concentration dependent in neonatal fibroblasts (Fig. 3B). Specific bands corresponding to the molecular masses of MMP13 (57–55/48–45 kDa; type I collagenase), MMP2 (72/66 kDa; gelatinase A), and MMP 9 (95/88 kDa; gelatinase B) were increased by treatment with H$_2$O$_2$ and XXO in both neonatal and adult fibroblasts (Fig. 4). Notably, H$_2$O$_2$ and XXO increased both the proenzyme and active enzyme bands for MMP13, MMP2, and MMP9.

All MMP activities were inhibited by the metal chelators EDTA and 1,10 phenanthroline, but not the serine protease inhibitor PMSF (data not shown), confirming their identity as MMPs.

Inhibition of SOD and G6PD increases oxidative stress in cardiac fibroblasts. Treatment of neonatal fibroblasts with 100 μM DDC for 24 h inhibited total SOD activity −54 ± 19% (n = 3; P < 0.05) and increased superoxide production 87 ± 5% (n = 3; P = 0.013; Fig. 5A). Treatment of neonatal fibroblasts with 10 μM DHEA for 24 h inhibited G6PD activity by 23 ± 6% (n = 5; P = 0.015) and increased H$_2$O$_2$ production.
Effects of SOD and G6PD inhibition of collagen synthesis and MMP activity. Treatment (24 h) of neonatal cardiac fibroblasts with 100 μM DDC or 10 μM DHEA increased total MMP activity by 33 ± 4% (n = 11; P < 0.001; Fig. 6C) and 64 ± 20% (n = 7; P = 0.001; Fig. 6D), respectively. The effects of DDC and DHEA on collagen synthesis and MMP activation were inhibited by the SOD/catalase mimetic EUK-134 (Fig. 6, A–D).

**Discussion**

The major new finding of this study is that ROS may have profound effects on collagen metabolism in cardiac fibroblasts by affecting both synthesis and the activity of degradative enzymes. H₂O₂ and XXO each decreased collagen synthesis (as measured by collagenase-sensitive [³H]proline incorporation) and decreased the abundance of mRNAs for procollagens α₁(I), α₂(I), and α₁(III). Likewise, H₂O₂ and XXO caused an increase in MMP activity as measured by in-gel zymography. These effects were mimicked by inhibition of endogenous antioxidant systems and were reversed by an SOD/catalase mimetic.

ROS inhibit collagen synthesis. H₂O₂ and XXO each decreased collagenase-sensitive [³H]proline incorporation. This effect was not due to a generalized depression in protein synthesis since it occurred without a decrease in overall protein synthesis as reflected by [³H]leucine incorporation. The synthesis of collagen is regulated at the transcriptional and posttranslational levels (20). H₂O₂ and XXO decreased the levels of mRNA for procollagens α₁(I), α₂(I), and α₁(III), indicating that the ROS-stimulated decrease in collagen synthesis was due, at least in part, to a decrease in mRNA transcription and/or stability.

In cardiac fibroblasts, prostaglandin E₂ (4) and phorbol esters are known to decrease collagen synthesis (13). On the other hand, several other stimuli are known to increase collagen synthesis in cardiac fibroblasts, including endothelin (14), aldosterone (4), mechanical stretch (6), transforming growth factor-β₁ (TGF-β₁) (13), and angiotensin (4). TGF-β₁ and angiotensin also stimulate the proliferation of cardiac fibroblasts.
ROS stimulate collagen degradation. Collagen degradation is regulated by the activity of extracellular MMPs, which is determined by both transcriptional and posttranslational mechanisms. Posttranscriptional regulation occurs through the activation of latent proenzymes (pMMPs) by factors such as serum (39), heparin (40), and prostaglandin E2 (4). Conversely, the activation of MMPs is opposed by the endogenous tissue inhibitors of metalloproteinase (TIMPs) (22).

H2O2 and XXO each increased total MMP activity as measured by in-gel zymography in both neonatal and adult fibroblasts. H2O2 and XXO increased the bands corresponding to MMP13, MMP2, and MMP9. The increases were due to both pMMPs and active MMPs, suggesting that the effects of ROS were mediated at both the transcriptional and posttranscriptional levels. ROS have previously been shown to cause direct activation of latent pMMPs in conditioned media from cardiac fibroblasts in vitro (38, 41). In this study, H2O2 and XXO were added to fresh medium in which pMMPs had not had time to accumulate. Therefore the increase in pMMPs and the activation of pMMPs in our experiments was likely not due to the oxidative burst of the stimuli, which lasted 1–2 h (data not shown).

Regulation of MMPs by oxidative stress has been shown in noncardiac cells. Hyperoxia increases expression of pMMP2 and pMMP9 mRNA in rat lung (25). XXO increases expression of pMMP2 and decreases expression of TIMP2 in dermal fibroblasts (19). H2O2 increases expression of pMMP2, pMMP9, and MMP14 (which is responsible for pMMP2 activation) proteins and activates pMMP2 in human venous endothelial cells (2).

Inhibition of endogenous antioxidant systems. Antioxidant enzymes including SODs, catalase, and peroxidases regulate ROS by maintaining superoxide and H2O2 at low levels. DDC and DHEA each decreased collagen synthesis and increased MMP activity. DDC is a Cu chelator that inhibits CuZn-SOD and extracellular SOD (16). DHEA inhibits G6PD, which catalyzes...
the formation of NADPH during the conversion of glucose 6-phosphate to 6-phosphogluconate. NADPH is needed for the activity of both glutathione peroxidase and catalase, which are important enzymes in the conversion of \( \text{H}_2\text{O}_2 \) to water. The role of G6PD as an antioxidant enzyme has been shown by targeted disruption and overexpression experiments (24, 26, 29). DDC and DHEA caused modest increases in ROS, and their effects on collagen synthesis and MMP activity were prevented by EUK-134, an antioxidant SOD/
catalase mimetic, suggesting that their effects were mediated by ROS (1). The ability of DDC and DHEA to mimic the effects of H$_2$O$_2$ and XOO further indicate that the effects of ROS demonstrated here can occur at levels of ROS that can be made by the cardiac fibroblast.

**Implications.** ROS are increased in failing myocardium (21). There may be increased production of ROS due to mechanical strain (7), stimulated by substances such as angiotensin (23) or inflammatory cytokines (23), decreased activity of mitochondrial electron transport (18), and/or decreases in antioxidant systems (e.g., SOD and glutathione peroxidase) (17). The demonstration that ROS can cause both a decrease in fibrillar collagen synthesis and an increase in MMP activity suggests that ROS could play an important role in the pathophysiologic remodeling of the heart.

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