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ALCOHOL MODULATION OF MMP AND TIMP EXPRESSION IN THE HEART FAVORS COLLAGEN ACCUMULATION

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Abstract

Background—Chronic alcohol consumption has been shown in human and animal studies to result in collagen accumulation, myocardial fibrosis, and heart failure. Cardiac fibroblasts produce collagen and regulate extracellular matrix (ECM) homeostasis through the synthesis and activity of matrix metalloproteinases (MMP) and tissue inhibitors of MMPs (TIMP), with the balance of MMPs/TIMPs determining the rate of collagen turnover. Dynamic changes of MMP and TIMP expression were reported in alcohol induced hepatic fibrosis; however, the effect of alcohol on MMP/TIMP balance in the heart and cardiac fibroblasts is unknown. We hypothesized that alcohol exposure alters cardiac fibroblast MMP and TIMP expression to promote collagen accumulation in the heart.

Methods—Cardiac fibroblasts isolated from adult rats were cultured in the presence of alcohol (12.5–200 mM) for 48 hrs. MMP, TIMP, and collagen type I and III expression were assayed by Western blot analysis. Hydroxyproline (HPro) was used as a marker of collagen production. The in vivo cardiac effects of ethanol were determined using rats exposed to ethanol vapor for two weeks, resulting in blood alcohol levels of 150–200 mg/dl. Cardiac collagen volume fraction (CVF), as well as MMP, TIMP and collagen expression, was assessed.

Results—Ethanol exposed rats exhibited upregulation of TIMP-1, -3 and -4 in the heart, with no significant increases in MMPs. Cardiac fibroblasts exhibited transformation to a profibrotic phenotype following exposure to alcohol. These changes were reflected by increased α -smooth muscle actin and collagen I and III expression, as well as increased collagen secretion. In vivo ethanol exposure also produced fibrosis, indicated by increased CVF and expression of collagens.

Conclusion—Alcohol exposure modulates cardiac fibroblast MMP/TIMP expression favoring a profile associated with collagen accumulation. Our data suggest that this disrupted MMP/TIMP profile may contribute to the development of myocardial fibrosis and cardiac dysfunction resulting from chronic alcohol abuse.

Keywords

extracellular matrix remodeling; ethanol; fibrosis; heart; proteinase

Introduction

Moderate alcohol consumption has been associated with a lower risk of coronary heart disease (Mukamal et al., 2005; Costanzo et al., 2010). However, reports on the impact of higher levels of alcohol intake on cardiovascular risk factors have not been consistent (Mukamal et al., 2005; Costanzo et al., 2010). Most studies indicate that chronic alcohol abuse contributes to the etiology of heart disease (Walsh et al., 2002). Several mechanisms have been proposed to contribute to alcohol-induced myocardial dysfunction (Rubbiati et al., 2000), including oxidative stress, mitochondrial and sarcoplasmic reticulum abnormalities in myocytes, cardiomyocyte hypertrophy, and cardiac fibrosis (Ferrans et al., 1965; Brown et al., 1999; Djoussé and Gaziano, 2007).

Cardiac fibroblasts, which constitute approximately 70% of the total number of cells in the heart, regulate extracellular matrix (ECM) homeostasis (Borg and Baudino, 2011). The synthesis of collagen types I and III, which form a supporting network for cardiac cell alignment, interaction and communication, is a key function of cardiac fibroblasts. Fibroblasts can modify collagen production and degradation by modulating the expression and activity of matrix metalloproteinases (MMPs), which degrade collagen, and tissue inhibitors of metalloproteinases (TIMPs) (Truter et al., 2009). TIMPs are endogenous MMP inhibitors that provide post-translational control of MMP activity by binding to MMP pro- and active forms (Nagase et al., 2006).

Under normal conditions, the activity of most MMPs is low or negligible in cardiac tissue. However, conditions associated with increased expression of inflammatory cytokines, growth hormones, and oxidative stress lead to activation of MMPs (Siwik et al., 2001; Lindsey et al., 2005). Upregulation of MMP-2, MT1-MMP and MMP-9 have been identified in the hypertrophied heart, various cardiomyopathies, and heart failure (Moshal et al., 2005; Bergman et al., 2007; Lindsey and Zamilpa, 2010). Clinical studies have demonstrated that increased MMP-2, MT1-MMP and MMP-9 contribute to myocardial fibrosis by activating pro-fibrotic signaling molecules and are associated with heart dysfunction (Zile et al., 2011). Given their established role in cardiac remodeling, our assessments focused on these key MMPs. TIMP-1, -2, -3, and -4 are expressed in the heart, and elevations of TIMP expression are associated with fibrosis in the heart and other tissues (Nagase et al., 2006; Truter et al., 2009). There is in vitro evidence of TIMP specificity for certain MMPs, such as TIMP-1/MMP-9, TIMP-2/MMP-2, and TIMP-4/MT1-MMP; however, the interaction of MMPs and TIMPs is not as simple as enzyme and inhibitor. For example, both MT1-MMP and TIMP-2 are involved in the activation of pro-MMP-2 (Murphy et al., 1992). In this study, the effects of alcohol on expression of cardiac MMPs and TIMPs were assessed using in vitro and in vivo exposure models.

Although an increase of cardiac interstitial fibrosis has been reported in patients with alcoholic cardiomyopathy (Soufen et al., 2008), the role of altered MMP and TIMP

expression in cardiac fibroblasts of chronic alcoholics has not been investigated. However, many reports suggest that alcohol-mediated dysregulation of MMP/TIMP balance may be an underlying mechanism in several pathological conditions. Dynamic disarray of the MMP/TIMP balance has been reported in alcohol-induced liver fibrosis (Xu et al., 2004) and in alcohol-mediated cerebral dysfunction (Haorah et al., 2008). Moreover, alcohol reduces MMP-2 expression in smooth muscle cells (Fiotti et al., 2008) and increases MMP-9 activity in plasma of alcohol-fed rats (Koken et al., 2010). Whether alcohol affects the regulation of collagen synthesis or the balance of MMP and TIMP expression by cardiac fibroblasts has not been investigated.

We and others previously reported that myocardial hypertrophy and heart dysfunction are associated with increased collagen type I and III expression (Voloshenyuk and Gardner, 2010; Mukherjee and Sen, 1991). Similar increases in collagen expression have been reported in human alcoholic cardiomyopathy (Soufen et al., 2008). However, whether alcohol produces these alterations in collagen expression by altering cardiac fibroblast production of collagen, MMPs or TIMPs has not been previously investigated.

The aim of this study was to examine the effects of alcohol exposure on the cardiac expression of collagens, MMPs and TIMPs using isolated cardiac fibroblasts and a rodent model of alcohol abuse. Our findings provide insight into likely mediators of ECM remodeling that may contribute to myocardial fibrosis resulting from alcohol abuse.

Materials and Methods

In vitro cardiac fibroblast alcohol exposure model

Adult cardiac fibroblasts were isolated from Sprague-Dawley rats (~10 wks age) by enzymatic digestion as previously described (Voloshenyuk et al., 2011) and detailed in Supplemental Material. All experimental procedures were approved by Louisiana State University Health Sciences Center Institutional Animal Care and Use Committee. Fibroblasts were incubated in the presence of alcohol (12.5–200 mM) for 48 h. An equal volume of fibroblast media was added to the control cells and fibroblasts were incubated for 48 hrs at the same conditions as alcohol treated cells.

In vivo rodent alcohol exposure model

Male Sprague-Dawley rats (~10 wks age) were exposed daily to intermittent (14 hr ON/10 hr OFF) ethanol vapor for two weeks in vapor chambers (La Jolla Alcohol Research, Inc., La Jolla, CA), as previously described (Gilpin, et al., 2008). Vapor settings were adjusted to produce blood-alcohol levels (BALs) between 150–200 mg/dl (Analox Instruments). This combination of EtOH exposure pattern and dose allows animals to achieve daily intoxication and withdrawal during the exposure, as in the human condition. Controls were exposed to ambient room air. At the experimental endpoint, hearts were removed under isoflurane anesthesia (4%) for molecular and histological analyses.

Protein expression

Western blot analyses of protein expression in fibroblast lysates and LV homogenates were performed as previously described (Voloshenyuk et al., 2011). Primary antibodies and reagents are listed in Supplemental Material. Densitometries were normalized by either GAPDH or β -actin expression, or Ponceau-S staining of protein.

MMP activity assay by zymography

30 μ g of protein from each cellular sample was separated by electrophoresis in nonreducing conditions on a 10% SDS-PAGE gel containing gelatin (1 mg/ml) from porcine skin (Sigma Chemicals, St Louis, MO), as previously described (Siwik et al., 2001).

Hydroxyproline (Hpro)

Two ml of fibroblast conditioned media were hydrolyzed in 6N HCl at 110 °C for 24 hours and followed by hydroxyproline determination, as described in Supplemental Material (Stegemann, 1958).

Statistical analysis

All results are expressed as the Mean $\pm$ SEM. One-way ANOVA was used to determine the differences between control and experimental conditions. Post hoc analysis was Bonferroni's multiple comparison test, with $P < 0.05$ considered significant. The immunoblotting data were normalized by GAPDH or β -actin expression, and expressed as a percentage of the average values for the appropriate control group. No significant differences in GAPDH or β -actin expression were found between the study groups. Data processing and statistical analyses were performed using Microsoft Excel and Graphpad analysis software (version 5.0, Prism, San Diego).

RESULTS

In vitro: alcohol differentially modulates expression and activity of MMP in cardiac fibroblasts

Expression and activity of MMP-2 and -9 were assessed, as these MMPs are linked to adverse cardiac ECM remodeling and their activation occurs at the end of the MMP activation cascade. MT1-MMP expression was also assessed, due to its association with fibrosis. As shown in Figure 1A, 12.5–25 mM of alcohol did not alter pro- (72 kDa) or active- (68 kDa) forms of MMP-2. However, following exposure to 50–200 mM alcohol, expression of both forms was significantly increased. MT1-MMP expression was markedly elevated only after exposure to 100–200 mM alcohol (3-fold versus control; $P < 0.05$, Figure 1B). MMP-2 activity in cardiac fibroblast culture media was significantly higher than control following 48 h of 50 mM and 100 mM alcohol exposure (25–50% increase; $P < 0.05$, Figure 1C). However, at the highest dose of alcohol, 200 mM, MMP-2 activity was not different from control. MMP-9 expression and activity were not detectable in fibroblasts from any of the experimental conditions. This is consistent with previous reports that MMP-9 expression is increased during inflammation and heart failure, and rarely in the normal myocardium (Okada et al., 2010).

In vitro: alcohol increased TIMP expression in a concentration-specific pattern in cardiac fibroblasts

Expression of TIMP-1, an inhibitor of MMP-9, was increased following 50–200 mM alcohol concentration (Figure 2A). Expression of TIMP-2, a well-known MMP-2 inhibitor, was not altered by alcohol 12.5–50 mM, but was significantly increased 3-fold versus control after exposure to 100 and 200 mM alcohol (Figure 2B). TIMP-3 showed significant upregulation following exposure to 25–50 mM alcohol, and was further increased after 100–200 mM alcohol exposure (Figure 2C). TIMP-4 was not detected in cardiac fibroblasts.

In vitro: alcohol produced a pro-fibrotic phenotype in cardiac fibroblasts

α -smooth muscle actin (SMA) is a marker of increased synthesis of profibrotic proteins in cardiac fibroblasts and has been shown to be increased in fibroblasts undergoing transition from normal to the myofibroblast-like phenotype (Teunissen et al, 2007). As shown in Figure 2D, fibroblasts exposed to 50–200 mM alcohol had greater expression of α -SMA ($p < 0.05$ vs control). Collagen type I and type III are the main structural fibrillar components of the heart; however, each has different physical properties. Collagen type I confers a stiffness and resistance to stretch, while collagen type III has greater distensibility (Mukherjee D, Sen S, 1991). To better understand the mechanism by which alcohol contributes to myocardial fibrosis, we assessed the type of collagen expressed by fibroblasts exposed to increased concentrations of alcohol. As shown in Figure 3A, western blot analysis found significant upregulation of both collagen types in cardiac fibroblasts exposed to 50–200 mM alcohol. Hydroxyproline (HPro) is a reliable marker of total collagen content (Mukherjee D, Sen S, 1991), and was measured in the fibroblast media to determine the amount of collagen secreted by fibroblasts. As shown in Figure 3B, HPro concentration was significantly increased in cardiac fibroblast conditioned media of cells exposed to 50–200 mM of alcohol. Concentration of HPro in the control fibroblasts media was 4.55 ± 0.43 $\mu\text{g/ml}$.

In vivo exposure to alcohol did not significantly increase cardiac MMP expression

LV freewall homogenates were assessed for protein expression for the active forms of MMP-2, -9 and MT1-MMP by western blot. In contrast to our findings in isolated fibroblasts, two weeks of alcohol exposure had no significant effect on MMP expression (Figure 4). The proforms of MMP-2 and -9 followed similar trends as the active forms, with no significant increase versus controls (data not shown).

In vivo exposure to alcohol differentially regulated cardiac TIMP expression

TIMP-1, -3, and -4 expression were significantly increased in hearts of alcohol-exposed rats ($p < 0.05$ versus Air; Figure 5), with TIMP-4 exhibiting the greatest increase (56% versus Air). Cardiac TIMP-2 expression was not significantly increased by alcohol.

In vivo exposure to alcohol increased cardiac collagen

Much like our findings in cultured cardiac fibroblasts, alcohol caused increased expression of both collagen types I and III in the hearts of exposed rats ($p < 0.05$ versus Air; Figure 6A). Cardiac expression of α -SMA was also significantly increased by alcohol (Figure 6B).

These increases in expression were associated with increased LV collagen staining in alcohol-exposed rats, as assessed by collagen volume fraction (CVF; Figure 6C).

Discussion

We investigated the effects of alcohol on cardiac production of collagen, and MMP and TIMP enzymes using a rodent model of in vivo exposure and cultured cardiac fibroblasts. Alcohol differentially modulated expression and activity of MMPs and TIMPs. In our cellular studies, we found that exposure to 50 mM ethanol promoted MMP-2 expression and activation, but concordant upregulation of TIMP-2 expression occurred only at higher concentrations. In contrast, in vivo exposure to alcohol did not increase MMP-2 or TIMP-2 expression. Our cellular results demonstrated that alcohol upregulated fibroblast expression of TIMP-1 and TIMP-3, without a parallel activation of MMP-9. Likewise in cardiac tissue from alcohol-exposed rats, TIMP-1 and -3 expression were significantly increased, with no increase in MMP-9. Fibroblasts expression of MT1-MMP was only increased at very high doses of alcohol, and was not significantly elevated in cardiac tissue. Though not detected in isolated fibroblasts, TIMP-4 was increased in hearts of alcohol-exposed rats. Although the cellular and in vivo data are largely in agreement, it is interesting to note that alcohol increased the fibroblast expression of MMPs primarily at doses at or above 50 mM. This level of alcohol is reaching the upper limit of physiologic dosing, which may explain why MMPs were not elevated in vivo in rats with BALs of 150–200 mg/dl (~32–43 mM). The cardiac MMP/TIMP ratio clearly favored collagen accumulation in our in vivo exposure model. The discordant upregulation of MMPs and TIMPs was associated with profibrotic transformation of cardiac fibroblasts, as evidenced by expression of α -SMA and increased collagen type I and III expression, as well as increased collagen secretion by fibroblasts. Likewise, alcohol-exposed rats exhibited increased LV collagen staining and expression of collagens and α -SMA. Taken together these results provide evidence for alcohol-mediated alterations in collagen synthesis and identify increased cardiac TIMPs as a potential mechanism underlying alcoholic cardiomyopathy and fibrosis.

Our collagen expression results are in agreement with reports that clinical alcoholic cardiomyopathy is associated with increased collagen expression and total collagen content (Soufen et al, 2008). This change in collagen can directly affect cardiac structure and function, and may play a role in the progression of alcoholic cardiomyopathy (Brown et al., 1999; Brower et al, 2006; Collins et al., 2009; Graham et al, 2008; Mukamal et al., 2005; Piano, 2002; Walsh et al., 2002). Although few animal studies focus on the effects of ethanol on the ECM, our findings are in agreement with those of Law, et al, (2012) who reported fibrosis in the hearts of alcohol-fed mice. Thus, we speculate that alcohol-dependent alterations of the collagenous ECM may contribute to the underlying pathophysiological mechanisms of alcoholic cardiomyopathy.

The importance of interaction between the MMPs and TIMPs has been demonstrated by several studies. TIMP-1 has high affinity and rate of MMP-9 inhibition, while TIMP-3 effectively inhibits MMP-9, MMP-2, and MT1-MMP (Nagase, et al., 2006). Increased expression or an imbalance of MMPs and TIMPs plays a critical role in myocardial ECM remodeling, and is associated with collagen accumulation in numerous cardiomyopathies

(Lindsey and Lee, 2000; Graham et al., 2008). Previous reports in the literature support the involvement of alcohol-mediated alteration of MMP/TIMP balance in the pathophysiology of fibrotic changes associated with alcohol abuse. Xu et al. (2004), demonstrated that the alcohol dependent increase of TIMP-1 expression contributes greatly to the progression of liver fibrosis. Moreover, increased MMP-2 expression and activity in response to acute alcohol exposure was reported in brain microvascular endothelial cells (Haoran et al., 2008). In contrast, moderate and long term alcohol consumption decreases MMP-2 activity in human sera, isolated smooth muscle cells (Fiotti et al, 2008), and in liver of alcohol-fed rats (Vizzutti et al., 2010). We also demonstrated differential effects of alcohol concentration on MMP-2 activity. Alcohol at lower concentration (100 mM) activated MMP-2 in cardiac fibroblasts, whereas at 200 mM, no changes were noted. Similar results have been obtained with breast cancer cells, in which low alcohol concentrations (<100 mM) activate MMP-2, whereas higher concentrations (100–200 mM) inhibit MMP-2 activity (Aye et al., 2004; Luo, 2006). Because MMP-2 degrades fibrillar collagen breakdown products in addition to newly synthesized collagen types I and III (Nagase et al., 2006), we can speculate that the attenuated MMP-2 activity may predispose the heart to collagen accumulation and contribute to myocardial fibrosis. Unlike our cellular data, our in vivo findings indicate that MMP-2 was not increased by ethanol. These differences may be associated with dosing of ethanol or duration of exposure, as mentioned above.

The dose-dependent alcohol effects on fibroblast MMP-2 expression and activity could also be related to its differential effects on TIMP-2 expression. TIMP-2, at low concentration, has a unique property of activating MMP-2 through formation of a tri-molecular complex with pro-MMP2 and MT1-MMP, and inhibits MMP-2 at higher concentration (Murphy et al., 1992). This is in accordance with our findings that increased alcohol concentration enhanced TIMP-2 expression and prevented MMP-2 activation in the fibroblasts.

The increased secretion of collagen by fibroblasts at higher alcohol concentration (100–200 mM) may be explained also by enhanced expression of membrane-bound MT1-MMP. Spinale et al., (2010) demonstrated that cardiac specific overexpression of MT1-MMP increases collagen accumulation and leads to myocardial fibrosis following myocardial infarction. Similar results of increased MT1-MMP and concomitant fibrosis were also recently reported in a murine model of pressure overload (Zile et al., 2012). Despite these in vitro findings, we found no significant elevation of cardiac MT1-MMP in rats exposed to ethanol. As before, these dissimilarities could be due to the differences in alcohol dose, as MT1-MMP expression was only increased at high doses (100–200 mM), and the in vivo dosing was <50 mM. Regardless, the balance of MMPs/TIMPs in our in vivo studies favored collagen deposition. Our data suggests that alcohol-dependent activation of cardiac fibroblasts may promote collagen accumulation and contribute to myocardial remodeling in alcohol abusers. Ethanol caused increased expression of α -SMA, a marker of fibroblasts activation and transformation to myofibroblasts, in both isolated fibroblasts and hearts of exposed rats. These findings are in agreement with those of Law and Carver, 2013, who found that ethanol causes activation of cardiac fibroblasts by a transforming growth factor (TGF)- β dependent mechanism.

Study Limitations

Our assessment of cardiac MMPs was limited to MMP-2, -9 and MT1-MMP. However, many other MMPs are expressed in the myocardium, including MMP-1, -3, -7, -8, and -13, which may play a role in alcohol induced fibrosis. Nevertheless, our data indicate ventricular collagen accumulation and increased expression of collagens in the hearts of ethanol-exposed rats. While our data suggest that increased cardiac TIMPs contribute to alcoholic fibrosis, our studies did not identify specific mechanisms. Future studies are warranted to address mechanisms of increased cardiac TIMPs and fibrosis, including alcohol induced oxidant stress, the potential role of ethanol metabolites, and the profibrotic transformation and activation of cardiac fibroblasts.

Conclusion

Our studies provide evidence of alcohol-induced fibrosis in both isolated cardiac fibroblasts and a rodent model of alcohol abuse. These data are the first to demonstrate that alcohol modifies MMP and TIMP expression by cardiac fibroblasts and in the hearts of exposed rats to favor collagen accumulation. These data also provide insight into the ECM-dependent molecular mechanisms that contribute to alcohol-induced myocardial fibrosis. Future studies will explore potential therapeutic approaches for prevention of alcohol-mediated myocardial remodeling and fibrosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. MMP-2 and MT1-MMP protein expression in cardiac fibroblasts, and MMP-2 activity in cell-conditioned media

Values reflect a minimum of three independent experiments (* $P < 0.05$ vs. control). Values are mean $\pm$ SEM protein densities normalized to actin expressed as percent of untreated (0 mM ALC) control cardiac fibroblasts. (A) MMP-2 expression in cardiac fibroblasts determined by Western blot analysis following exposure to alcohol (12.5–200 mM) for 48 hrs. (B) MT1-MMP expression in cardiac fibroblasts determined by Western blot analysis following alcohol treatment (12.5–200 mM) for 48 hrs. (C) MMP-2 activity determined by

zymography in cardiac fibroblast culture media following exposure to alcohol (12.5–200 mM) for 48 hrs.

Figure 2. TIMP-1, TIMP-2, TIMP-3, and α-smoothmuscle actin (SMA) protein expression in cardiac fibroblasts

(A) TIMP-1, (B) TIMP-2, (C) TIMP-3, and (D) α-SMA protein expression in cardiac fibroblasts determined by Western blot analysis following exposure to alcohol (ALC; 12.5–200 mM) for 48 hrs. Values are mean $\pm$ SEM. Protein densities were normalized to β-Actin or Ponceau stain and expressed as percent of vehicle treated control fibroblasts (0 mM ALC). Values reflect a minimum of three independent experiments (* $P < 0.05$ vs. control; † $P < 0.05$ vs. 100 mM of ALC.).

Figure 3. Collagen type I and III expression and collagen secretion in cardiac fibroblasts culture
(A) Collagen I and III expression in cardiac fibroblasts determined by Western blot analysis following exposure to alcohol (0–200 mM) for 48 hrs. Values are mean $\pm$ SEM protein densities normalized to actin expressed as percent of control (0 mM ALC) cardiac fibroblasts. **(B)** Hydroxyproline (HPro) concentration in cardiac fibroblast conditioned media. HPro is an indicator of collagen secretion and was measured following exposure to 50–200 mM ALC for 48 hrs. Values are mean $\pm$ SEM expressed as percent of control (0 mM ALC) cardiac fibroblasts. Values reflect a minimum of three independent experiments (* $P < 0.05$ vs. control).

Figure 4. Cardiac MMP expression of air- and ethanol-exposed rats

Left ventricular (LV) freewall homogenates were assessed for MMP expression by western blot. Ethanol (EtOH) did not significantly increase cardiac expression of (A) MMP-2 (active; 68 kDa), (B) MMP-9 (active; 90 kDa), or (C) MT1-MMP (63 kDa) relative to air-exposed rats (n=6 Air; n=7 EtOH for all western blot analysis of LV tissue). (D) Representative blots. Densitometry normalized to GAPDH (37 kDa) and then expressed as percentage of air-exposed controls (mean $\pm$ SE).

Figure 5. Cardiac TIMP expression from air- and ethanol-exposed rats

TIMP protein expression was determined in LV freewall homogenates by western blot. Ethanol (EtOH) significantly increased cardiac expression of (A) TIMP-1 (31 kDa), (C) TIMP-3 (36 kDa), and (D) TIMP-4 (26 kDa), but not (B) TIMP-2 (24 kDa). Densitometry was normalized to GAPDH and then expressed as percentage of Air-exposed controls (* $p < 0.05$). (E) Representative blots. Vertical line in TIMP-4 blot demarcates two distinct western blots.

Figure 6. Ethanol produced a profibrotic cardiac response

(A) Collagen I and III protein expression was determined in LV freewall homogenates by western blot (75 and 125 kDa, respectively). Ethanol (EtOH) significantly increased the cardiac expression of both collagens I and III, as well as (B) α-smooth muscle actin (SMA; 43 kDa). Densitometry was normalized to β-actin (43 kDa) and expressed as percentage of Air-exposed controls (* $p < 0.05$). (C) Ethanol increased LV interstitial collagen volume fraction (CVF) in exposed rats. Fixed (4% paraformaldehyde) mid-LV sections were stained with collagen-specific picrosirius red. Avoiding perivascular collagen, twenty images were collected per slide, and collagen staining expressed as % total myocardial area (* $p < 0.05$; $n = 6$ Air; $n = 7$ EtOH).