Cyclical mechanical stretch modulates expression of collagen I and collagen III by PKC and tyrosine kinase in cardiac fibroblasts

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The Extracellular Matrix (ECM) provides the physical microenvironment in which cells live and a substrate for cell anchorage. It serves as a tissue scaffold and is a dynamic structure whose organization and composition modulate various cellular processes including cell proliferation, migration, differentiation, and survival. The cardiac ECM consists of the main components collagen type I and III. The fibroblasts are responsible for the expression and degradation of ECM proteins.

The different patterns of ECM remodeling in the heart depend on altered mechanical and chemical conditions and can contribute to cardiac dysfunction. Several groups of factors including growth factors, such as TGF-β, angiotensin II, and PDGF, as well as cytokines, affect the collagen expression and/or the growth of fibroblasts. Myocardial hypertrophy, in response to changes in mechanical tension, expression and/or the growth of fibroblasts, affects the collagen of ECM proteins. Fibroblasts are responsible for collagen expression and degradation. The cardiac ECM consists of the main components collagen type I and III. The fibroblasts are responsible for the expression and degradation of ECM proteins.

The experimental protocols for rats were approved by the Martin Luther University Halle-Wittenberg Institutional Animal Care and Use Committee and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, (National Institutes of Health publication no. 85–23, revised 1996).

Cell culture and stretch. Fibroblasts were isolated from both atrial and ventricular tissue of adult male rat (292 ± 14 g body wt; n = 20) hearts (1.14 ± 0.11 g heart wt) by means of retrograde perfusion of collagenase-containing solutions. Details have been reported previously. After the perfusion, the cell suspension was centrifuged at 700 rpm for 5 min at room temperature. The cell pellet was resus-
pended in DMEM/medium 199 (Earle’s salts) in the ratio 4:1 and 10% FCS (Sigma) containing 1% penicillin/streptomycin (Sigma) and 10 μg/ml amikacin (Sigma). Cells were grown to confluency and then passed once to culture on the Bioflex culture plates coated with collagen I within 24 h. For the experiments, each well of cells got fresh DMEM/medium 199 containing either one of four different serum concentrations (0%, 0.5%, 5%, 10% FCS). The used inhibitors were 1 μM RO-31-8220 (40) or 5 μM chelerythrine chloride (28) for PKC inhibition; 1 μM herbinycin A (46), 5 μM SU6656 (7), or 25 μM SU4984 (36) for tyrosine kinase inhibition; and 3 μM H89 (41) or 3 μM KT5720 (37) for PKA inhibition (all inhibitors from Calbiochem) without or with 10% FCS. For the stretch experiments, the Bioflex culture plates were put in the gasket with the Loading Stations of the Flexercell Strain Unit (Flexercell; McKeepes, PA) in a tissue incubator (5% CO₂, 37°C). The stretch was carried out with the Bioflex Loading Stations with a diameter of 25 mm per well, resulting in an equally uniform stretch in all directions. The parameter of elongation had to apply to every direction. It was not in existence in the different levels of stretch. That means every cell obtains the same strength of stretch. The Flexercell computer system connected the unit with a vacuum pump and controlled the stretch parameters (2). The unit cyclically stretched the foil with the adherent fibroblasts at a frequency of 0.33 Hz and duration of 24 h. Three different elongations (3%, 6%, 9%) were investigated. The control groups were handled in the same way but without cyclical deformation. Each set of experiments was performed from at least four different fibroblast isolations. Every resulting value of each experimental set is related to its own control value.

RNase protection assay. Total RNA isolation was performed according to a modified phenol/guanidiniumthiocyanat method of Chomczynski and Sacchi (18) using Trizol (Gibco-BRL). Five micrograms of total RNA was used in the RNase protection assay (RPA) with the probe template set labeled with RiboQuant In Vitro Transcription Kit (final probe concentration: 4 × 10⁶ cpm/μl; Pharmingen) and [α-32P]UTP (3,000 Ci/mmol; Amer sham), as described by the manufacturer. After hybridization (56°C; 12–16 h), the unhybridized riboprobes were digested with a mixture of RNases A and T1 (RiboQuant RPA kit; Pharmingen), according to the manufacturer’s instructions. Protected probes were displayed by electrophoresis on a denaturing gel containing 5% polyacrylamide/8 M urea followed by visualization with the Molecular Imaging (Bio-Rad). The densitometric quantification of the individual bands of the RPA assays was performed by the Multi-Analyst program version 1.1 (Bio-Rad). The probe template set contained the following cDNA (probe length in bp/protected): rat collagen I (504/449), rat collagen III (286/211), rat collagen II (190/142), rat collagen IV (213/156), rat collagen XVII (213/156), rat collagen XVIII (213/156), rat collagen I (369/269), and GAPDH (225/161) (228/82) (8).

Immunofluorescence. The cultured fibroblasts were fixed by a solution containing 4% paraformaldehyde (pH 7.4). After being permeabilized with 1% Triton X-100 and rinsed with PBS containing 3% wt/vol bovine serum albumin. As primary antibodies, rabbit antibodies against vimentin (ab7783; Abcam), discoidin domain receptor 2 (DDR2; ab5520; Abcam) and collagen I (ab292; Abcam and 234167; Calbiochem), as well as mouse antibodies against β-actin (A5441; Sigma), nonmuscle heavy chain myosin (ab684; Abcam), α-smooth muscle-specific actin Ab-2 (CP47; Calbiochem), and collagen III (C7805; Sigma) were used in a dilution of 1:50 and incubated for 24 h at 4°C. After being washed, cells were incubated with secondary antibodies against rabbit or mouse connected with fluorescence label Oregon Green 488 (Molecular Probes) for 5 h at room temperature, followed by being washed and mounted onto glass slides. The fibroblasts were analyzed by confocal microscopy (Radiance 2000; Bio-Rad), and the images were processed by using the software MetaMorph Imaging System (Microsoft).

Determination of collagen secretion. Collagen I and collagen III secretion was determined by ELISA. Media and collagen standards (Sigma) were incubated for 24 h in 96-well Nunc-ImmuNo-Maxisorp plates (Nalge Nunc International) followed by washing and blocking with 2% bovine serum album. Subsequently, the wells were incubated with rabbit antibody against collagen I or III (1:1,000; Biotrend) for 1 h at room temperature. After three washes with 0.05% Tween in PBS, horseradish peroxidase-conjugated secondary antibody (1:5,000; Biotrend) was applied for 1 h at room temperature. After three further washes, the wells were incubated with o-phenylenediamine (Sigma), and the reaction was stopped after 15 min with 1 N H₂SO₄. The absorbance at 490 nm was determined by using a multimwell-multilabel reader (25). The protein content of fibroblast lysates was analyzed with the Bio-Rad protein assay. The content of collagen I and collagen III in the media was related to the protein content of the fibroblasts.

Statistical analysis. Data are expressed as means ± SD. The differences were assessed by one-way ANOVA combined with the Bonferroni test. A value of P < 0.05 was considered to be statistically significant.

RESULTS

Characterization of fibroblasts culture. The cultured fibroblasts used in collagen-expressing experiments were identified as a pure culture of fibroblasts showed by labeling of vimentin and the collagen receptor DDR2 in Fig. 1, A and B. In all cells, a diffused distribution of vimentin was detected. A comparable pattern to vimentin was obtained by labeling with DDR2. A lot of DDR2 spots were seen in many cells. Stress fibers consisting of β-actin were recognized only in few border regions (Fig. 1C). Otherwise, the labeling of β-actin showed a diffused distribution in all cells. The pattern of nonmuscle myosin labeling revealed unarranged fibers in all fibroblasts (Fig. 1D) which did not localize in the border regions. The identity of α-smooth muscle actin-positive cells resulted in < 10% α-smooth muscle actin-positive fibroblasts (Fig. 1E). The occasional visible α-smooth muscle actin fibers did not allow terming these cells as myofibroblasts. The residual light of the cells of Fig. 1E was used to show the fibroblasts in Fig. 1F. The pattern of collagen I, including extracellular filaments and intracellular labeling of procollagen I, could be presented in Fig. 1G. The intracellular distribution of procollagen I was separately shown in Fig. 1H. Collagen III could be only weakly detected (Fig. 1I).

Response to different serum concentrations. The depletion of serum increased the expression of collagen I and collagen III mRNA (Fig. 2). The mRNA of collagen I was increased by 56% 24 h after change from 10% to 0.5% FCS and by 72% after change to 0% FCS (n = 15). The mRNA of collagen III was much more increased by a factor of 2.8 24 h after change from 10% to 0.5% FCS and by a factor of 3.1 after change to 0% FCS (n = 15). The ratio of collagen I to collagen III was diminished from a factor of 6.5 using 5% or 10% serum and by a factor of 3.6 after serum removal (0% or 0.5% FCS).

Response to different elongation of cyclical mechanical stretch. The response to cyclical mechanical stretch (CMS) was investigated by 0%, 5%, and 10% FCS. Fig. 3 shows parts of RPA gels as a representative gel picture to illustrate the specificity of the decisive probes by 0% and 10% FCS. In absence of serum, the level of collagen I mRNA was not changed by elongations of 3% and 6% CMS. However, an elongation of 9% CMS increased the expression of collagen I mRNA by a factor of 1.70 (Fig. 4A). Similarly, the collagen III mRNA was
mRNA increased by a factor of 1.64 by an elongation of 9% CMS and was unchanged by elongations of 3% and 6% CMS (Fig. 4B). With the use of 5% FCS, there was no effect of collagen I and collagen III mRNA by all three different CMS elongations (Fig. 4, A and B). In the presence of 10% serum, the levels of collagen I and collagen III mRNA were unchanged by an elongation of 3% CMS. This high concentration of FCS caused a decrease of collagen I and collagen III mRNA by an elongation of 6% CMS (for collagen I mRNA 38% and for collagen III mRNA 21%) and of 9% CMS (for collagen I mRNA 26% and for collagen III mRNA 28%), as shown in Fig. 4, A and B. The analysis of MMP-2, TIMP-2, and collagen mRNA showed only an increase of all three mRNAs by an elongation of 9% CMS in the absence of serum (Fig. 4C). In all other samples, the levels of mRNA of MMP-2, TIMP-2, and collagen were unchanged.

**Response to CMS: influence of PKC, tyrosine kinase and PKA in the absence of serum.** As a first step, the signaling pathway was investigated in the absence of serum. The inhibition of PKC by 1 μM RO-31-8220 caused a reduction of collagen I and collagen III mRNA (Fig. 5A). The stretch-induced increase of collagen I and collagen III mRNA by an elongation of 9% CMS was prevented by PKC inhibition. The inhibition of PKC with 5 μM chelerythrine caused the same effects on the collagen I and collagen III mRNA (Fig. 6A) as the PKC inhibition by RO-31-8220. The stretch-induced effects on collagen I and collagen III mRNA by an elongation of 9% was also prevented by PKC inhibition with chelerythrine.
The inhibition of PKA by using 3 μM H89 showed no effect on the levels of collagen I and collagen III mRNA (Fig. 5C). In response to CMS, the results were comparable to those without PKA inhibition. The same results were analyzed for MMP-2 and TIMP-2 mRNA levels. The exception was the colligin mRNA, which was not increased by 9% elongation of CMS. These results could be confirmed by using 3 μM KT5720 for PKA inhibition (Fig. 6D). The mRNA levels of collagen I and collagen III, MMP-2, TIMP-2, and colligin showed the same results in absence or application of CMS by 9% elongation using KT5720 compared with PKA inhibition with H89.

Response to CMS: influence of PKC, tyrosine kinase and PKA in the presence of 10% serum. As a second step, the signaling pathway was investigated in the presence of 10% FCS. The inhibition of PKC by Ro-31-8820 or chelerythrine did not modify the levels of collagen I and collagen III mRNA (Figs. 7A and 8A). The stretch caused decrease of collagen I and collagen III mRNA by 6% and 9% CMS, respectively, was prevented by PKC inhibition with both inhibitors. PKC inhibition using Ro-31-8820 or chelerythrine did not influence the levels of MMP-2, TIMP-2, and colligin mRNA in absence or the application of CMS.

The inhibition of tyrosine kinase with herbimycin A did not change the levels of collagen I and collagen III mRNA (Fig. 7B). The same results on the collagen I and collagen III mRNA levels were analyzed by the inhibition of Src family kinase and by the inhibition of tyrosine kinase activity of FGFR1 (Fig. 8, B and C). The stretch caused decrease of collagen I and collagen III mRNA by 6% and 9% CMS, respectively, was prevented by tyrosine kinase inhibition with herbimycin A, SU6656, or SU4984. The MMP-2 mRNA expression increased during the tyrosine kinase inhibition by all three inhibitors in absence or application of CMS. The TIMP-2 and colligin mRNA was enhanced during the tyrosine kinase inhibition. But in response to CMS the tyrosine kinase inhibition by all three inhibitors did not change the levels of TIMP-2 and collagen mRNA compared with control.

The inhibition of PKA by H89 did not modify the levels of collagen I and collagen III mRNA (Fig. 7C). The PKA inhibition by KT5720 had no effect on the collagen I mRNA level, but reduced the collagen III mRNA level (Fig. 8D); this was a difference compared with PKA inhibition by H89. In response to CMS, the H89 effects on the collagen I and collagen III mRNA levels were comparable to those without PKA inhibition. The mRNA levels of collagen I and collagen III were reduced during the PKA inhibition by KT5720 in response to 9% CMS, but a synergistic effect of PKA inhibition by KT5720 and CMS on the collagen III mRNA level was not comparable to the effect of PKC inhibition with Ro-31-8820. The PKC inhibition by Ro-31-8820, as well as by chelerythrine reduced the mRNA levels of MMP-2, TIMP-2, and colligin. This reduction of the MMP-2, TIMP-2, and colligin mRNA was abolished by an elongation of 9% CMS during PKC inhibition by both inhibitors.

The inhibition of the tyrosine kinase using 1 μM herbimycin A reduced the levels of collagen I and collagen III mRNA in the absence or application of CMS by all three different elongations (Fig. 5B). Reduced mRNA levels of collagen I and collagen III resulted also in the specific inhibition of Src family kinase by 5 μM SU6656, as well as the tyrosine kinase inhibition of fibroblast growth factor receptor 1 by 25 μM SU4984 (Fig. 6, B and C). CMS by 9% elongation did not influence the reduced mRNA levels of collagen I and collagen III in the presence of SU6656 or SU4984. The tyrosine kinase inhibition by herbimycin A, SU6656, or SU4984 did not affect the MMP-2, TIMP-2, and colligin mRNA expressions. In response to 9% CMS, the mRNA expressions of MMP-2, TIMP-2, and colligin increased also in the presence of herbimycin A, SU6656, or SU4984.

Fig. 2. Expression of collagen I mRNA (A) and collagen III mRNA (B) in relating to GAPDH mRNA in response to different serum concentrations (means ± SD; n = 15) in cardiac fibroblasts within 24 h.

Fig. 3. Representative parts of RNase protection assay gels to show collagen I mRNA, collagen III mRNA, and GAPDH mRNA without stretch as control (C) and in response to different stretch elongations (S) using 0% and 10% FCS.
detectable. PKA inhibition by H89 did not influence the levels of MMP-2, TIMP-2, and colligin mRNA in the absence or application of CMS. The same results were analyzed for MMP-2 and TIMP-2 mRNA levels by PKA inhibition with KT5720. The exception was the colligin mRNA that was decreased by 9% elongation of CMS, which was also a difference to PKA inhibition with H89.

Response to CMS: extracellular collagen I and collagen III protein levels. The investigations of the protein levels of collagen I and collagen III in the extracellular environment revealed an increase of collagen I by a factor of 1.28 and of collagen III by a factor of 1.37 in response to CMS by an elongation of 9% in the absence of serum (Fig. 9). The inhibition of PKC by 1 μM RO-31-8220, as well as by 5 μM chelerythrine, reduced the extracellular protein levels of collagen I and collagen III. In the presence of PKC inhibition, CMS by 9% elongation did not cause an increase of the extracellular collagen I and collagen III protein levels. The inhibition of tyrosine kinase using 1 μM herbimycin A decreased the extracellular protein levels of collagen I and collagen III. These reduced collagen I and collagen III protein levels did not change under the influence of 9% CMS. In the presence of 5 μM SU6656, the Src family kinase inhibitor, an effect on the extracellular collagen I and collagen III protein levels could be not detected. The protein level of collagen I could even increase in the presence of SU6656 caused by 9% CMS. But, the presence of SU6656 prevented the CMS-caused effect on the protein level of collagen III.

DISCUSSION

This study investigated the induction of the main components of ECM, which are collagen I and collagen III in heart in dependence on the strength of the stretch in fibroblasts from adult animals. The dependence of ECM induction on the strength of strain is important because a distribution of different strain strength in the heart was demonstrated after myocardial infarction (20), and the changed strain conditions were created during the development of cardiac hypertrophy caused by volume or pressure overload (40, 50). The altered ECM induction by changed strain strength influenced the scaffold of the ventricular heart tissue and can consequently cause cardiac dysfunction (4). The adult ventricular fibroblasts were chosen in an in vitro model to exclude possible paracrine mechanisms proceeded from ventricular myocytes on the fibroblasts and with it connected ECM gene expression. Therefore, the influence of growth factors on ECM induction was on the one hand excluded and, on the other hand, investigated in different concentrations by using serum in an indefinable composition. The activity of the fibroblasts, depending on the pressure and volume proportions in the heart, is also decided by mechanical conditions (6, 15, 40). A permanent volume overload altered the fibroblast function even in following culture (23). The involvement of the signaling pathways in the signal transduction from the mechanical stimulus to the ECM induction was the focus of our interest.

We found in the absence of growth factors that only a very strong strength of strain by 9% elongation was able to increase the mRNA of collagen I and collagen III. This 9% elongation in all directions means an enlargement of the cell area of 18%. To study the direct effect of mechanical load on the cell...
function, different strain devices have been developed (31, 52). In these published in vitro experiments, the response of the cells was modulated by the nature of the matrix on which the cells had grown and by the manner of the strain deformation. Moreover, the response of the cells appears to be cell type and tissue specific, as well as influenced by the presence of serum (5, 8, 17). Consequently, it is difficult to compare the results of the reports using neonatal or fetal rat cardiac fibroblasts that found an increase of collagen I after 48 h of CMS or only of collagen III after 24 h of CMS (11, 14) with our results using

Fig. 5. Signaling pathways in the absence of serum under the influence of PKC inhibition with 1 μM RO-31-8220 (A), tyrosine kinase inhibition with 1 μM herbimycin A (B), and PKA inhibition with 3 μM H89 (C) on expression of collagen I mRNA (black bars), collagen III mRNA (top, white bars), MMP-2 mRNA (bottom, white bars), TIMP-2 mRNA (hatched bars) and colligin mRNA (double-hatched bars) in response to different stretch elongations within 24 h (means ± SD; n = 4–7); *P < 0.05 vs. control without stretch and serum; §P < 0.05 vs. control without stretch but in the presence of inhibitor.

Fig. 6. Signaling pathways in the absence of serum under the influence of PKC inhibition with 5 μM chelerythrine (A), tyrosine kinase inhibition with 5 μM SU6656 (B), as well as PKA inhibition with 3 μM KT5720 (D) on expression of collagen I mRNA (black bars), collagen III mRNA (top, white bars), MMP-2 mRNA (bottom, white bars), TIMP-2 mRNA (hatched bars), and colligin mRNA (double-hatched bars) in response to stretch by 9% elongation within 24 h (means ± SD; n = 4–7); *P < 0.05 vs. control without stretch and serum; §P < 0.05 vs. control without stretch but in the presence of inhibitor.
adult cardiac fibroblasts in the first passage and our strain device. Another in vitro study using a culture of adult rat cardiac fibroblasts in a form of a cell line showed an increase of collagen III only by 3% static strain (31). Therefore, it is not possible at present to give a complete explanation of the stretch-regulated ECM expression. The different conditions of stretch and culture cause a different character of fibroblasts, particularly the differentiation and the growth of the fibroblasts.

In the second part, we investigated which signaling pathways were involved in this stretch-induced increase of collagen I and collagen III by 9% elongation. We had inhibited three...
enzymes of different signaling pathways, and we found that the inhibition either of PKC or tyrosine kinase was sufficient to prevent the induction of collagen I and collagen III. From this result, we can conclude that at least these two signaling pathways are necessary for the activation of this ECM expression. Stimulation of PKC by mechanical load was reported for neonatal cardiac myocytes, as well as tyrosine kinase inhibition with 1 μM herbimycin A and 5 μM SU6656, in response to stretch by 9% elongation in the absence of serum within 24 h (means ± SD; n = 3); *P < 0.05 vs. control without stretch and inhibitor; §§P < 0.05 vs. stretched effect without inhibitor; §P < 0.05 vs. control in the presence of inhibitor.

For the simulation by the influence of growth factors, we used different concentrations of fetal calf serum. Serum causes changes in the basal protein synthesis level and in the release of the autocrine-mediated growth factors. Subsequently, the oversupply of growth factors changes the activity and the protein level of the enzymes of signal transduction pathways. We found that a reduction of the serum concentration increased the mRNA of collagen I and collagen III. The response of adult cardiac fibroblasts to mechanical stretch showed that CMS decreased the mRNA levels of collagen I and collagen III in the presence of 10% serum. Thus, we can hypothesize that growth factors presented in calf serum and mechanical load exert a synergistic effect on the collagen synthesis in adult cardiac fibroblasts. A report about the effect of high serum concentrations or specific growth factors in combination with mechanical stretch had suggested such a synergy, but it does not elucidate whether this synergy involves similar or diverse pathways (11). The inhibition of the PKC or the tyrosine kinase prevented the decrease of the collagen I and collagen III mRNA levels in the presence of 10% serum. The inhibition of the PKA had no effect on the mRNA levels of collagen I and collagen III. Therefore, we can assume that the growth factors and mechanical load regulate the collagen expression by the similar mechanisms. The comparison with other studies shows that the response of fibroblasts diverges relating to their original tissue, the serum concentrations and the mechanical regimens (5, 54).

A further aspect in the regulation of ECM is the family of enzymes that degrade ECM components that are the MMPs and their natural inhibitors called tissue inhibitors of MMPs (TIMPs). Many studies investigated the role of MMPs and TIMPs in cardiac matrix remodeling (21, 33). Enhanced expression and activity of MMPs, including MMP-2, was detected after myocardial infarction (19, 46). MMP-2 was shown to be associated with cardiac fibroblasts and activated by mechanical tension and transmural pressure (9, 16, 33, 48). Our results corresponded with these reports. The mRNA levels of MMP-2, TIMP-2, and colligin, which is the chaperone of collagen I, was increased by CMS but also only by an elongation of 9%. The inhibition of the PKC, tyrosine kinase, or PKA had no effect on this stretch-induced increase of MMP-2 and TIMP-2 mRNA levels, even if the PKC reduced the mRNA basic levels of MMP-2, TIMP-2, and colligin. Under the influence of 10% serum, there was no stretch, as well as PKC- or PKA-mediated effect on the MMP-2 and TIMP-2 mRNA levels, but the inhibition of the tyrosine kinase increased the mRNA levels of MMP-2, TIMP-2, and colligin. CMS abolished the enhanced levels of TIMP-2 and colligiln mRNA, but the MMP-2 mRNA level remained enhanced under the influence of stretch. The important role of the protein tyrosine kinase for the MMP-2 production or activation has been described previously (43, 53).

ECM plays an essential role in the cardiovascular function. Changes in the ECM can contribute to myocardial dysfunction and are regulated by cardiac fibroblasts (4). Mechanical load was detected as one stimulus for ECM and MMP expression in vivo and in vitro (5, 8, 17, 33, 49). Our study extends the
quantity of results about the regulation of ECM by mechanical stretch. Our results suggest that the induction of ECM is dependent on the strength of strain and at least two different signaling pathways are involved in this induction process. In these experiments, we used cultured cardiac fibroblasts from the ventricles of adult rats. The results showed that the response of these fibroblasts to CMS is influenced by the serum concentration. In conclusion, the regulation of ECM can be attributed to the sensitive response of adult cardiac fibroblasts relating to their mechanical and chemical conditions.

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REFERENCES


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