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RESEARCH ARTICLE

Irregular pacing of ventricular cardiomyocytes induces pro-fibrotic signalling involving paracrine effects of transforming growth factor beta and connective tissue growth factor

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Aims Atrial fibrillation is the most prevalent sustained arrhythmia associated with arrhythmic ventricular contractions, incident heart failure, increased morbidity and mortality. The relationship between arrhythmic contractions and ventricular remodelling is incompletely understood. The aim of this study was to characterize the influence of irregular contractions on pro-fibrotic signalling in neonatal rat ventricular cardiomyocytes (NRVM).

Methods and results Neonatal rat ventricular cardiomyocytes were paced via field stimulation at 3 Hz for 24 h. Irregularity was created by pseudorandomized variation of stimulation intervals and compared to regular pacing. Treatment of neonatal cardiac fibroblasts (NCF) with medium of irregularly paced NRVM increased protein expression of collagen I ($206 \pm 62\%$, $P = 0.0121$) and collagen III ($51 \pm 37\%$, $P = 0.0119$). To identify the underlying mechanism, expression of pro-fibrotic connective tissue growth factor (CTGF) and transforming growth factor beta (TGF-β) was assessed. In irregularly paced NRVM, increased protein expression of CTGF ($80 \pm 22\%$, $P = 0.0035$) and TGF-β ($122 \pm 31\%$, $P = 0.0022$) was associated with enhanced excretion of both proteins into the medium. Electron paramagnetic resonance spectroscopy revealed an increased production of reactive oxygen species ($46 \pm 21\%$, $P = 0.0352$) after irregular pacing accompanied by increased 8-hydroxydeoxyguanosine staining ($214 \pm 53\%$, $P = 0.0011$). Irregular pacing was associated with elevated mRNA levels of anti-oxidative superoxide dismutase 1 ($25 \pm 7\%$, $P = 0.0175$), superoxide dismutase 3 ($20 \pm 7\%$, $P = 0.0309$), and catalase ($20 \pm 7\%$, $P = 0.046$).

Conclusion These data demonstrate that irregular pacing is an important inductor of pro-fibrotic signalling in NRVM involving paracrine effects of CTGF and TGF-β as well as increased oxidative stress. Thus, irregularity of the heart beat might directly be involved in the progression of maladaptive remodelling processes in atrial fibrillation.

Keywords Arrhythmia • Fibrosis • Atrial fibrillation • Oxidative stress

Introduction

Atrial fibrillation (AF) is the most prevalent sustained arrhythmia and is highly associated with incident heart failure.[1] Heart failure is a multifactorial, systemic disorder in which structural and molecular mechanisms are activated leading to progression of left ventricular dysfunction and to clinical signs and symptoms.[2] AF can be the result of myocardial stress and dilatation in response to elevated ventricular filling pressures, but could, in turn, trigger maladaptive mechanisms by causing ventricular arrhythmia in

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conditions such as hypertension, valve diseases, coronary heart disease, or heart failure.[2]

The potential influence of irregular contractions on the progression of maladaptive interstitial remodelling involving fibrosis and pro-fibrotic signalling is not understood. A central regulator of pro-fibrotic signalling is transforming growth factor beta (TGF-β). TGF-β is highly associated with the development of cardiac fibrosis.[3] TGF-β induces production of collagen I and III in fibroblasts involving pro-fibrotic connective tissue growth factor (CTGF) signalling. CTGF is produced by cardiomyocytes and fibroblasts and mediates the proliferation, migration, and adhesion of fibroblasts.[4] Oxidative stress is known to influence the development of cardiac fibrosis.[5] Reactive oxygen species (ROS) play a role in remodelling, myocardial dysfunction, and apoptosis.[6] In turn, the myocardium has the possibility of protecting itself from oxidative stress by upregulation of anti-oxidative systems like superoxide dismutases (SOD) 1–3, while the instable product H_2O_2 is transformed into H_2O and O_2 by catalase.[7,8] The interleukin (IL)-related protein ST2 and its ligand IL-33 are the link between oxidative stress and the progression of fibrosis.[9] The membrane-bound isoform ST2L serves an anti-fibrotic function by inhibiting pro-fibrotic pathways of angiotensin II and phenylephrine.[9] The soluble STS (sST2) acts like a decoy receptor for the ligand IL-33. An upregulation of sST2 by oxidative stress mediates pro-fibrotic signalling.[10]

Despite the association between AF and ventricular fibrosis,[11] it is not understood whether arrhythmic contractions as a major feature of AF have a direct influence on fibrosis. Therefore, we established a cell culture model with isolated neonatal rat ventricular cardiomyocytes (NRVM) and neonatal cardiac fibroblasts (NCF) to investigate the influence of arrhythmic contractions of ventricular cardiomyocytes on paracrine pro-fibrotic signalling under controlled rate conditions.

Material and methods

Isolation of neonatal rat ventricular cardiomyocytes and cardiac fibroblasts

Neonatal cardiomyocytes were isolated from 3–5-day-old male and female Sprague–Dawley rats as described previously.[12,13] In brief, after extirpation of the heart, the ventricular tissue was digested in a mixture of collagenase II (Worthington Biochemical, Cell Systems) and pancreatin (Sigma-Aldrich Chemie, München, Germany). To separate myocytes from fibroblasts the cell lysate was filtered (Greiner Bio-One, M180054) and incubated in F10 medium (Gibco, Invitrogen, Karlsruhe, Germany) containing 10% horse serum, 5% foetal calf serum and 1% penicillin/streptomycin. After 1 h the fibroblasts were separated from the cardiomyocytes due to adherence to the cell culture dish. Cardiac fibroblasts were cultured until cells were ~80–90% confluent. Cardiomyocytes were counted using a Neubauer haemocytometer and 600 000 NRVM were seeded and incubated in six-well plates (BD Falcon 6-Well Plate 353846, BD, Franklin Lakes, NJ, USA).

Pacing of neonatal rat ventricular cardiomyocytes

Four days after isolation, NRVM were spontaneously beating at about 1 Hz and then divided into three groups. Group one served as an internal control (Ctrl) without electrical stimulation. The second group was paced regularly (Reg) and compared to the third group, which was paced irregularly (Irreg). Pacing rate in both groups was kept stable at 3 Hz under regular and irregular conditions for 24 h, respectively.

We used the cell culture stimulator C-PACE EP (Cell Culture EP Stimulator, IonOptix Limited, Dublin, Ireland) and carbon electrodes (C-Dish CLD6WBFC, IonOptix Limited, Dublin, Ireland) (*Figure 1*). Following the pacing protocol, cell culture medium was collected for further experiments and NRVMs were harvested for gene and protein analyses.

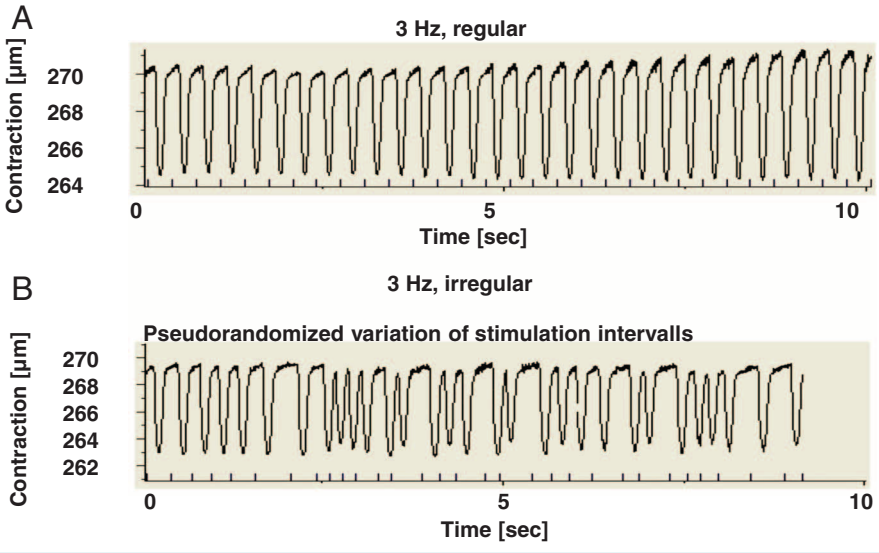

Figure 1 Representative contraction analysis of regularly (*A*) and irregularly (*B*) paced neonatal rat ventricular cardiomyocytes.

Treatment of neonatal cardiac fibroblasts with medium of regular and irregularly paced neonatal rat ventricular cardiomyocytes

Neonatal cardiac fibroblasts were visually controlled and at 90% confluence splitted and incubated. To investigate the interaction of NCFs and NRVMs, confluent NCFs were incubated with medium obtained from paced NRVMs derived from the same hearts and incubated for another 48 h. Thereafter, NCFs were harvested and protein expression was analysed.

Inhibition of TGF-β signalling was performed by selective blockade of the TGF-β receptor via A83-01 (Tocris, #2939). Therefore, the stock of 25 mmol/L was diluted with dimethyl sulfoxide to a working solution of 0.5 mmol/L. Medium obtained from irregularly paced NRVMs was mixed with A83-01working solution to an end-concentration of 0.5 μmol/L and used to incubate NCFs. After 48 h NCFs were harvested for protein isolation and Western blot analysis.

Western blot analysis

Proteins (50 μg/lane) of NRVM or NCF were isolated and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis as described previously.[13] Proteins were transferred to nitrocellulose membranes using a Biorad Mini Trans Blot System (Bio-Rad Laboratories, Hercules, CA, USA) (350 mA, 2 h). Membranes were incubated with primary and peroxidase conjugated secondary antibodies. Antibody-bound proteins were visualized by enhanced chemiluminescence using ECL Western blotting detection reagents (GE Healthcare, Munich, Germany). Band intensities were analysed by densitometry with UVP Labworks (version 4.6.00.0**,** UVP, LLC, Upland, CA, USA) and referred to glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH, Milipore, MAB374). For protein detection, the following antibodies were used: anti-CTGF (Abcam, ab6992); anti-p-SMAD3 (Cell Signalling Technology, #9520); anti-SMAD3 (Cell Signalling Technology, #9513); anti-p-SMAD2 (Cell Signalling Technology, #3104S); anti-SMAD2 (Cell Signalling Technology, #5339); anti-SMAD4 (Cell Signalling Technology, #38454); anti-TGF-β (Santa Cruz Biotechnology, Inc., sc-130348); anti-IL-33; (Abcam, ab118503); anti-ST2 (Abcam, ab72778); secondary antibodies: goat-anti-rabbit IgG (BioRad, #172-1019); goat-anti-mouse IgG (BioRad, #170-656).

For Western blots analyses in cell culture, 10 μL of medium was used and continued as described above. As loading control for Western blots with medium, Ponceau-red staining (Ponceau-S-solution, Serva; #33427.01) was used.

Reverse transcriptase polymerase chain reaction

Total RNA isolation, reverse transcription, and quantitative polymerase chain reaction (PCR) were performed according to standard techniques. Measurement of mRNA was performed as described by Lavall *et al.*[14] Real-time PCR was conducted in a StepOne plus thermocycler (Applied Biosystems, Darmstadt, Germany) using SYBR Green PCR Mastermix (Applied Biosystems). Signals were normalized to corresponding 18S as control. No template controls were used to monitor for contaminating amplifications. The ΔCt was used for statistical analysis and $2^{-\Delta\Delta Ct}$ for data presentation. Sequences for sense and antisense primers were as follows: sST2 (IL1RL1)_for: GGT GTG ACC GAC AAG GAC T rev: TTG TGA GAG ACA CTC CTT AC, IL-33_for: ACC TGC GCT TGT TTC CTT CT rev: TTC AGT CAC AGG TGC GAA CT, SOD1_for: ACA CAA GGC TGT ACC ACT GC, rev: CCA CAT TGC CCA GGT CTC C, SOD3_for: AGG TCG AAT GTG CGC AGA TAA rev: GAA CAC CCC AAG GGA GTT CTC, Catalase_for: CCC AGA AGC CTA AGA ATG CAA rev: TCC CTT GGC AGC TAT GTG AGA 18S_for: TTG ATT AAG TCC CTG CCC TTT GT rev: CGA TCC GAG GGC CTC ACTA.

Electron paramagnetic resonance spectroscopy

Reactive oxygen species were measured by electron paramagnetic resonance spectroscopy as described.[15] Krebs–Henseleit buffer and diethyldithiocarbamate were added to the cell lysate to stabilize the oxide radicals.

8-Hydroxydeoxyguanosine stain of paced neonatal rat ventricular cardiomyocytes

After pacing, cardiomyocytes were fixed in 4% formalin. After treatment with the primary antibody for 8-hydroxydeoxyguanosine (8-OHdG), the cells were incubated overnight in 4°C. Next day, cells were treated with a secondary antibody conjugated with tetramethylrhodamine and incubated overnight at 4°C. Next day, cells were treated with another secondary antibody conjugated with fluorescein isothiocyanate. 8-OHdG fluorescence was detected via microscopy. In every six-well three fields of vision were chosen randomly. A red tone was depicted for the red fluorescending 8-OHdG. The fluorescending area was related to the whole area of the field of vision. For the analysis, Nikon Instruments Software (NIS)-Elements (BR 3.2, Nikon instruments) was used.

Statistical analysis

All values are expressed as mean ± standard error of the mean. Groups were tested for normal distribution. Student's *t*-tests, Mann–Whitney U tests and one-way ANOVA for multiple comparisons were applied. Post hoc comparisons were performed with the Bonferroni's multiple comparison test. Data were statistically analysed with GraphPad Prism software 6.0 (GraphPad Software Inc., San Diego, CA, USA). All results are shown in percent of control. Differences were considered significant at $P < 0.05$.

Results

Irregular pacing of neonatal rat ventricular cardiomyocytes led to increased expression of collagen I and III in neonatal cardiac fibroblasts

We treated NCFs with medium obtained from NRVM subjected to 24 h pacing at 3 Hz under regular vs. irregular pacing conditions. After 48 h of incubation, we investigated the protein expression of collagen I and III in the cell lysates. In NCFs, treatment with medium from irregularly paced NRVMs led to an increased expression of collagen I by 206 ± 62% ($P = 0.0121$) (*Figure 2A*)

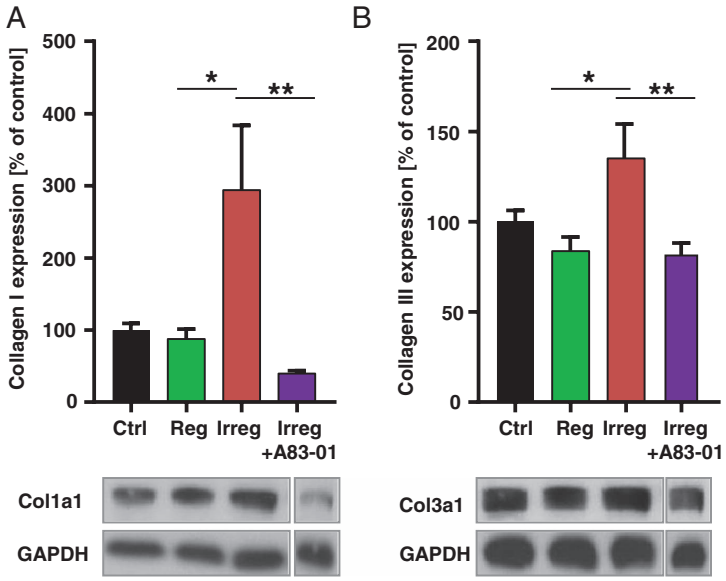

Figure 2 Effect of irregular pacing on expression of collagen I (*A*) and collagen III (*B*) in neonatal cardiac fibroblasts. Neonatal cardiac fibroblasts were treated with medium obtained from regularly and irregularly paced neonatal rat ventricular cardiomyocytes in the presence or absence of transforming growth factor beta (TGF-β) inhibitor A83-01. Western blot analysis and quantification of collagen I (*A*) and collagen III (*B*) ($n = 7–9$). All data are depicted as mean ± standard error of the mean (*$P < 0.05$; **$P < 0.01$). Col1a1, Collagen 1a1; Col3a1, Collagen 3a1; Ctrl, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Irreg, irregular pacing; Reg, regular pacing.

and of collagen III by 51 ± 37% ($P = 0.0119$) (*Figure 2B*) compared to NCFs that were incubated with medium of regularly paced NRVMs. To assess the contribution of TGF-β on mediating activation of collagen expression, we co-incubated NCFs with the specific TGF-β signalling inhibitor A83-01. Treatment of NCFs with medium from irregularly paced NRVM containing 0.5 μmol/L A83-01 prevented increase of collagen I expression by 254 ± 65% ($P = 0.0025$) (*Figure 2A*) and of collagen III by 52 ± 14% ($P = 0.0077$) (*Figure 2B*). Interestingly, neither irregular pacing nor selective inhibition of TGF-β signalling by A83-01 affected expression or phosphorylation status of the intracellular TGF-β signal molecules SMAD2, SMAD3 and SMAD4. Furthermore, the ratios of p-SMAD2/SMAD2 and p-SMAD3/SMAD3 showed no significant difference between regular and irregular pacing (online supplementary *Figure S1*). In addition, expression of α-smooth muscle actin was not increased after treatment with medium of paced cardiomyocytes and was not influenced by inhibition of TGF-β signalling (online supplementary *Figure S2*).

Irregular pacing of neonatal rat ventricular cardiomyocytes was associated with increased expression and secretion of transforming growth factor beta and connective tissue growth factor in the medium

Next, we investigated the influence of irregular pacing on intracellular expression of pro-fibrotic signalling molecules TGF-β and CTGF in NRVMs. Western blot analysis of the cell lysate demonstrated an increased protein expression of TGF-β by 122 ± 31% ($P = 0.0022$) (*Figure 3A*) and of CTGF by 80 ± 22% ($P = 0.0035$) (*Figure 3B*). To determine paracrine secretion of pro-fibrotic signalling proteins TGF-β and CTGF, Western blot analyses of cell culture medium obtained 24 h after regular and irregular pacing were performed. Irregular pacing induced an increased secretion of TGF-β by 99 ± 23% ($P = 0.0005$) and of CTGF by 70 ± 24% ($P = 0.0194$) into the medium (*Figure 3C* and *3D*).

Oxidative stress was increased in neonatal rat ventricular cardiomyocytes after irregular pacing.

To analyse ROS production in NRVM after 24 h of irregular pacing electron paramagnetic resonance spectroscopy was used. Irregular pacing led to increased ROS production by 46 ± 21% ($P = 0.03$) in NRVM (*Figure 4A* and *4B*). To further characterize this finding, 8-OHdG was stained as a potent marker for ROS-mediated DNA damage in NRVM. An increase in 8-OHdG was observed following irregular pacing by 214 ± 53% ($P = 0.0011$) (*Figure 4C*).

Anti-oxidative proteins were upregulated after irregular pacing in neonatal rat ventricular cardiomyocytes

To clarify whether anti-oxidative systems are activated in response to oxidative stress after irregular pacing,

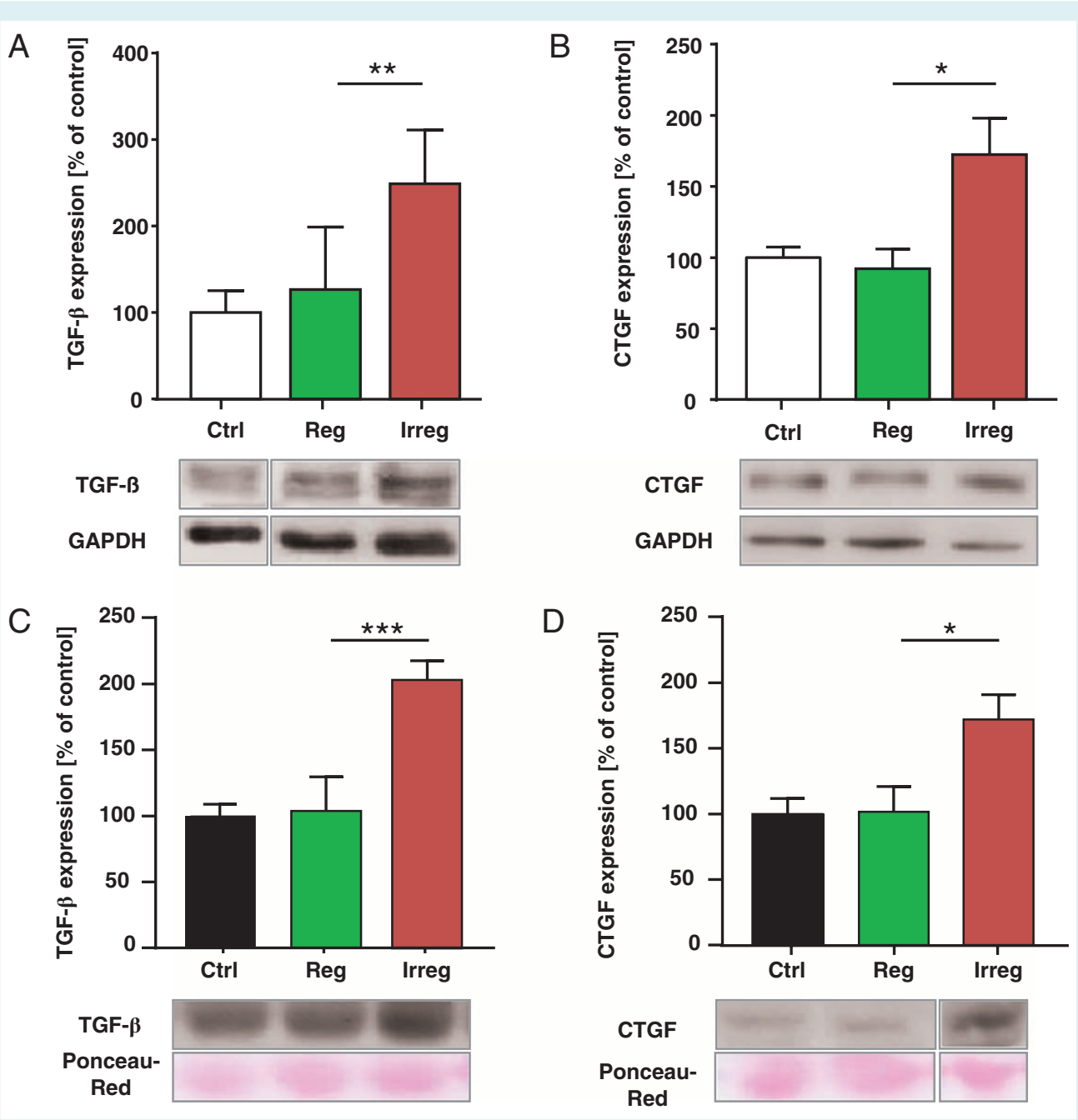

Figure 3 Expression and secretion of pro-fibrotic transforming growth factor beta (TGF-β) and connective tissue growth factor (CTGF) in regularly and irregularly paced neonatal rat ventricular cardiomyocytes. Representative Western blot analysis and quantification of intracellular protein expression of (*A*) TGF-β (n = 6–12) and (*B*) CTGF (n = 6–15) in neonatal rat ventricular cardiomyocytes and secretion of (*C*) TGF-β (n = 13–23) and (*D*) CTGF (n = 13–23) into the medium after 24 h pacing. All data are depicted as mean ± standard error of the mean (*P < 0.05; **P < 0.01; ***P < 0.001). Ctrl, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Irreg, irregular pacing; Reg, regular pacing.

real-time PCR gene expression analysis was performed. Our results demonstrated an upregulation of SOD1 mRNA by 25 ± 7% (P = 0.017) (*Figure 5A*) and of SOD3 by 20 ± 7% (P = 0.031) (*Figure 5B*). Furthermore, catalase mRNA level was increased by 20 ± 7% (P = 0.046) (*Figure 5C*) in irregularly paced NRVMs.

ST2/interleukin-33 system is sensitive to irregular pacing

In NRVMs, irregular pacing led to increased levels of IL-33 mRNA by 83 ± 16% (P = 0.0005) (*Figure 6A*) and of sST2 mRNA by 52 ± 15% (P = 0.0104) (*Figure 6B*). To explore the

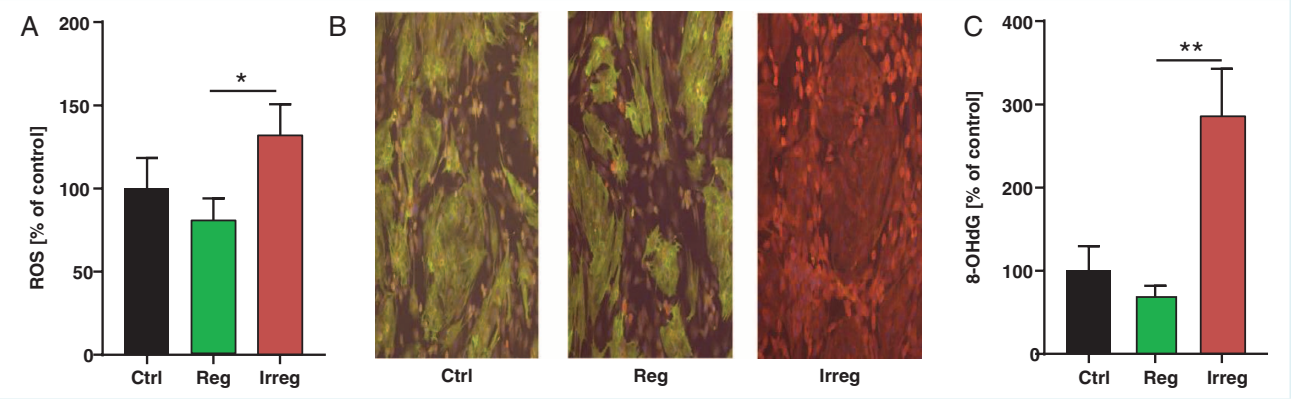

Figure 4 Effect of irregular pacing on biomarkers for oxidative stress in neonatal rat ventricular cardiomyocytes. (*A*) Quantification of reactive oxygen species (ROS) concentration measured by electron paramagnetic resonance spectroscopy ($n = 36$). (*B*) Representative staining and (*C*) quantification of 8-hydroxydeoxyguanosine (8-OHdG) ($n = 11-12$). All data are depicted as mean ± standard error of the mean (*$P < 0.05$; **$P < 0.01$). Ctrl, control; Irreg, irregular pacing; Reg, regular pacing.

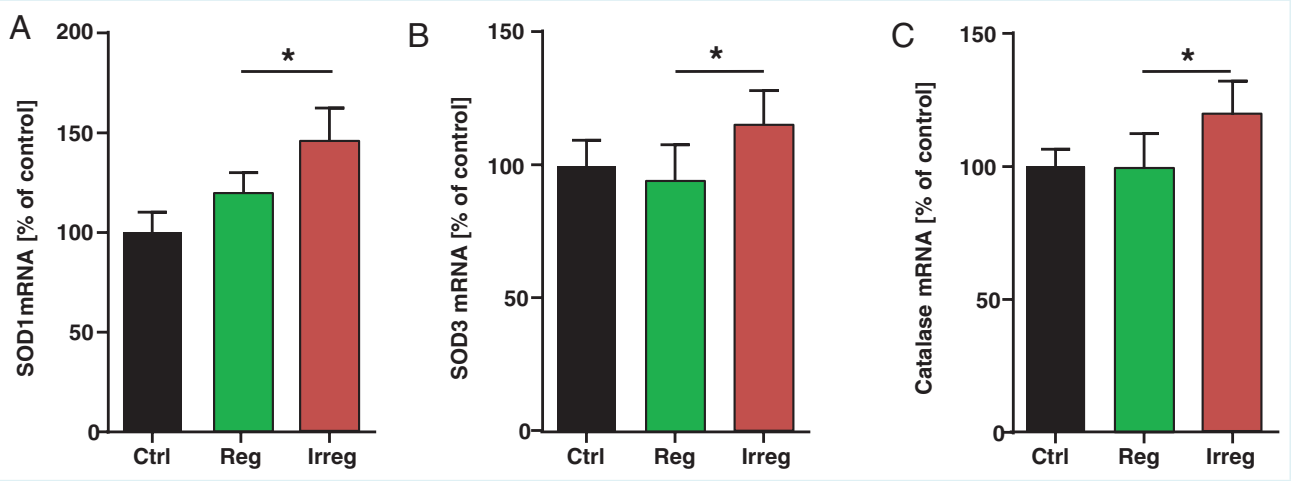

Figure 5 Effect of irregular pacing on biomarkers of the anti-oxidative system in neonatal rat ventricular cardiomyocytes. Quantification of mRNA expression by real-time polymerase chain reaction of (*A*) superoxide dismutase 1 (SOD1), (*B*) superoxide dismutase 3 (SOD3) and (*C*) catalase ($n = 6$). All data are depicted as mean ± standard error of the mean (*$P < 0.05$). Ctrl, control; Irreg, irregular pacing; Reg, regular pacing.

relationship between these two mediators on protein level, we performed Western blots. ST2 protein expression increased by 75 ± 27% ($P = 0.0313$) (*Figure 6D*) upon irregular pacing compared to regular pacing. However, we could not detect a significant increase in IL-33 protein levels after irregular pacing (*Figure 6C*).

Discussion

Based on previous reports demonstrating increased expression of CTGF protein[16,17] in patients who suffered from AF for more than 3 months,[16,17] we developed a novel cell culture based model to analyse the effect of irregular pacing under controlled rate conditions on the onset of pro-fibrotic signalling cascades in ventricular cardiomyocytes. Here, we show two important novelties. Firstly, irregular contracting cardiomyocytes activate expression of pro-fibrotic signal molecules, represented by TGF-β and CTGF, already after a short arrhythmia episode of 24 h. Secondly, irregular pacing enhanced paracrine secretion of TGF-β and CTGF by cardiomyocytes, thus possibly signalling increased collagen production in cardiac fibroblasts. Blockade of TGF-β signalling in NCFs prevented collagen expression, clearly indicating a crucial role of TGF-β in arrhythmia-induced fibrosis. Interestingly, intracellular TGF-β signalling was not accompanied by changes in the expression of components of the canonical SMAD pathway. Several non-SMAD-associated pathways in TGF-β signalling had been described[18] and identification of the underlying mechanism will be a task for future studies.

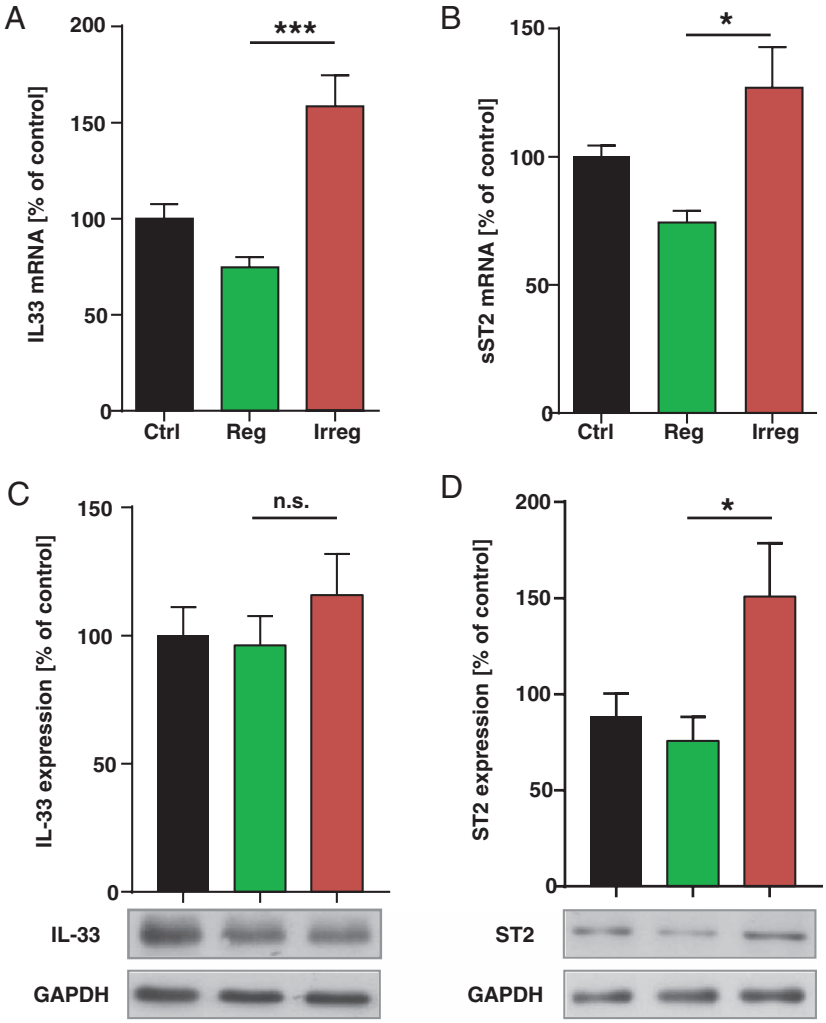

Figure 6 Effect of irregular pacing on expression of interleukin-33 (IL-33) and ST2 in neonatal rat ventricular cardiomyocytes. Quantification of mRNA expression by real-time polymerase chain reaction of (*A*) IL-33 and (*B*) ST2 ($n = 5–6$). Representative Western blot analysis and quantification of (*C*) IL-33 and (*D*) ST2 protein ($n = 10–11$). All data are depicted as mean ± standard error of the mean (*$P < 0.05$, ***$P < 0.001$). Ctrl, control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Irreg, irregular pacing; Reg, regular pacing.

In our model, irregular contractions were associated with increased oxidative stress followed by an activation of the anti-oxidative system in NRVM. Oxidative stress not only leads to the development of cardiac fibrosis but also exerts direct arrhythmic effects.[19] Therefore, we explored parameters for oxidative stress like ROS levels and 8-OHdG as a marker of ROS-mediated DNA damage, in response to irregular pacing. 8-OHdG is a reliable marker for oxidative stress in neonatal rat cardiomyocytes.[20] Both 8-OHdG and ROS levels were significantly increased after irregular pacing of NRVM. This finding indicates that oxidative stress not only leads to arrhythmia as previously described[19] but, in turn, arrhythmia also leads to oxidative stress. As a result of these findings we suggest that there is a vicious circle between arrhythmia and oxidative stress promoting each other. In response to increased oxidative stress, cardiomyocytes activate anti-oxidative proteins like catalase and SOD to reduce toxic oxidative stress.[21] These observations are in line with our results, demonstrating enhanced mRNA levels of catalase as well as SOD already after 24 h of irregular pacing. ROS excess persisted after irregular pacing as the anti-oxidative system seems not to be effective enough to completely neutralize oxidative stress after irregular pacing.

One possible link between oxidative stress and fibrosis might be represented by the ST2/IL-33 system. IL-33 is the ligand for the ST2 receptor in cardiomyocytes and fibroblasts.[22] The transmembrane bound form of ST2L and IL-33 are upregulated in response to mechanical stimulation or injury.[22] It has already been shown that the interaction between ST2L and IL-33 is part of an anti-fibrotic signalling pathway.[23] The soluble form sST2 is upregulated by oxidative stress.[24] sST2 acts as a decoy receptor for IL-33, which inhibits the anti-fibrotic signalling of ST2L.[25] This study showed that IL-33 mRNA as well as sST2 mRNA was upregulated in cardiomyocytes

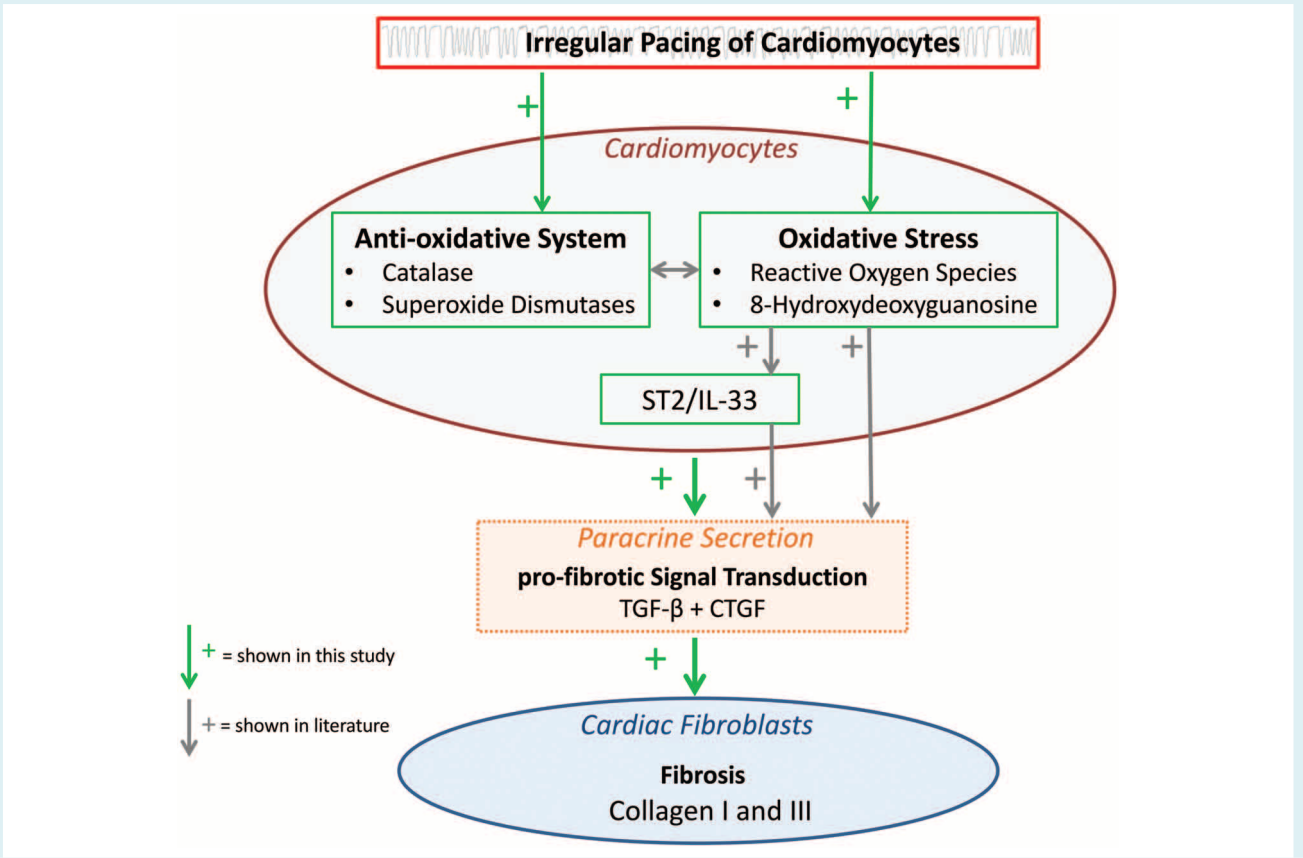

Figure 7 Schematic overview of the mechanisms and their connection investigated in this study in irregularly paced cardiomyocytes. Twenty-four hours of irregular pacing of neonatal rat ventricular cardiomyocytes lead to increased pro-fibrotic signalling and oxidative stress resulting in increased production of fibrotic collagen I and III in neonatal cardiac fibroblasts involving increased paracrine secretion of transforming growth factor beta (TGF-β) and connective tissue growth factor (CTGF). Furthermore, irregular pacing led to increased production of reactive oxygen species and 8-hydroxydeoxyguanosine, as parameters of oxidative stress in neonatal rat ventricular cardiomyocytes. Moreover, catalase and superoxide dismutases are increased by irregular pacing. Soluble ST2, which is induced by oxidative stress amongst others, is also increased under irregular pacing. These parameters, increased by irregular pacing, are associated with fibrosis mediated by pro-fibrotic signalling in neonatal rat ventricular cardiomyocytes and neonatal cardiac fibroblasts. IL-33, interleukin-33.

after arrhythmic stimulation. In agreement with increased mRNA concentration, ST2 protein expression was upregulated after irregular pacing but IL-33 was not. As sST2 acts as a decoy receptor, we suggest that arrhythmia causes an inhibition of anti-fibrotic signal transduction via ST2/IL-33. Another conceivable mechanism of ROS-mediated collagen production was described by Park *et al.*,[26] demonstrating oxidative stress to directly activate fibrogenic CTGF expression. Heart rate is a significant predictor of outcome in heart failure.[27] Further studies showed that the beneficial effects of beta-blockers on outcome are associated with heart rate reduction in sinus rhythm but not in AF,[28] independent of heart rate[29] and ejection fraction[30] at baseline. In heart failure with preserved ejection fraction, heart rate is associated with outcome only in sinus rhythm but not in AF and the event rate was higher in patients with AF.[31] Here, we suggest that the irregularity of the heart beat has a pathophysiological impact to promote fibrotic interstitial remodelling induced directly by arrhythmia.

Limitations

We used a cell culture model with neonatal rat cardiomyocytes and cardiac fibroblasts. Methodical setting was optimized for the aim of this investigation. However, it was impossible in this setting to investigate the effect of irregular pacing explicitly on atrial myocytes or adult myocytes due to unreliable stability and viability of these cells. By using this model, only paracrine fibrogenic mediators can be analysed. We are aware that the syncytium formed by cultured cardiomyocytes only partially reflects the full spectrum of the extracellular matrix, electrical modulators, mechanical junctions, or membrane nanotubes that are present in the organ.[32] Cardiac fibroblasts were shown to facilitate electrical coupling via expression of functional gap junctions between myocytes and fibroblasts.[33] The impact of arrhythmic field stimulation on cardiac fibroblasts still has to be elucidated. In this cell culture model, we focused on TGF-β dependent signal transduction and its effects on fibroblasts as a central signalling pathway. Effects of other signalling

pathways on cardiomyocytes and fibroblasts cannot be excluded. Therefore, the results of this study cannot be directly transferred to intact human heart tissue.

Conclusions

This study shows that irregular pacing of cardiac myocytes elevates oxidative stress and initiates pro-fibrotic signalling towards cardiac fibroblasts through increased excretion of CTGF and TGF-β, initiating expression of collagen I and III. Arrhythmia might represent an important mechanism in the development of fibrosis and progressive remodelling in ventricular tissue in AF (*Figure 7*).

Supplementary Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Expression of members of the SMAD pathway in neonatal cardiac fibroblasts (NCF) after treatment with medium from regularly and irregularly paced neonatal rat ventricular cardiomyocytes (NRVM). Quantification and representative Western blot analysis of SMAD2 (*A*), SMAD3 (*B*), SMAD4 (*C*), p-SMAD2 (*D*), p-SMAD3 (*E*), ratio of p-SMAD2/SMAD2 (*F*) and ratio of p-SMAD3/SMAD3 (*G*) in NCF after treatment with medium from paced NRVM with or without TGF-β signalling inhibitor A83-01 ($n = 8–18$).

Figure S2. Expression of α-smooth muscle actin (α-SMA) in neonatal cardiac fibroblasts (NCF) after treatment with medium from regularly and irregularly paced neonatal rat ventricular cardiomyocytes (NRVM). To determine fibrogenic response of cardiac fibroblasts to TGF-β stimulation, protein expression of α-SMA was analysed by Western blot to determine differentiation of fibroblasts into myofibroblasts. Quantification and representative Western blot of α-SMA in NCF after treatment with medium from paced NRVM with or without TGF-β signalling inhibitor A83-01 ($n = 9–15$) are shown.

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Conflict of interest: none declared.

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