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Heart diseases

ROLE OF CONNEXIN 43 IN MYOCARDIAL SCAR FORMATION, ADVERSE LEFT VENTRICULAR REMODELING AND HEART FAILURE

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1. Project summary

Background

Connexin 43 (Cx43) is an essential protein for cardiac physiology, allowing the existence of electrical coupling between neighboring cells. In addition, Cx43 plays important roles in propagation of cell injury during myocardial ischemia-reperfusion, as well as in cardioprotection by ischemic preconditioning. Furthermore, Cx43 is involved in scar formation and in the healing process in other tissues such as the skin or cornea, but its role in cardiac scar formation is unknown. Previous results obtained in bone tissue suggested that there could be a relationship between Cx43 and lysyl oxidase (LOX), the first enzyme that catalyzes the formation of collagen crosslinks.

Aims

(1) To study the role of Cx43 in myocardial collagen deposition, left ventricular remodeling and heart failure in murine models of (a) pressure overload induced by chronic treatment with angiotensin II (AngII) or (b) post-infarction myocardial remodeling; (2) to analyze the involvement of LOX in the observed effects, and the relationship that may exist between both proteins; and (3) to characterize the mechanisms involved.

Methods and results

To analyze the role of Cx43 in myocardial fibrosis we have used $Cx43^{fl/fl}$ mice (normal expression of Cx43) and an inducible Cx43 knock-out model, $Cx43^{Cre\text{-}ER(T)/fl}$ (content of Cx43 of 50%), treated with vehicle or 4-hydroxytamoxifen (4-OHT) to induce a global deletion of the floxed Cx43 allele.

Some of the animals were infused, simultaneously and for 14 days, with saline or AngII (1000 ng/Kg/min, to induce hypertrophy and fibrosis), using subcutaneously implanted osmotic pumps. AngII treatment induced a similar hypertrophic response in all groups, independently of Cx43 levels. Myocardial collagen content, analyzed by Picrosirius Red staining, was low in all groups treated with saline (n=8-9/group), but was significantly increased in all those infused with AngII (n=8-10/group, $p<0.05$). However, animals with partial Cx43 deficiency ($Cx43^{Cre\text{-}ER\ (T)/fl}$ mice treated with vehicle) exhibited an exaggerated fibrotic response that was reversed in mice treated with 4-OHT, which abolishes Cx43 expression. The exaggerated fibrotic response observed in partially

deficient $Cx43^{Cre\text{-}ER(T)/fl}$ mice was associated with increased p38 MAPK activation and was not evident in $Cx43^{+/-}$ heterozygous mice with a similar partial deficiency, thus suggesting that this effect is independent of Cx43 expression levels. However, normalization in collagen deposition observed in $Cx43^{Cre\text{-}ER(T)/fl}$ mice treated with 4-OHT, after AngII infusion, was correlated with an increase in metalloproteinase 9 (MMP9) activity, in IL-6 and NOX2 expression (two inflammation markers), and with an enhanced macrophage content, as well as with a reduced fibroblast differentiation capacity.

To analyze the fibrotic response and myocardial remodeling after infarction, additional groups were subjected to transient coronary occlusion (45 min) followed by reperfusion (14 days). Our results show that postinfarct scar area, determined by staining with Picrosirius Red, was significantly lower in both $Cx43^{Cre\text{-}ER(T)/fl}$ animals treated with either vehicle (50% Cx43 expression) or 4 -OHT (<5%), compared with results obtained in $Cx43^{fl/fl}$ animals (15.78±3.42 and 16.54±2.31% vs. 25.40±3.14 and 22.43±3.88% in mice treated with vehicle and 4-OHT, respectively, p=0.027 for genotype, two-way ANOVA). The reduction in body weight and ventricular dilation were significantly attenuated in both Cx43-deficient groups. This protective effect on scar size was correlated with a reduction in the expression of pro-TGFβ1 in these animals. Additionally, these results were compared with those obtained in animals overexpressing LOX. This comparison indicates that there is no clear relationship between Cx43 and LOX, and that the initial findings found (LOX overexpression in Cx43-deficient animals) was probably due to a compensatory response.

Conclusions

Cx43 plays an important role in myocardial fibrosis and in adverse left ventricular remodeling. A reduction in Cx43 expression attenuates collagen deposition both after pressure overload with AngII, and after myocardial infarction. In the first case, the mechanisms are related to a lower fibroblast differentiation capacity, greater MMP9 activity and greater accumulation of macrophages in the tissue. In the second case, the effects are associated with a lower expression of pro-TGFβ1.

2. Results obtained

1. Role of connexin 43 in myocardial collagen deposition, adverse left ventricular remodeling and heart failure induced by pressure overload after chronic treatment with AngII.

1.a. Left ventricular hypertrophy

Treatment with AngII for 14 days induced an increase in cardiac weight/body weight ratio (CW/BW), indicative of cardiac hypertrophy, of similar magnitude in all groups, regardless of Cx43 expression (100 % of Cx43 expression in $Cx43^{fl/fl}$ animals, 50% in $Cx43^{Cre\text{-}ER(T)/fl}$ mice and <5% in $Cx43^{Cre\text{-}ER(T)/fl}$ mice injected with 4-OHT). The lack of influence of Cx43 on the hypertrophic response to AngII was confirmed by measurements of cardiomyocyte sectional area as well as by echocardiography. In line with these data, AngII caused a significant induction of the hypertrophic marker ANP in all groups (two-way ANOVA, $p<0.001$). Similar results were obtained in a different mice strain (heterozygous $Cx43^{+/-}$) with a partial Cx43 deficiency similar to that found in $Cx43^{Cre\text{-}ER(T)/fl}$ animals.

1.b. Fibrotic response

Myocardial interstitial collagen content was low in all experimental groups treated with saline, ranging from 2% to 3% (Figure 1). In contrast, treatment with AngII for 14 days induced an increase in collagen content in $Cx43^{fl/fl}$ mice injected with oil from 2.67±0.32% to 7.30±1.19% ($p<0.05$) (Figure 1a). However, the increase in collagen deposition in response to AngII was significantly higher in $Cx43^{Cre\text{-}ER(T)/fl}$ animals treated with oil, which expressed 50% of normal Cx43 content (Figure 1b).

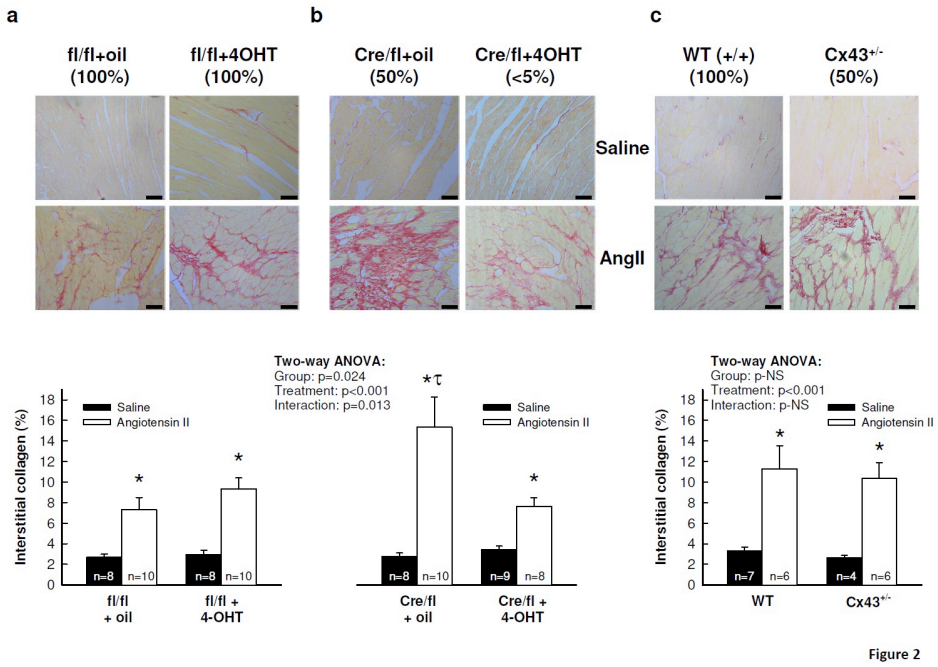

Figure 1. Cardiac fibrosis induced by chronic treatment with AngII in $Cx43^{fl/fl}$, $Cx43^{Cre\text{-}ER(T)/fl}$ and $Cx43^{+/-}$ mice. * ($p<0.05$) indicates significant differences compared to the corresponding saline-treated group. τ ($p<0.05$) indicates significant differences compared to remaining AngII-treated groups.

To assess whether the marked increase in collagen content in response to AngII that was observed in $Cx43^{Cre\text{-}ER(T)/fl}$ + oil mice could be explained by the partial Cx43 deficiency that is present in these animals, we repeated this experiment in another model with a similar Cx43 deficiency ($Cx43^{+/-}$ mice). Unexpectedly, treatment with AngII did not increase collagen content in $Cx43^{+/-}$ mice above levels found in their wild-type littermates (Figure 1c). This may suggest that findings obtained in $Cx43^{Cre\text{-}ER(T)/fl}$ + oil mice were not in fact directly related to the reduction in Cx43 expression.

The effects of AngII on collagen deposition observed in $Cx43^{fl/fl}$ mice injected with oil were not modified when animals of this genotype were treated with 4-OHT (Figure 1a). In contrast, the marked increase in collagen levels observed in $Cx43^{Cre\text{-}ER(T)/fl}$ mice, with a moderate Cx43 deficiency, was reversed when Cx43 deletion was almost complete ($Cx43^{Cre\text{-}ER(T)/fl}$ treated with 4-OHT) (Figure 1b), attaining collagen values close to those found in AngII-treated $Cx43^{fl/fl}$ mice (Figure 1a).

1.c. Mechanisms involved

1.c.1. Paradoxical overexpression of mRNAs encoding for proteins involved in collagen synthesis and degradation in Cx43-deficient mice.

We analyzed the expression of mRNA encoding proteins involved in the collagen synthesis (COL1A1, TGFβ1, P4HA1), maturation (LOX) and degradation (TIMP1, TIMP2), both in wild-type animals ($Cx43^{fl/fl}$) and in Cx43-deficient mice, and infused with saline or AngII. Hearts from animals treated with saline, both from $Cx43^{fl/fl}$ mice and from $Cx43^{Cre\text{-}ER(T)/fl}$ animals treated with oil (50% Cx43 expression) had similar mRNA levels of all analyzed markers. In contrast, hearts from $Cx43^{Cre\text{-}ER(T)/fl}$ animals injected with 4-OHT had a marked induction of COL1A1, TGFβ1, LOX and TIMP1.

AngII treatment increased the expression of these four mRNAs in $Cx43^{fl/fl}$ mice and $Cx43^{Cre\text{-}ER(T)/fl}$ animals injected with oil, but not in mice with a marked Cx43 deficiency ($Cx43^{Cre\text{-}ER(T)/fl}$ + 4-OHT). In this last group, mRNA levels for COL1A1, TGFβ1 and LOX, although still high, were significantly lower than those found in mice from this group when infused with saline.

1.c.2. The increase in collagen deposition in response to AngII observed in hearts from animals partially-deficient for Cx43 ($Cx43^{Cre\text{-}ER(T)/fl}$ + oil) correlates with an enhanced activation of p38 MAPK.

Increased collagen deposition induced by AngII treatment in $Cx43^{Cre\text{-}ER(T)/fl}$ mice injected with oil (50% Cx43 expression) was associated with an enhanced activation of p38 MAPK. This increased phosphorylation of p38 MAPK was not observed in $Cx43^{+/-}$ mice treated with AngII, which did not show the enhanced collagen deposition (Figure 1c).

1.c.3. Normalization of collagen content in hearts from $Cx43^{Cre\text{-}ER(T)/fl}$ mice injected with 4-OHT and treated with AngII is associated with increased metalloproteinase 9 (MMP9) activity.

Gelatin zymography of mice myocardial samples allowed us to demonstrate an increased MMP9 activity in animals with almost total absence of Cx43 ($Cx43^{Cre\text{-}ER(T)/fl}$ + 4-OHT), compared to remaining groups, both after saline or AngII treatment. No significant differences were observed for MMP2 activity.

1.c.4. Deletion of Cx43 in Cx43$^{Cre\text{-}ER(T)/fl}$ mice injected with 4-OHT is associated with enhanced expression of inflammation markers.

Deletion of Cx43 after treatment with 4-OHT in Cx43$^{Cre\text{-}ER(T)/fl}$ mice induced an enhancement in myocardial mRNA levels for IL-6 and NOX2, both in animals infused with saline or with AngII. In addition, by immunohistochemical staining of cardiac sections we demonstrated that these hearts showed an increased expression of both the macrophage marker LAMP-2/Mac-3 and MMP9, either under saline or AngII infusion.

1.c.5. Studies in isolated fibroblasts.

Cardiac fibroblasts isolated from animals with marked Cx43 deficiency (Cx43$^{Cre\text{-}ER(T)/fl}$ + 4-OHT) depicted an abnormal phenotype, including reduced size and highly refractory nuclei. In addition, these cells showed a decrease in expression of α-SMA and SM22α, two markers of cell differentiation.

1.d. Comparison between results obtained in Cx43$^{Cre\text{-}ER(T)/fl}$ animals with those obtained in mice overexpressing LOX.

Overexpression of human LOX in a transgenic mice model accelerates cardiac remodeling and aggravates dysfunction and hypertrophy induced by chronic treatment with AngII. In these animals, and contrary to what happened in Cx43-deficient animals treated with 4-OHT, we observed a greater fibrotic response, although they shared an increased myocardial inflammatory infiltrate, and an exacerbated expression of proinflammatory markers. These results seem to exclude a clear relationship between Cx43 and LOX.

2. Role of Connexin 43 in myocardial healing, adverse left ventricular remodeling and heart failure after myocardial infarction.

2.a. Postinfarct scar area

Area of postinfarct scar, as determined by Picrosirius Red staining 14 days after transient coronary occlusion (45 min), was significantly reduced in mice with Cx43 deficiency (Cx43$^{Cre\text{-}ER(T)/fl}$). This was true both in oil-treated animals (50% Cx43 expression) and in those injected with 4-OHT (<5% Cx43 expression), as compared with results obtained in Cx43$^{fl/fl}$ animals, which have normal Cx43 levels (16.10±2.16%

for both groups of Cx43-deficient animals vs. 23.91±2.44% for both $Cx43^{fl/fl}$ groups, p=0.022, Student's t test) (Figure 2).

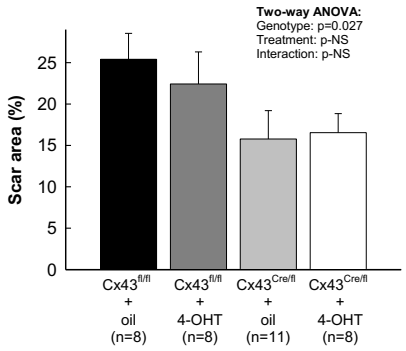

Figure 2. Infarct size, with respect to total area, determined by Picrosirius Red staining, in cardiac sections obtained from the 4 experimental groups.

2.b. Changes in expression of healing markers by Western Blot.

Two-way ANOVA showed a significant increase in myocardial expression of NF-κB, pro-TGFβ1, and the active form of TGFβ1, together with a very slight increase in SMAD2/3, in animals submitted to transient coronary occlusion and reperfusion for 14 days. However, in Cx43-deficient animals (both in those treated with 4-OHT or oil), the increase in pro-TGFβ1 was lower, and in fact it did not reach statistical significance as compared with their corresponding control groups.

2.c. Comparison between results obtained in $Cx43^{Cre\text{-}ER(T)/fl}$ animals with those obtained in mice overexpressing LOX.

LOX overexpression did not modify the size of the postinfarct myocardial scar, as determined by the area of fibrosis within the area at risk through Picrosirius Red staining. Similarly, LOX overexpression did not alter collagen deposition in remote myocardial regions (6.57±0.85 vs. 7.38±0.45% in WT and TgLOX, respectively, p-NS). These data again do not support a clear relationship between Cx43 and LOX.

3. Relevance with possible future implications

Our study demonstrates that connexin 43 (Cx43) deficiency causes a reduction in collagen deposition, that is to say, in the development of myocardial fibrosis during adverse left ventricular remodeling.

In the case of left ventricular remodeling induced by pressure overload, as happened during treatment with angiotensin II, Cx43 deficiency was associated with a reduction in interstitial fibrosis, an effect that was linked to enhanced metalloproteinase 9 activity, greater inflammatory infiltrate and lower capacity of fibroblasts to differentiate. Probably due to peculiarities of the animal model used, a very marked Cx43 deficiency is necessary to observe this beneficial effects. In any case, our data support previous results obtained in other tissues, such as the skin or the cornea, in which it was shown that a reduction in Cx43 levels or functionality (by means of creams with inhibitory peptides, for example) causes a better and faster healing process after various types of injuries.

In the case of post-infarct left ventricular remodeling, we have demonstrated that both a moderate and a marked reduction in Cx43 levels causes a decrease in scar size. This effect was associated with a lower expression of pro-TGFβ1, which could explain these findings, at least in part. The time limitations of the model (whose mortality would increase if we prolonged follow-up for more than 14 days after reperfusion) made it impossible to find out what happens in the long term. But it seems likely that this lower post-infarct scarring would result in a reduction in adverse left ventricular remodeling and, therefore, in a lower appearance of heart failure.

Therefore, development of drugs capable of decreasing the functionality or expression of Cx43 could become a very important tool to reduce the development of fibrosis and the associated appearance of heart failure. It should be considered that cardiovascular diseases remain the leading cause of death globally, and among them myocardial infarction is the leading cardiovascular disease. In the case of an acute myocardial infarction, the improvement in current treatments has led to enhanced patients survival after the acute episode. However, this survival occurs at the expense of reduced cardiac function, due to development of myocardial hypertrophy and fibrosis, with the consequent appearance of heart failure. Likewise, hypertension is a very

common comorbidity in cardiovascular patients, which ends up leading to ventricular remodeling with the appearance of fibrosis. Any treatment aimed at reducing the appearance of fibrosis, an orphan target today, will have a great impact on the quality of life of these patients and would reduce the costs of the healthcare system.

However, it must be recognized that the application of therapies aimed at reducing the function and/or expression of Cx43 will not be simple. A reduction in Cx43 levels has been associated with a high incidence of arrhythmias. This would be an important limitation. To solve this problem there are several possibilities. In the case of infarction, inhibitors or siRNA could be administered directly, and selectively, into the area at risk, with intracoronary catheters, which is a feasible maneuver, as we have shown in other studies. It would be more complex in the case of hypertension, which affects the entire organ. In this sense, subsequent studies should analyze whether these effects on the development of fibrosis are due to their participation as channels within the gap junctions (with the consequent affectation of arrhythmias), or independently of them, such as acting as a regulatory transcriptional factor. In this case, therapies aimed at specifically targeting this function could be designed.

Therefore, the conclusions of this project are:

A. Regarding ventricular remodeling after pressure overload induced by chronic treatment with AngII.

(1) A moderate Cx43 deficiency (50%) causes a huge increase in collagen deposition after treatment with angiotensin II, which appears independent of such Cx43 deficiency, and is associated with an increase in p38 MAPK activation.

(2) A marked Cx43 deficiency results in a reduction in collagen deposition over that expected for that genotype after treatment with angiotensin II. This effect is associated with increased MMP9 activity, increased inflammatory infiltrate and a reduced ability of fibroblasts to differentiate.

B. Regarding ventricular remodeling after myocardial infarction.

(1) Cx43 deficiency (both moderate and marked) causes a reduction in postinfarct scar size. This effect is associated with lower expression of pro-TGFβ1.

4. Scientific bibliography generated

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