International Journal of Cardiology 252 (2018) 163–166

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

Short communication

Evidence for functional PAR-4 thrombin receptor expression in cardiac fibroblasts and its regulation by high glucose PAR-4 in cardiac fibroblasts

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ARTICLE INFO

Article history:
Received 15 May 2017
Received in revised form 27 September 2017
Accepted 5 October 2017

Keywords:
Thrombin
Protease-activated receptor
Cardiac
Fibroblast
Diabetes
High glucose

ABSTRACT

Background: Thrombin promotes cardiac fibroblast proliferation and fibrosis via protease-activated receptor (PAR-1). PAR-4 is reportedly absent in cardiac fibroblasts. In smooth muscle cells, PAR-4 expression is also low but increases upon hyperglycemia and contributes to vascular remodelling in diabetic mice. We examined if PAR-4 is a glucose-responsive gene with remodelling-related functions in cardiac fibroblasts.
Methods and results: Cardiac PAR-4 increased in mice with streptozotocin- or diabetogenic diet (DD)-induced diabetes. PAR-4 mRNA and protein were detectable in cardiac fibroblasts from chow-fed mice and increased in high (HG, 25 mM) vs. low glucose (LG; 5.5 mM) cultures. Conversely PAR-4 mRNA was higher in fibroblasts from DD-fed mice but reduced in LG cultures. Cardiac fibroblasts in HG culture responded more strongly to thrombin or PAR-4 activating peptide in terms of migration (wound-scratch assay), remodelling-associated gene expression (interleukin 6, alpha smooth muscle actin) and oxidative stress (dihydroethidium fluorescence).
Conclusion: PAR-4 is expressed in mouse cardiac fibroblasts and is dynamically regulated by extracellular glucose in vitro and diabetes in vivo, thereby impacting on fibroblast functions relevant for cardiac remodelling. These findings add further evidence for the usefulness of the recently developed PAR-4 antagonists in clinical settings.

1. Introduction

Thrombin inhibition protects against diabetic cardiomyopathy (DCM) and downregulates cardiac protease-activated receptors PAR-1 and PAR-4 [1]. PAR-1 contributes to remodelling after myocardial infarction (MI) by driving cardiomyocyte hypertrophy [2] and fibroblast proliferation [3,4], PAR-4 by blocking adenosine pathways and promoting cardiomyocyte apoptosis [5,6]. PAR-4 is reportedly absent in cardiac fibroblasts [7,8]. Smooth muscle cells also express near-indetectable levels of PAR-4 at rest, but dramatic upregulation upon hyperglycemic stress contributes to vascular remodelling in diabetic mice [9,10]. We therefore examined PAR-4 as a candidate glucose-responsive gene with remodelling-related functions in cardiac fibroblasts.

2. Methods

2.1. Materials

Human thrombin was from American Diagnostica GmbH (Pfungstadt, Germany), D-glucose, streptozotocin (STZ) and dihydroethidium (DHE) from Sigma (München, Germany), PAR-4 activating peptide (AP) from Tocris Bioscience (Bristol, UK), all other materials from Merck (Darmstadt, Germany) or Roth (Karlsruhe, Germany) unless otherwise stated.

2.2. In vivo protocols

Male C57Bl6/J mice aged 6–8 weeks were subjected to streptozotocin (STZ)-induced type 1 diabetes [10], or diabetogenic diet (DD, #S7200-E010, Ssniff Spezialdiäten GmbH, Soest, Germany) for the indicated intervals. PAR-4$^{-/-}$ mice were generously provided by Dr. Justin Hamilton, Australian Centre for Blood Diseases, and bred in house. The studies had the approval of the local animal experimentation ethics committee.

2.3. In vitro protocols

Ventricular fibroblasts isolated from chow vs. DD-fed mice by enzymatic digestion (Liberase™, Sigma) were cultured to P1 in Dulbecco's Modified Eagle Medium (DMEM) containing low (LG, 5.5 mM) glucose and 10% fetal calf serum (FCS, GibcoBRL, Rockville, MD). Cells adhered for 24 h in LG-DMEM, synchronised for 48 h in 1% FCS then switched to either LG or high glucose medium (HG, 25 mM) for 72 h. In separate studies, cardiac fibroblasts were cultured in LG vs. HG medium to P2, synchronized for 24 h then stimulated for PAR-4 expression or functional studies as indicated.

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[3] This author performed surgical interventions and contributed to data analysis and drafting of the manuscript.
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https://doi.org/10.1016/j.ijcard.2017.10.019
0167-5273/

2.4. Realtime PCR and western blotting

RNA extraction, reverse transcription, realtime PCR and western blotting were performed as described [10].

2.5. Functional cell-based assays

Fibroblast migration was assessed by wound-scratch assay and counted by a blinded observer as described [9]. Oxidative stress was assayed in cells loaded with DHE (10 μM in DMEM) for 20 min in the dark followed by kinetic mode measurement in a fluorescent plate reader (Ex 535 nm/Em 635 nm). Data show background-corrected AUC over 10 min of measurement.

2.6. Statistical analysis

Data are presented as mean ± SEM, normalised to controls as indicated. Statistical analysis utilised Wilcoxon matched rank test or one-way analysis of variance, with Dunnet's post-hoc test for multiple comparisons, as appropriate. $P < 0.05$ was accepted as significant.

3. Results

3.1. Diabetes upregulates PAR-4 in cardiac fibroblasts

Cardiac PAR-4 mRNA was upregulated by STZ-treatment (3 weeks, Fig. 1A) and DD (8 weeks, Fig. 1B, all n = 4). PAR-4 mRNA was detectable in cardiac fibroblasts isolated from mice after 11 weeks of chow vs. DD (Fig. 1C). Cells from chow-fed mice and maintained in LG medium (n = 6) significantly upregulated PAR-4 mRNA upon switch to HG medium for 72 h (n = 3). Modest elevations were seen in cells from DD-fed mice exposed to HG medium (n = 5, P = 0.1), switching to LG medium reduced PAR-4 to levels in chow-fed LG controls (n = 7). Cardiac fibroblasts from normal mice and cultured in LG conditions incrementally upregulated PAR-4 when culture medium was switched to medium glucose (MG, 10 mM) or HG for 24 h; conversely, in parallel HG cultures, PAR-4 expression dropped to LG control levels within 24 h of switching culture medium to MG or LG conditions (all n = 4, Fig. 1D). PAR-4 protein expression was 3-fold higher in HG vs. parallel LG cultures (Fig. 1E, n = 3).

3.2. High glucose potentiates PAR-4 responsiveness in cardiac fibroblasts

Thrombin (1 U/mL, 24 h) upregulated IL-6 and alpha smooth muscle actin (αSMA) mRNA in HG but not LG fibroblasts from WT mice (all n = 4, Fig. 2A, B). Periostin gene expression increased modestly (n = 4, Fig. 2C). These stimulatory effects of thrombin were absent in PAR-4$^{-/-}$ cardiac fibroblasts (all n = 4, Fig. 2A–C.) Migratory capacity was reduced in HG vs. LG fibroblasts; PAR-4AP (100 μM) or thrombin (1 U/mL) significantly stimulated fibroblast movement into the cleft, more strongly in HG cultures (all n = 4, representative images in Fig. 2D, pooled data in Fig. 2E). PAR-4AP (100 μM) did not stimulate intracellular superoxide over short-term (30 min) exposure in either LG or HG fibroblasts (Fig. 2E) but doubled DHE fluorescence in HG fibroblasts over 24 h. LG cells remained unresponsive (Fig. 2F, all n = 4).

3.3. Supplemental results

Cardiac PAR-4 was induced in mice with MI or transaortic constriction. Simulated ischemia (SI), angiotensin II or Hyper-IL-6, a fusion protein of interleukin 6 and its soluble receptor upregulated PAR-4 in mouse cardiac fibroblasts. SI and deferoxamine (DFO) both induced hypoxia-inducible factor (HIF)1α in cardiac fibroblasts but DFO did not regulate PAR-4 (all n = 3–6, Supplementary figure).

4. Discussion

PAR-4 is both detectable in mouse cardiac fibroblasts and dynamically regulated by glucose in vitro and diabetes in vivo. Accordingly, thrombin-stimulated responses are enhanced in cardiac fibroblasts pre-exposed to high glucose. Supplementary data show cardiac PAR-4 upregulation also in MI and pressure overload, which is reproduced in isolated cardiac fibroblasts with SI and the pro-fibrotic factors Ang II

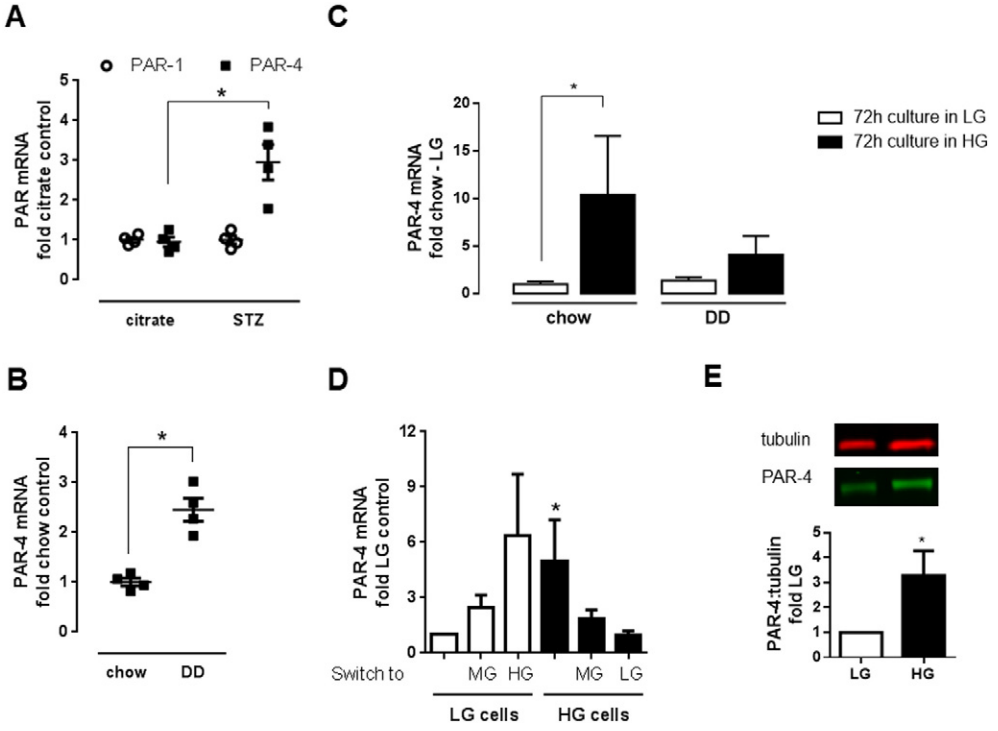

Fig. 1. *PAR-4 upregulation in settings associated with cardiac fibrosis.* (A) Cardiac PAR-1 or PAR-4 mRNA expression in mice with diabetes induced by STZ or (B) diabetogenic diet (DD). All n = 3–4. (C) PAR-4 mRNA expression in cardiac fibroblasts isolated from mice fed chow or DD and cultured in low glucose (LG, 5.5 mM) or high glucose (HG, 25 mM) medium for 72 h (n = 3–7). (D) PAR-4 regulation in normal mouse cardiac fibroblasts cultured under LG vs. HG conditions then switched to medium containing either LG, medium glucose (MG, 10 mM) or HG for 24 h (all n = 4). *P > 0.05 vs. respective controls.

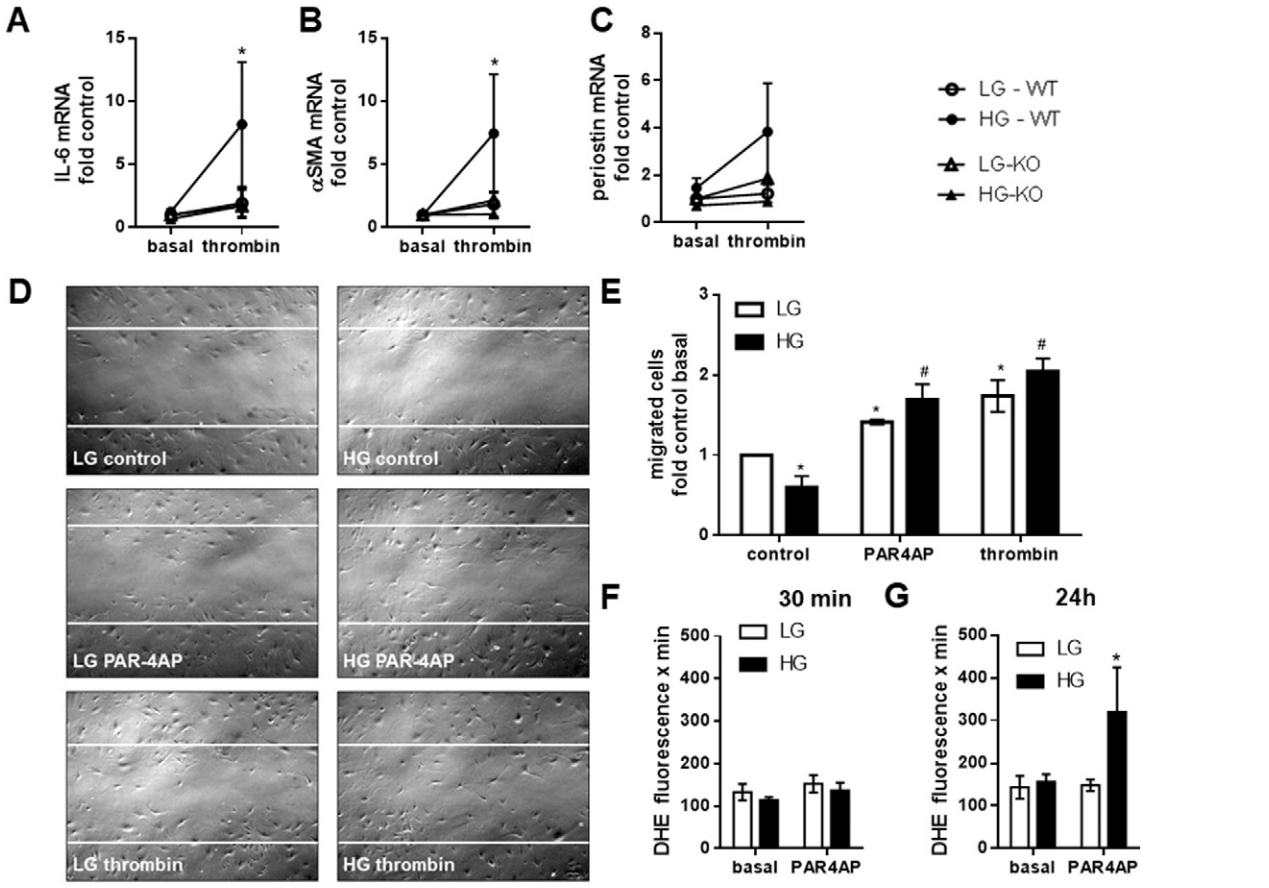

Fig. 2. *Increased PAR-4 responsiveness in cardiac fibroblasts cultured under HG conditions.* Thrombin (1 U/mL, 24 h)-stimulated changes in mRNA expression of interleukin-6 (IL-6, A), alpha smooth muscle actin (αSMA, B) and periostin (C) in wildtype (WT) or PAR-4$^{-/-}$ (KO) mouse cardiac fibroblasts maintained in low glucose (LG, 5.5 mM) or high glucose (HG, 25 mM) medium. Representative images (D) and pooled data (E) of wound-scratch analysis of basal and stimulated (thrombin, 1 U/mL, PAR-4 activating peptide, 100 μM) migration of cardiac fibroblasts cultured in LG or HG medium (72 h). Dihydroethidium (DHE) fluorescence, a measure of intracellular oxidant stress, in LG or HG cardiac fibroblasts stimulated with PAR-4 activating peptide (100 μM) for 30 min (F) or 24 h (G). All n = 4, *P < 0.05 vs. unstimulated LG control, $^{\#}$P < 0.05 vs. respective LG stimulated control.

and IL-6 [11,12]. HIF1α, identified by promoter analysis (Transfac®) as a putative PAR-4 regulatory transcription factor, does not appear to contribute to PAR-4 induction in cardiac fibroblasts.

Two studies have so far examined PAR-4 involvement in cardiac remodelling [5,6], both focussed on cardiac myocyte functions and performed in non-diabetic mice. Given that PAR-4 antagonists are candidate low-risk antiplatelet agents, investigation of their potential impact on organ systems such as the heart is warranted. We here show that PAR-4-mediated wound closure, oxidative stress and expression of remodelling-related genes are potentiated in cardiac fibroblasts maintained in HG conditions, implying a role for PAR-4 in the early remodelling process in the diabetic heart. Our supplemental findings imply that PAR-4 upregulation contributes to adverse remodelling in other settings also.

In conclusion, hyperglycemic cardiac fibroblasts are identified as previously unrecognised target cells for PAR-4-activating proteases. Since the heart is a major target for diabetic end-organ damage, to which cardiac fibroblasts critically contribute, and PAR-4 is also upregulated in pressure overload and MI, our findings could be relevant for the application of the emerging PAR-4 antagonists for indications beyond platelet inhibition.

5. Study limitations

The present findings are preliminary and validation of PAR-4 expression and function in cardiac fibroblasts in other models of cardiac remodelling is required. For translational relevance, PAR-4 needs also to be demonstrated in human cardiac fibroblasts. We hope to provide a first indication of a novel site of action of PAR-4 and trigger further studies in this context.

Conflict of interest

The authors report no relationships that could be construed as a conflict of interest.

Grant support

This work was supported in part through grants from the Ernst und Berta Grimmke-Stiftung Düsseldorf (ACF, Project 3/09) and the Deutsche Forschungsgemeinschaft (ACF, project RO 3921/2-1). SK was supported by an intramural grant from the medical faculty of the Heinrich-Heine-University Düsseldorf.

Acknowledgments

We thank Prof. Jens. W. Fischer for the generous provision of samples from mice subjected to LAD occlusion and Hyper-IL-6-stimulated cardiac fibroblasts.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijcard.2017.10.019.

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