Cardiovascular Research (2013) **100**, 211–221
doi:10.1093/cvr/cvt181

Inhibition of endothelial nitric oxide synthase induces and enhances myocardial fibrosis

Andrey Kazakov[1]*, Rabea Hall[2], Philippe Jagoda[1], Katrin Bachelier[1], Patrick Müller-Best[1], Alexander Semenov[1], Frank Lammert[2], Michael Böhm[1], and Ulrich Laufs[1]

[1]Klinik für Innere Medizin III, Kardiologie, Angiologie und Internistische Intensivmedizin, Universitätsklinikum des Saarlandes, Homburg, Saar 66424, Germany; and [2]Klinik für Innere Medizin II, Gastroenterologie, Hepatologie, Endokrinologie, Diabetologie und Ernährungsmedizin des Saarlandes, Homburg, Saar 66424, Germany

Received 4 March 2013; revised 10 June 2013; accepted 13 July 2013; online publish-ahead-of-print 17 July 2013

Time for primary review: 28 days

Aims The endothelial nitric oxide synthase (eNOS) contributes to cardiac remodelling. We studied the role of eNOS in the development of myocardial fibrosis during cardiac overload.

Methods and results Ten-week-old male C57/Bl6 wildtype (WT) and eNOS mice ($eNOS^{-/-}$) were subjected to transverse aortic constriction (TAC, 360 μm) and WT were treated with L-N^G-nitroarginine methyl ester (L-NAME, 100 mg/kg/day) for 35 days. Inhibition of eNOS by L-NAME induced interstitial fibrosis, augmented replacement fibrosis, and induced apoptosis of cardiac fibroblasts and cardiomyocytes. L-NAME and $eNOS^{-/-}$ markedly increased the fibrosis induced by TAC and enhanced the myocardial prevalence of $CXCR4^{pos}$ fibroblasts. Myocardial stromal-derived factor-1 (SDF-1) expression was up-regulated by L-NAME and down-regulated after TAC. Blood pressure lowering by co-treatment with hydralazine (250 mg/L/day) did not reverse the L-NAME effects.

In mice transplanted with green fluorescent protein $(GFP)^{pos}$ bone marrow, L-NAME increased the percentage of GFP^{pos} fibroblasts in the myocardium to 45–70%. Strain-mismatched BMT of $eNOS^{-/-}$-BM increased and of WT-BM decreased the percentage of $CXCR4^{pos}$ fibroblasts in all groups. The number of fibrocytes ($CD45^{pos}$ collagen I^{pos} cells) in the peripheral blood and in the bone marrow was increased both by TAC and L-NAME. L-NAME but not the inhibitor of inducible NOS 1400 W and of neuronal NOS 7-nitroindazole increased hydroxyproline and collagen Iα1. L-NAME up-regulated SDF-1 mRNA in cultured neonatal rat cardiac fibroblasts as well as their migratory capacity.

Conclusion eNOS inhibition induces and enhances cardiac fibrosis independently of blood pressure by activating SDF-1/CXCR4, extracellular matrix production in cardiac fibroblasts and by increasing recruitment of fibrocytes from the bone marrow.

Keywords Cardiac fibrosis • eNOS inhibition • Fibrocytes • SDF-1

1. Introduction

Endogenous nitric oxide (NO) regulates myocardial remodelling.[1–8] A central component of cardiac remodelling is myocardial fibrosis. Growing evidence suggests that endothelial nitric oxide synthase (eNOS) is involved in regulation of intracardiac fibroblast activity and in mobilization and trafficking of fibrocytes.[9–11] However, the precise mechanisms of the involvement of eNOS in the development of cardiac fibrosis under different pathological conditions are not completely understood.[3–8] Whereas some studies demonstrate a deteriorating effect,[3,4] the others show protective role of eNOS during increased cardiac afterload.[5–8]

Recent findings suggest that in addition to the resident cardiac fibroblasts circulating bone marrow (BM)-derived fibroblast (fibrocytes) may contribute to cardiac fibrogenesis.[12–16] These cells constitute a BM-derived fibroblast population that circulates in the peripheral blood.[12] The mobilization and recruitment of fibrocytes into tissues in profibrotic conditions are regulated by chemokines such as the stromal-derived factor-1 (SDF-1, CXCL12) whose receptor CXCR4 is highly expressed in fibrocytes.[17–20] Circulating fibrocytes have been discussed as a prognostic disease marker and as a potential therapeutic target.[18–21]

Therefore, the aim of the study was to examine the role of systemic eNOS inhibition in the cellular mechanisms of cardiac fibrogenesis during cardiac afterload.

* Corresponding author. Tel: +49 68411621331; fax: +49 68411623434, Email: andrej.kasakow@uniklinikum-saarland.de

2. Methods

2.1 Animals, transverse aortic constriction, and administration of L-NAME and hydralazine

The study was approved by the animal Ethics Committee of the Universität des Saarlandes and conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Pub. No. 85–23, revised 1996). Ten-week-old male C57/Bl6 and eNOS$^{-/-}$ mice (B6129/P2-Nos3, Charles River Laboratories, Sulzfeld, Germany) were housed under standard conditions. The inhibitor of NO synthases (NOSs) L-N^G-nitroarginine methyl ester (Sigma-Aldrich, Germany; L-NAME, 100 mg/kg/day p.o. via drinking water) inhibits eNOS ~100-times more potently than inducible NOS (iNOS) [Ki values of 15 nM, 39 nM, and 4.4 μM for neuronal NOS (nNOS), eNOS, and iNOS, respectively].[22] The antihypertensive drug hydralazine (Sigma-Aldrich, Germany) was administrated at the dose 250 mg/L/day in the drinking water. Untreated wild-type animals (WT) served as a control. For surgery and left ventricle (LV)-pressure measurements animals were intraperitoneally anaesthetized with ketamine (100 mg/kg body weight) and xylazine (10 mg/kg). Anaesthetic monitoring was performed by testing of rear foot reflexes before and during procedures, observation of respiratory pattern, mucous membrane colour, and responsiveness to manipulations throughout the procedures. After orotracheal intubation using a 20G catheter, the tube was connected to a volume cycled rodent ventilator (Harvard Apparatus, USA) on supplemental oxygen with a tidal volume of 0.2 mL and respiratory rate of 110/min. The chest cavity was entered in the second intercostal space at the left upper sternal border through a small incision and aortic constriction was performed by tying a 7–0 nylon suture ligature against a 27G needle to yield a narrowing 360 μm in diameter and a transverse aortic constriction (TAC) of 65–70%. Control mice underwent a sham operation. The L-NAME administration in the TAC + L-NAME group was started at the day of surgery. After 5 weeks, LV-pressure measurements were performed with 1.4 Fr pressure-transducing catheter (Mikro Tip Catheter, Millar instruments, USA). Mice were sacrificed by i.p. injection of ketamine (1 g/kg body weight) and xylazine (10 mg/kg) and hearts were rapidly excised. Hearts were partly snap-frozen in liquid nitrogen and stored at −80°C and partly embedded in paraffin after fixation in PBS-buffered formalin (4%). Blood and BM were sampled. Blood pressure was measured on the tail artery of mice 4 weeks after surgery or beginning of L-NAME and hydralazine administration on five consecutive days using at least 20 repeating measurements per day (BP 2000 Series II Blood Pressure Analysis System, Visitech Systems, USA).

2.2 Bone marrow transplantation

Six-week-old C57Bl/6 WT mice, C57Bl/6-Tg(ACTbEGFP)1Osb mice [Jackson laboratory; expressing green fluorescent protein (GFP) ubiquitously] and eNOS$^{-/-}$ mice (B6129/P2-Nos3, Charles River Laboratories, Sulzfeld, Germany) were sacrificed by i.p. injection of ketamine (1 g/kg body weight) and xylazine (100 mg/kg) and marrow was obtained from the long bones. Bone marrow cells ($1–2 \times 10^7$) were transplanted into 10-week-old, male, lethally irradiated (total dose 9 Gy), recipient mice by injection into the tail vein 5 h after irradiation. The adequacy of anaesthesia and mouse killing were performed as described above. Both WT and eNOS$^{-/-}$ mice were transplanted with WT or eNOS$^{-/-}$ bone marrow.[8]

To monitor reconstitution of the BM, three animals per group were sacrificed 4 weeks after BMT and histological examination of BM sections was performed. The detailed information is described in the Supplementary material online. Four weeks after BMT, surgery, treatment with L-NAME or L-NAME and hydralazine were performed as described above.

2.3 Measurement of nitric oxide synthase activity

Myocardial tissues were homogenized and centrifuged. Activity of NOS in the supernatant was quantified by measuring the formation of radiolabelled L-[H^3]-citrulline from L-[H^3]-arginine (PerkinElmer, Germany) using the NOS Activity assay kit (Cayman Chemical, USA) according to the manufacturer's instructions. Activity of eNOS and nNOS was measured in the presence and activity of iNOS in the absence of 0.1 μM of calmodulin and 0.6 μM $CaCl_2$.

2.4 Fluorescence-activated cell sorter analysis

Cells from blood and BM samples were permeabilized with Cytofix/Cytoperm (Becton Dickinson, Germany) according to the manufacturer's instructions and double labelled with the following antibodies: biotin-conjugated anti-collagen I (Rockland, Germany) with streptavidin-FITC (Vector Laboratories, USA) and CD45-APC (Pharmingen, Germany). Isotype-identical antibodies served as controls (Becton Dickinson, Germany). The viable lymphocyte population was examined by flow cytometry (BD FACS Calibur™ instrument and BD CellQuest™ software).

2.5 Cell culture

Neonatal cardiac fibroblasts isolated from the minced ventricles of 5-day-old Sprague-Dawley rats by digestion with pancreatin and collagenase type II (Sigma-Aldrich, Germany) were re-suspended in Dulbecco's Modification of Eagle's Medium (DMEM; Life Technologies, USA) with 10% foetal calf serum (PAA Laboratories, Austria) for 1 h, whereupon attached cells were supplemented with the fresh medium. Cells at 85% confluence in cell culture dishes were digested by trypsinization and replanted. Fibroblasts from the second passage were used for all experiments. After 24 h in reduced serum medium, fibroblast cultures were treated with 30 μM L-NAME (Sigma-Aldrich, Germany), with 30 μM *N*-(3-(aminomethyl)benzyl) acetamidine, high-selective inhibitor of iNOS (1400 W,[23] Adipogen, Germany) and with 30 μM 7-nitroindazole, high-selective inhibitor of nNOS (Sigma-Aldrich, Germany) for 72 h. Cells were harvested for total protein- and RNA-isolation, used for migration assay or immunostained. To estimate collagen production, the level of hydroxyproline in the cell culture medium was determined by the hydroxyproline assay according to the manufacturer's instructions (QuickZyme Biosciences, The Netherlands). The purity of the fibroblast culture was confirmed by immunostainings which showed the presence of the fibroblast marker intracellular fibronectin (Abcam, UK) and the absence of endothelial cell marker podocalyxin (R&D Systems, USA), inflammation cell marker CD45 (Abcam, UK), and cardiomyocyte marker α-sarcomeric actin (Sigma-Aldrich, Germany). To estimate the extent of myofibroblast transformation, the number of cells double immunostained for α-smooth muscle actin (Sigma-Aldrich, Germany) and fibronectin per 300 fibronectin-positive cells were counted in each cell culture dish.

2.6 Migration assay

To assess the migratory capacity, 500 fibroblasts from each culture dish were transferred to modified Boyden chambers (BD Bioscience, Germany) in 24-well plates filled with 750 μL medium containing 10 μL SDF-1 (R&D Systems, Germany) for 24 h, whereupon were immunostained for intracellular fibronectin (Abcam, UK). Boyden chamber filters were cut out, placed on slides and mounted with fluorescent mounting medium (Vectashield, Vector Laboratories, USA). The whole filter was analysed using a Nikon E600 epifluorescence microscope (Nikon, Japan) with appropriate filters. Cells positive for fibronectin were counted.

2.7 Immunofluorescence analysis

To detect fibroblasts, cardiomyocytes, cycling cells, inflammatory cells, CXCR4, SDF-1α, and GFP immunostainings on 3 μm paraffin sections

of the LV were performed using heat-mediated antigen retrieval with citraconic anhydride solution followed by overnight incubation at 4°C with the first antibody and incubation with the appropriate secondary antibody at 37°C for 1 h. The detailed methods are described in the Supplementary material online.

2.8 Apoptosis detection

Apoptosis detection was performed on 3 μm thin sections of formalin-fixed heart sections with the ApopTag Peroxidase *In Situ* Oligo Ligation Kit (Millipore, Germany) according to the manufacturer's instructions. To evaluate apoptosis rate in cardiomyocytes and fibroblasts, specific immunostainings were performed after the apoptosis assay. The evaluation is described in details in the Supplementary material online.

2.9 Tissue morphometry

The procedures used for morphometric analyses are provided in detail in the Supplementary material online.

2.10 Real-time quantitative PCR for gene expression

Gene expression was measured by the real-time quantitative reverse transcriptase-polymerase chain reaction using the TaqMan system (AB Step One Plus, Applied Biosystems, Germany). Briefly, RNA from the cultured fibroblasts was extracted using the peqGOLD RNAPure (Peqlab Biotechnologie, Germany). Oligo (dT) primed cDNA synthesis was performed with a high capacity cDNA reverse transcription kit (Applied Biosystems, Germany). Expression of collagen Iα1 (Rn01463848_m1), SDF-1 (CXCL12) (Rn00573260_m1), and 18S rRNA was analysed with TaqMan gene expression assays-on-demand purchased from Applied Biosystems. For quantification, mRNA amount of the respective gene was normalized to the amount of 18S rRNA using the $2^{-\Delta\Delta CT}$ method.

2.11 ELISA for SDF-1α

To extract proteins for enzyme-linked immunosorbent assay (ELISA) and western Blot, samples of the LV and bone marrow were homogenized in RIPA lysis buffer (Sigma-Aldrich, Germany) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich, Germany). Protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Germany). SDF-1α expression in the LV was evaluated by an ELISA using an ELISA kit (R&D Systems, Inc., Germany) according to the manufacturer's instructions.

2.12 Western blot analysis

Protein extracts were separated by 12% SDS–PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Germany). Non-specific protein was blocked with 5% dried milk for 1 h and the membranes were incubated overnight at 4°C with primary antibody against eNOS (Thermo Scientific, USA), iNOS and nNOS (Abcam, UK) and at the next day with appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature in 5% milk. Visualization was performed with an enhanced chemiluminescence kit (Amersham, Germany). Autoradiography was carried out at 23°C, and the appropriate exposures were quantitated by densitometry.

2.13 Statistical analysis

Results are presented as means ± SEM. One-way ANOVA with a Fisher LSD *post hoc* test, Kruskall–Wallis ANOVA, or Mann–Whitney test was used where applicable. Values of $P < 0.05$ were considered significant.

3. Results

3.1 Systemic inhibition of eNOS induces and enhances cardiac fibrosis independently of the changes in parameters of LV haemodynamic and peripheral arterial blood pressure

L-NAME treatment (100 mg/kg/day, 35 days) decreased myocardial activity of constitutively expressed (eNOS and nNOS) isoforms of NOS and did not significantly change activity of iNOS (*Figure 1A* and *B*). In contrast, TAC (360 μm, 35 days) but not L-NAME reduced LV expression of eNOS protein and elevated iNOS expression (*Figure 1C* and *D*). Myocardial protein expression of nNOS was very weak and was not changed by TAC and L-NAME (*Figure 1E*).

Treatment with L-NAME increased peripheral arterial blood pressure and did not change parameters of LV haemodynamics. In contrast, TAC increased LVSP, LVEDP, dP/dt_{max}, dP/dt_{min}, the ratio of wet lung weight to tibia length, and decreased dP/dt_{min} and peripheral arterial blood pressure (*Table 1*). As expected, TAC induced both myocardial (increased ratio of heart weight to tibia length) and cardiomyocyte hypertrophy (increased cardiomyocyte cross-sectional area, CCSA), which was reduced by L-NAME administration (*Table 1*). In contrast, L-NAME treatment elicited only cellular hypertrophy, which was diminished by blood pressure lowering (*Table 1*).

Cardiac fibrosis was quantified morphometrically as a fractional area of collagen content in % of myocardial content using picrosirius red staining. Both L-NAME and TAC elicited interstitial fibrosis. However, only TAC led to a massive replacement fibrosis, which was further enhanced by L-NAME administration. L-NAME induced fibrosis was morphologically similar to the fibrosis in $eNOS^{-/-}$ mice. The augmentation of collagen synthesis was accompanied by an increase in the numbers of fibroblasts identified by immunostaining for intracellular fibronectin. The effects of L-NAME on cardiac fibrosis were not reversed by blood pressure lowering with hydralazine (*Figure 1F–J*).

3.2 Different role of BM-derived fibroblast in enhanced fibroblast turnover elicited by inhibition and deficiency of eNOS and aortic constriction

Both TAC and L-NAME increased apoptosis in cardiac fibroblasts and in cardiomyocytes (*Figure 2A–F*). Apoptosis in cardiomyocytes was more pronounced in the TAC groups and was accompanied by an increase in the number of cycling cardiomyocytes identified by expression of Ki67 (*Figure 2G–J*). In contrast, cardiac cell proliferation was not observed in mice treated with L-NAME.

To study a potential contribution of BM-derived fibroblasts in cardiac fibrosis, co-immunostaining for intracellular fibronectin and marker of circulating fibroblasts (fibrocytes) CXCR4 was performed. The percentage of $CXCR4^{pos}$ fibroblasts was similarly increased by L-NAME administration and systemic eNOS deficiency (*Figure 3A–G*). To assess the importance of eNOS in LV and BM for their recruitment into the heart, both WT mice and $eNOS^{-/-}$ mice were transplanted with WT or $eNOS^{-/-}$ BM and underwent TAC- or sham-operation. Strain-mismatched transplantation of WT BM decreased and of $eNOS^{-/-}$ BM increased the percentage of $CXCR4^{pos}$ fibroblasts both in SHAM and TAC mice (*Figure 3H*).

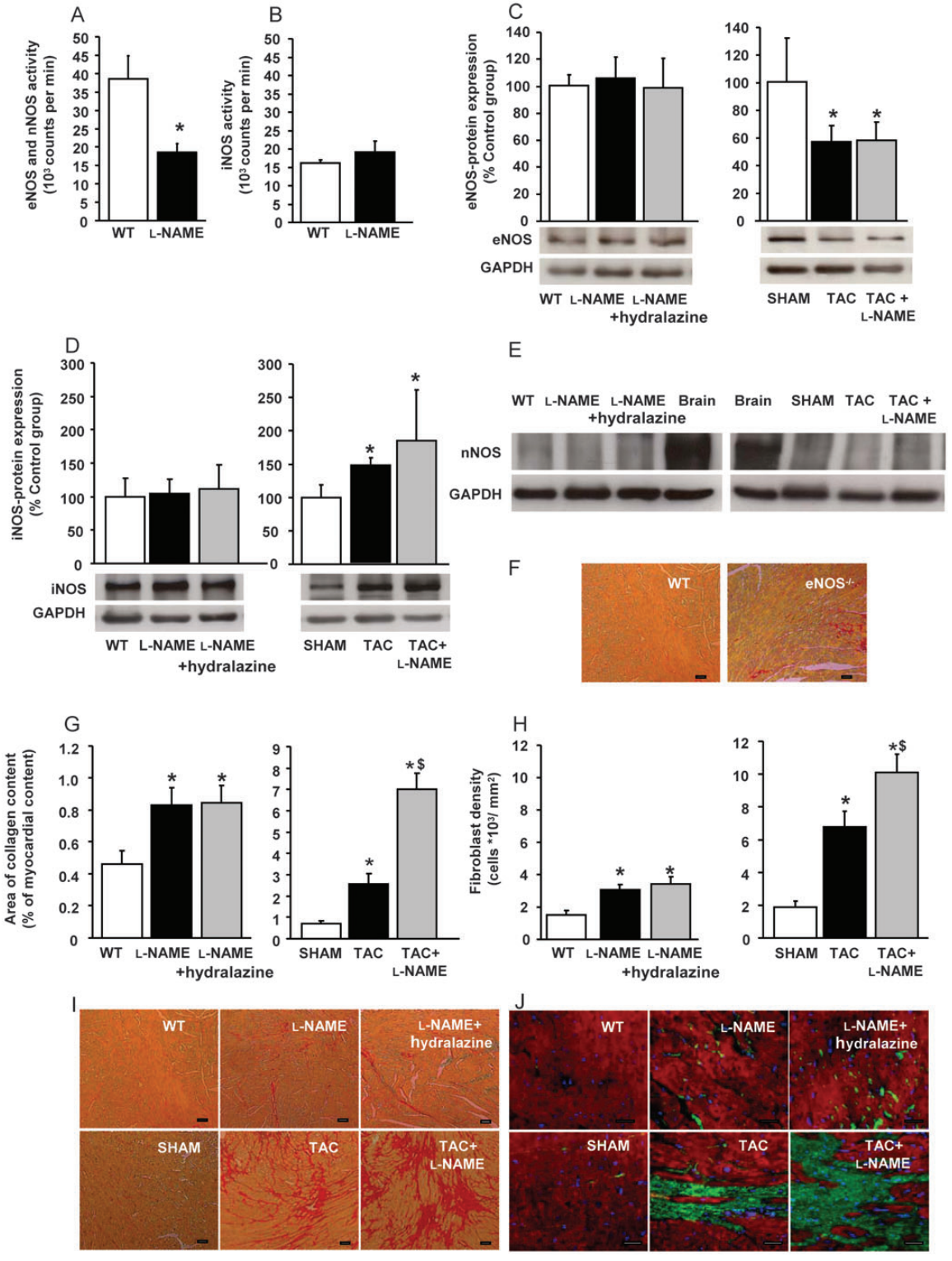

Figure 1 L-NAME treatment decreases myocardial activity of eNOS and nNOS but not of iNOS, induces and enhances cardiac fibrosis. L-NAME treatment, 100 mg/kg/day p.o., 35 days, decreases myocardial activity of eNOS and nNOS (*A*) but not of iNOS (*B*), $n = 3$ per group. TAC (360 μm, 35 days) but not L-NAME significantly reduces the expression of eNOS (*C*) and elevates of iNOS (*D*). Protein expression of eNOS and iNOS normalized against GAPDH. Myocardial protein expression of nNOS was very weak and was not changed by TAC and L-NAME (*E*). eNOS-knockout elicits cardiac fibrosis (*F*). L-NAME, combination of L-NAME and hydralazine 250 mg/L/day p.o., TAC and combination of TAC and L-NAME induce cardiac fibrosis (expressed as the fractional area of collagen content in % of myocardial content) (*G*) and increase the number of fibroblasts per mm^2 (*H*), $n = 7–10$ per group. $^*P < 0.05$ compared with corresponding control group: WT animals untreated with L-NAME or L-NAME and hydralazine (WT) or sham-operated animals (SHAM), $^\$P < 0.05$ compared with mice with TAC. Representative sections of the LV myocardium stained with picrosirius red (*I*) and co-immunostaining for myocytic α-sarcomeric actin (red) and the fibroblast marker intracellular fibronectin (green) (*J*). Nuclei are stained blue by DAPI. Bars = 30 μm.

Table 1 Anatomical and functional data

	WT	L-NAME	L-NAME + hydralazine	SHAM	TAC	TAC + L-NAME
Animal number	7	10	8	10	10	10
Heart rate (b.p.m.)	192 ± 19	237 ± 22	199 ± 18	212 ± 14	213 ± 19	300 ± 26§
SABP (mmHg)	115 ± 3	138 ± 2$^{*,\ddagger,\S,\|\|}$	117 ± 1.1†,§	110 ± 0.7	100 ± 3$^{*,\dagger,\ddagger,\|\|}$	113 ± 3§,†
DABP (mmHg)	80 ± 3	98 ± 3$^{*,\ddagger,\S,\|\|}$	80 ± 3†,§	83 ± 3	62 ± 3$^{*,\dagger,\ddagger,\|\|}$	85 ± 5§,†
LVSP (mmHg)	105 ± 3	107 ± 5	98 ± 6	85 ± 5	118 ± 6*	103 ± 18*
LV$dP/dt_{max}$ (mmHg/s)	2905 ± 170	2797 ± 237	2521 ± 279	2978 ± 185	3707 ± 162*,†,‡	3692 ± 573*,†,‡
LV$dP/dt_{min}$ (mmHg/s)	−2732 ± 216	−2485 ± 255	−2167 ± 242	−2693 ± 193	−3825 ± 200*,†,‡	−3621 ± 581*,†,‡
LVEDP (mmHg)	8.6 ± 0.37	8.6 ± 0.42	8.6 ± 0.4	7.0 ± 1.6	17.0 ± 1.6*,†,‡	17.6 ± 1.6*,†,‡
Lung fluid weight/TL (mg/mm)	9.2 ± 0.4	9.5 ± 0.5	9.5 ± 0.6	9.0 ± 0.5	12.5 ± 1.5*,†,‡	10 ± 0.8
Heart weight/TL (mg/mm)	8.0 ± 0.2	7.8 ± 0.1	8.8 ± 0.2	8.4 ± 0.3	12.3 ± 1.1*,†,‡	11.3 ± 0.9*,†,‡
CCSA (μm^2)	176 ± 5.2	234 ± 12*	170 ± 4†	130 ± 6.8	308 ± 19$^{*,\dagger,\ddagger,\|\|}$	191 ± 13.6*,§,†,‡

LV, left ventricle; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; SABP, systolic arterial blood pressure; DABP, diastolic arterial blood pressure; TL, tibia length; CCSA, cardiomyocyte cross-sectional area.
*$P < 0.05$ vs. corresponding control group.
†$P < 0.05$ vs. L-NAME.
‡$P < 0.05$ vs. L-NAME + hydralazine.
§$P < 0.05$ vs. TAC.
||$P < 0.05$ vs. TAC + L-NAME.

To further characterize the role of BM-derived fibroblasts in cardiac fibrosis induced by L-NAME, transplantation of GFP-positive BM (GFP^{pos} BMT) was performed. Fibrocytes were identified by co-immunostaining for intracellular fibronectin and GFP. The number per mm^2 and the percentage of GFP^{pos} fibroblasts were up-regulated by L-NAME similar to the number and the percentage of the $CXCR4^{pos}$ fibroblast in the non-BMT animals. The effect was not reversed by hydralazine (*Figure 3I–N*). Taken together, these data point towards a pivotal role of BM-derived fibroblasts and BM-eNOS for cardiac fibrosis under the conditions of systemic eNOS inhibition.

3.3 Differential regulation of SDF-1 content by L-NAME and TAC

Aortic ligation for 5 weeks decreased SDF-1α expression in the LV and BM (*Figure 4A* and *K*), whereas SDF-1α expression in LV was increased by L-NAME treatment. Cellular distribution of SDF-1 was different between groups: in the control groups SDF-1 was localized in cardiomyocytes and in the experimental groups the most prominent expression was observed in fibroblasts (*Figure 4B–J*).

3.4 Up-regulation of circulating fibrocytes by aortic constriction and eNOS inhibition and deficiency

The numbers of fibrocytes identified as $CD45^{pos}$ collagen I^{pos} cells were significantly increased by aortic banding and L-NAME administration both in the peripheral blood and in the BM (*Figure 5A* and *B*). The number of fibrocytes in the peripheral blood was similarly augmented in all treatment groups (*Figure 5A* and *C*). The highest number of fibrocytes in the BM was observed in the animals treated with L-NAME and L-NAME + hydralazine (*Figure 5B* and *D*). $eNOS^{-/-}$ increased the number CD45/collagen I-positive fibrocytes in the bone marrow but not in the peripheral blood (*Figure 5E–H*).

3.5 Inhibition of eNOS but not of iNOS and nNOS stimulates fibrotic response in isolated neonatal rat cardiac fibroblasts

To determine whether the profibrotic effect observed *in vivo* was related to inhibition of eNOS but not of iNOS or nNOS, isolated neonatal rat cardiac fibroblasts were treated with the inhibitor of eNOS and iNOS L-NAME, with the inhibitor of iNOS 1400 W and with the inhibitor of nNOS 7-nitroindazole for 72 h. Treatment with L-NAME but not with 1400 W or 7-nitroindazole increased the hydroxyproline level and mRNA expression of collagen Iα1 (*Figure 6A* and *B*). L-NAME enhanced and 1400 W diminished SDF-1 mRNA expression (*Figure 6C*). The number of myofibroblasts identified by co-immunostaining for α-smooth muscle actin and fibronectin was similar in L-NAME (47.6 ± 8.4%) and control groups (43 ± 11%). Boyden chamber assays showed that inhibition of eNOS but not of iNOS in neonatal rat cardiac fibroblasts increased their migratory capacity (*Figure 6D* and *E*).

4. Discussion

This study reports several novel findings. First, systemic eNOS inhibition induces and enhances cardiac fibrosis independently of the changes in the peripheral blood pressure and LV pressure. Secondly, inhibition of eNOS in cardiac fibroblasts both *in vivo* and *in vitro* induces a profibrotic response with increased production of extracellular matrix, SDF-1, and fibroblast migratory capacity. Thirdly, bone marrow-derived fibroblasts contribute to these profibrotic mechanisms.

Reduction of NO production in cardiovascular disease and heart failure is caused by enzyme inhibition, reduction of eNOS expression and NO inactivation.[1,2] Therefore, L-NAME treatment appears to be a suitable model to investigate the role of eNOS in cardiac fibrogenesis during cardiac afterload. L-NAME significantly decreased myocardial activity of constitutively expressed (endothelial and neuronal) isoforms of NOS but not of iNOS. In contrast, TAC but not L-NAME reduced eNOS and increased iNOS expression in the heart. Both L-NAME and TAC

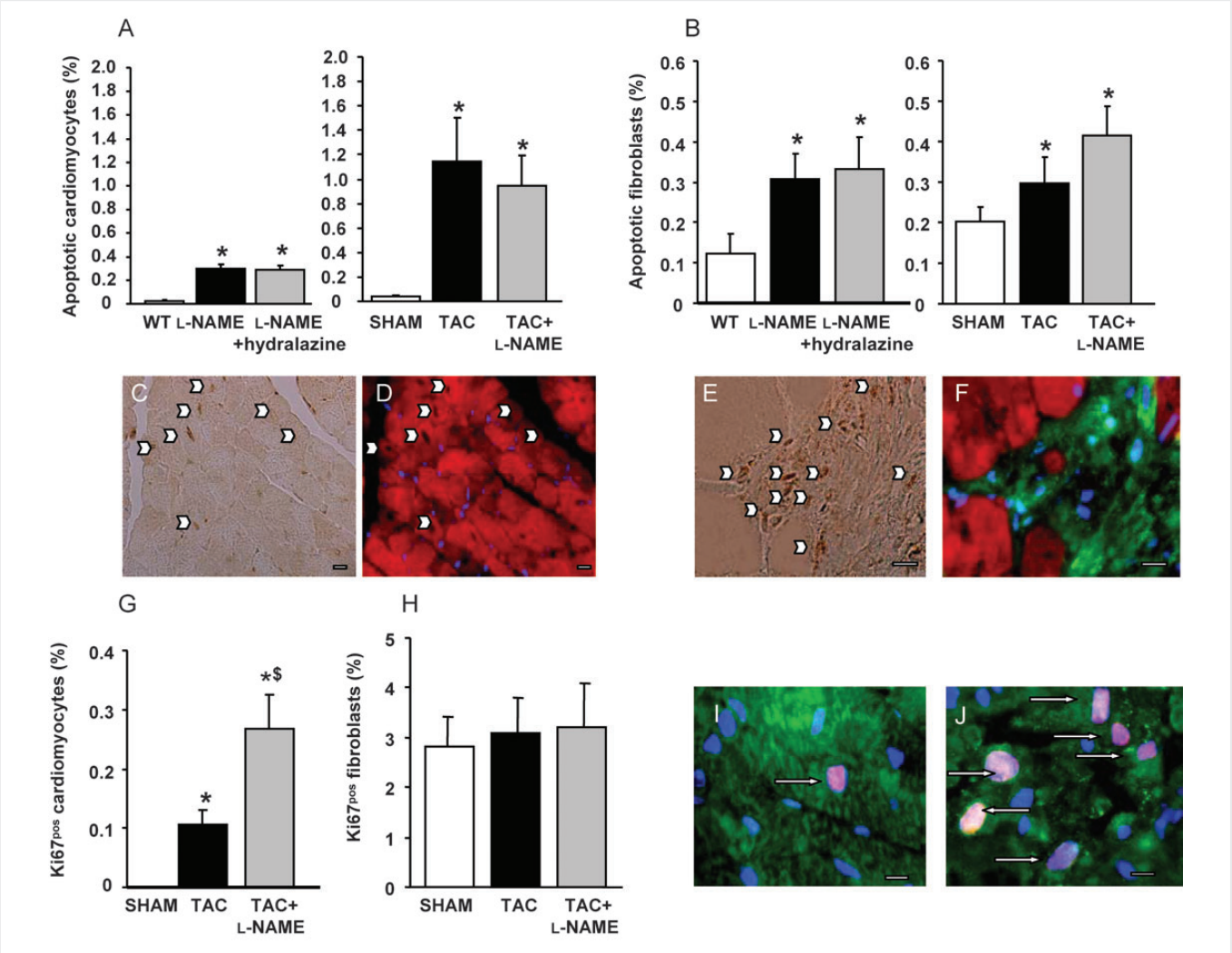

Figure 2 Effect of systemic eNOS inhibition on cardiac apoptotic and proliferative cells in normal and increased cardiac afterload. L-NAME and TAC increase the number and the percentage of apoptotic cardiomyocytes (*A*) and fibroblasts (*B*). The percentage of cycling cardiomyocytes (*G*) but not of fibroblasts was increased by TAC and was further enhanced by L-NAME (*H*), $n = 7–10$ per group. $^{*}P < 0.05$ compared with corresponding control group: WT or SHAM, $^{\$}P < 0.05$ compared with mice with TAC. (*C* and *D*) and (*E* and *F*) show the same section of the LV myocardium from mice after TAC. (*C*) and (*E*) show light microscopic staining for apoptosis (brown). (*D*) Fluorescence microscopy for the myocyte marker α-sarcomeric actin (red) and (*F*) shows fluorescence microscopy for the fibroblast marker intracellular fibronectin (green). Apoptotic nuclei are marked by arrowheads. Nuclei are stained blue by DAPI. Bars = 10 μm. (*I*) and (*J*) show examples of LV sections from TAC mice co-immunostained for the proliferation marker Ki67 (red) and the following cell-specific markers (in green): the myocyte marker α-sarcomeric actin (*I*), and the fibroblasts marker intracellular fibronectin (*J*). Cycling nuclei are marked by arrows. Nuclei are stained blue by DAPI. Bars = 10 μm.

elicited cardiomyocyte hypertrophy and apoptosis, which were more pronounced in the TAC mice where it was accompanied by cardiomyocyte cycling. L-NAME treatment reduced cardiomyocyte hypertrophy in TAC mice.[8] The morphology of the interstitial and replacement fibrosis induced by L-NAME were morphologically similar to the fibrosis in the eNOS$^{-/-}$ Sham and TAC mice observed in our previous work.[8] In agreement with the work of Ichinose *et al.*,[3] antihypertensive treatment with hydralazine did not reduce fibrosis caused by eNOS inhibition. Thus, both reduction and abolishing of NO production by eNOS induce and enhance cardiac fibrosis during different afterload conditions.

The augmentation of collagen synthesis was accompanied by an increase in the numbers of fibroblasts and enhancement of their turnover. Both TAC and L-NAME similarly elevated the percentage of apoptotic fibroblasts and did not influence their proliferation. In contrast, TAC diminished the percentage of the CXCR4^{pos} fibroblasts, whereas L-NAME administration, systemic and BM eNOS$^{-/-}$ increased it in all groups. BM chimera treated with L-NAME or L-NAME and hydralazine revealed an increase in the number and the percentage of GFP^{pos} fibroblasts compared with control. In accordance with these findings, the numbers of fibrocytes in the peripheral blood and in the BM were increased by L-NAME treatment and TAC compared with corresponding control groups. The highest numbers of fibrocytes in the bone marrow were observed in animals treated with L-NAME and L-NAME and hydralazine. eNOS$^{-/-}$ up-regulated the fibrocyte number in the bone marrow but not in the peripheral blood. Hence, their increased percentage in fibrosis may be explained by their mobilization at an earlier point in time. Furthermore, SDF-1 is the only chemokine

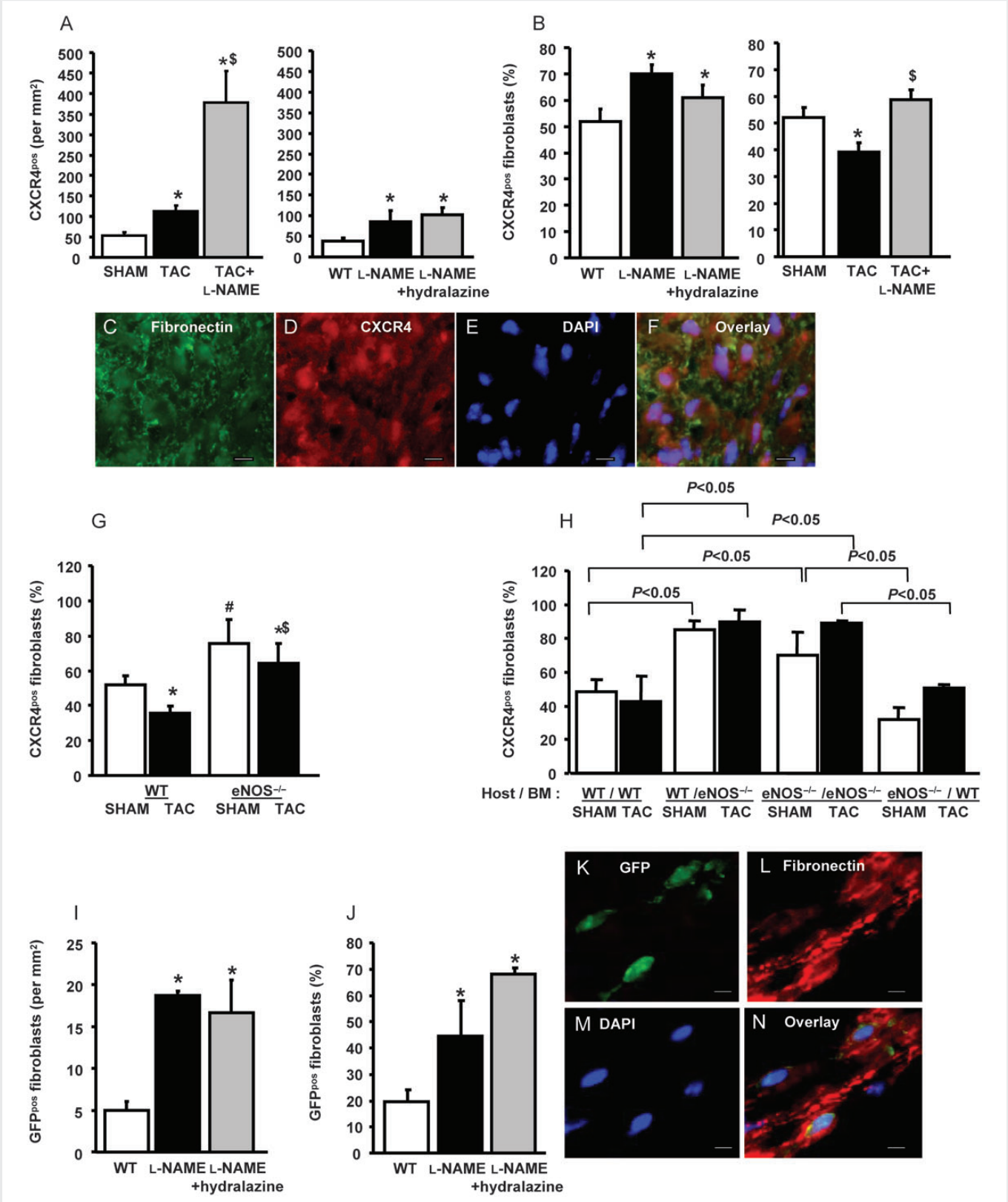

Figure 3 L-NAME, systemic, and bone marrow eNOS-deficiency increase the percentage of BM-derived fibroblasts in cardiac fibrosis. Treatment with L-NAME increases both the number per mm^2 (*A*) and the percentage (*B*) of CXCR4^{pos} fibroblasts in mice without BMT. Both systemic (*G*) and BM eNOS-deficiency (*H*) increase the percentage of CXCR4^{pos} fibroblasts. The number per mm^2 (*I*) and the percentage (*J*) of GFP^{pos} fibroblasts in GFP^{pos} BMT mice were increased by L-NAME, $n = 7–10$ per group. $^*P < 0.05$ compared with corresponding control group: WT or SHAM, $^\$P < 0.05$ compared with TAC, $^\#P < 0.05$ compared with WT SHAM. CXCR4^{pos} fibroblasts in a LV section from a TAC + L-NAME mouse: intracellular fibronectin (green, *C*), CXCR4 (red, *D*), nuclei-stained blue by DAPI (*E*) and the overlay of the three stainings (*F*). GFP^{pos} fibroblasts in a LV section from an L-NAME-treated GFP^{pos} BMT mouse: GFP (green, *K*), intracellular fibronectin (red, *L*), nuclei-stained blue by DAPI (*M*), and the overlay of the three stainings (*N*). Bars = 10 μm.

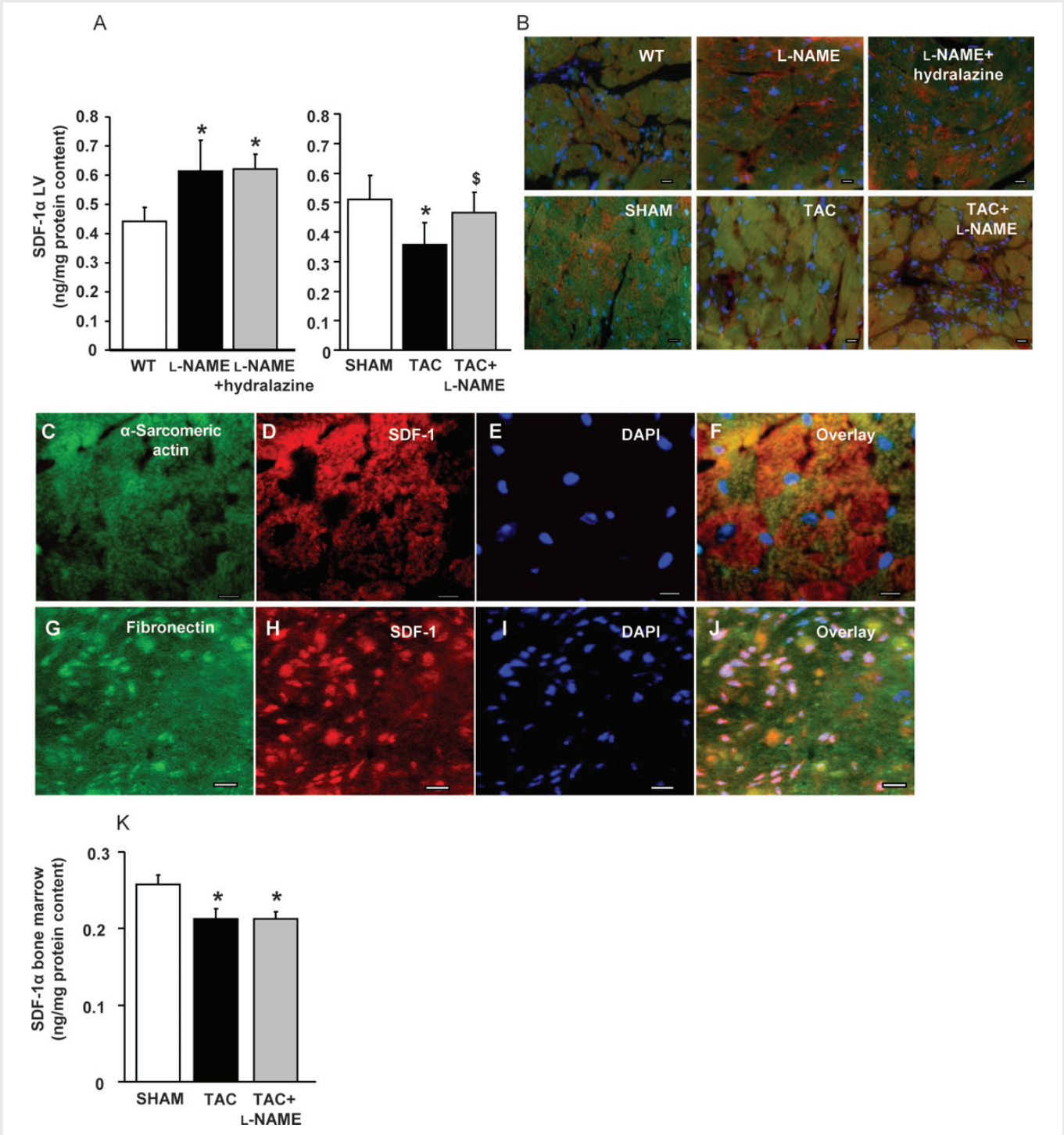

Figure 4 Effect of systemic eNOS inhibition on SDF-1 expression in normal and increased cardiac afterload. ELISA shows increase in SDF-1α expression in the LV in the L-NAME-treated groups and decrease in the TAC group in the heart (*A*) and in the bone marrow (*K*). $^{*}P < 0.05$ compared with corresponding control group: WT or SHAM, $^{\$}P < 0.05$ compared with TAC. Representative sections of the LV myocardium co-immunostained for SDF-1 (red) and myocytic α-sarcomeric actin (green) (*B*). Nuclei are stained blue by DAPI. Bars = 10 μm. In the control groups SDF-1 expression was observed only in cardiomyocytes and in the experimental groups was most prominent in the fibrotic areas. SDF-1 secretion by cardiomyocytes in an SHAM mouse: myocytic α-sarcomeric actin (green, *C*), SDF-1 (red, *D*), nuclei-stained blue by DAPI (*E*) and the overlay of the three stainings (*F*). SDF-1 secretion by fibroblasts in a TAC + L-NAME mouse: intracellular fibronectin (green, *G*), SDF-1 (red, *H*), nuclei-stained blue by DAPI (*I*), and the overlay of the three stainings (*J*).

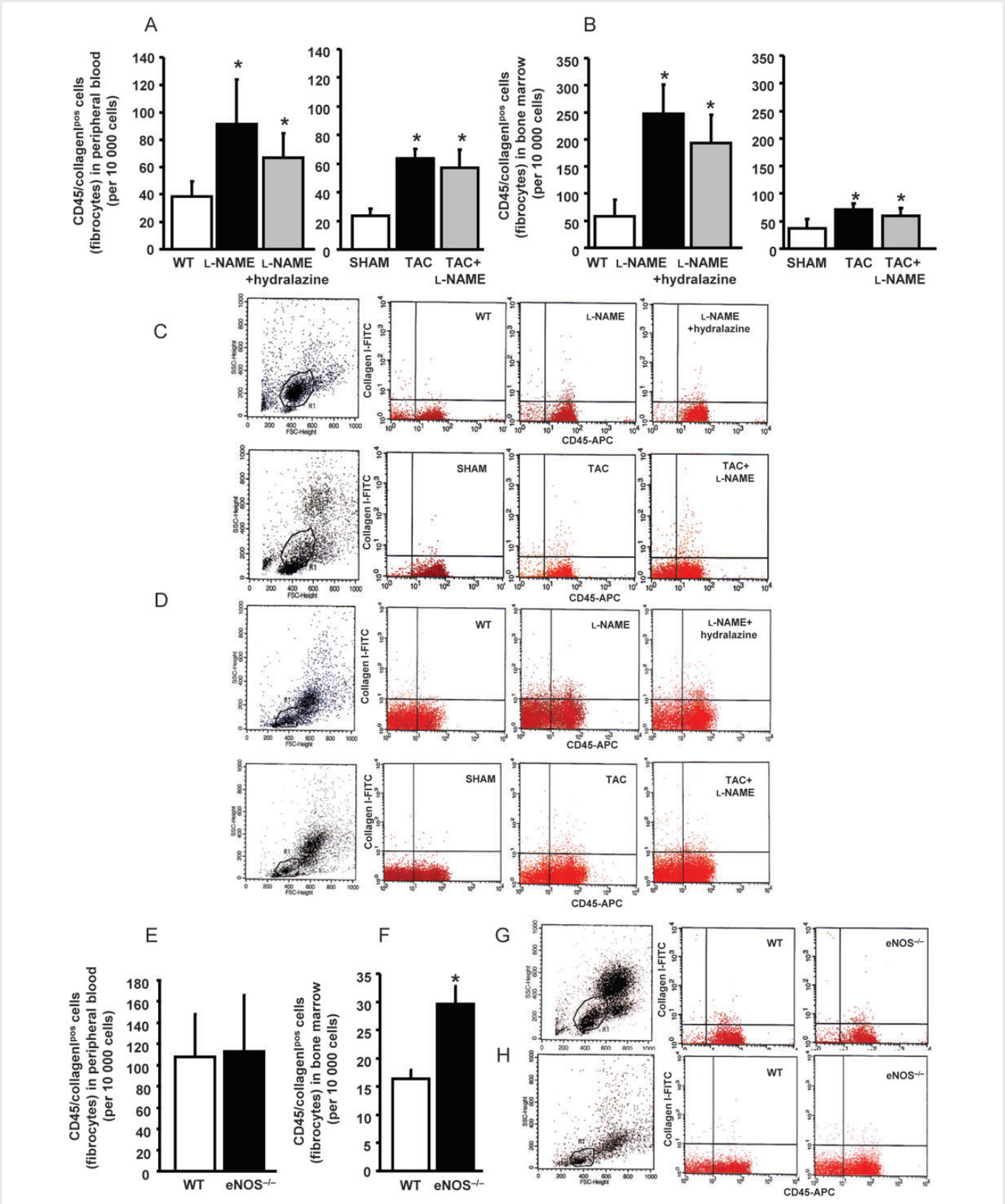

Figure 5 Both TAC and L-NAME increase the numbers of fibrocytes in the peripheral blood and in the BM. The numbers of CD45/collagen I-positive fibrocytes quantified by FACS analysis were increased in the peripheral blood (*A*) and in the BM (*B*) both by TAC and L-NAME, $n = 3–9$ per group. eNOS$^{-/-}$ increased the number of CD45/collagen I-positive fibrocytes in the bone marrow (*F*) but not in the peripheral blood (*E*). *$P < 0.05$ compared with corresponding control group: WT or SHAM. (*C* and *G*) and (*D* and *H*) show representative FACS blots for CD45 (*x*-axis) and collagen I (*y*-axis) in the peripheral blood and in the BM, respectively. Gated cells are marked by polygons. The double-positive cells in the upper right quadrant were counted as fibrocytes.

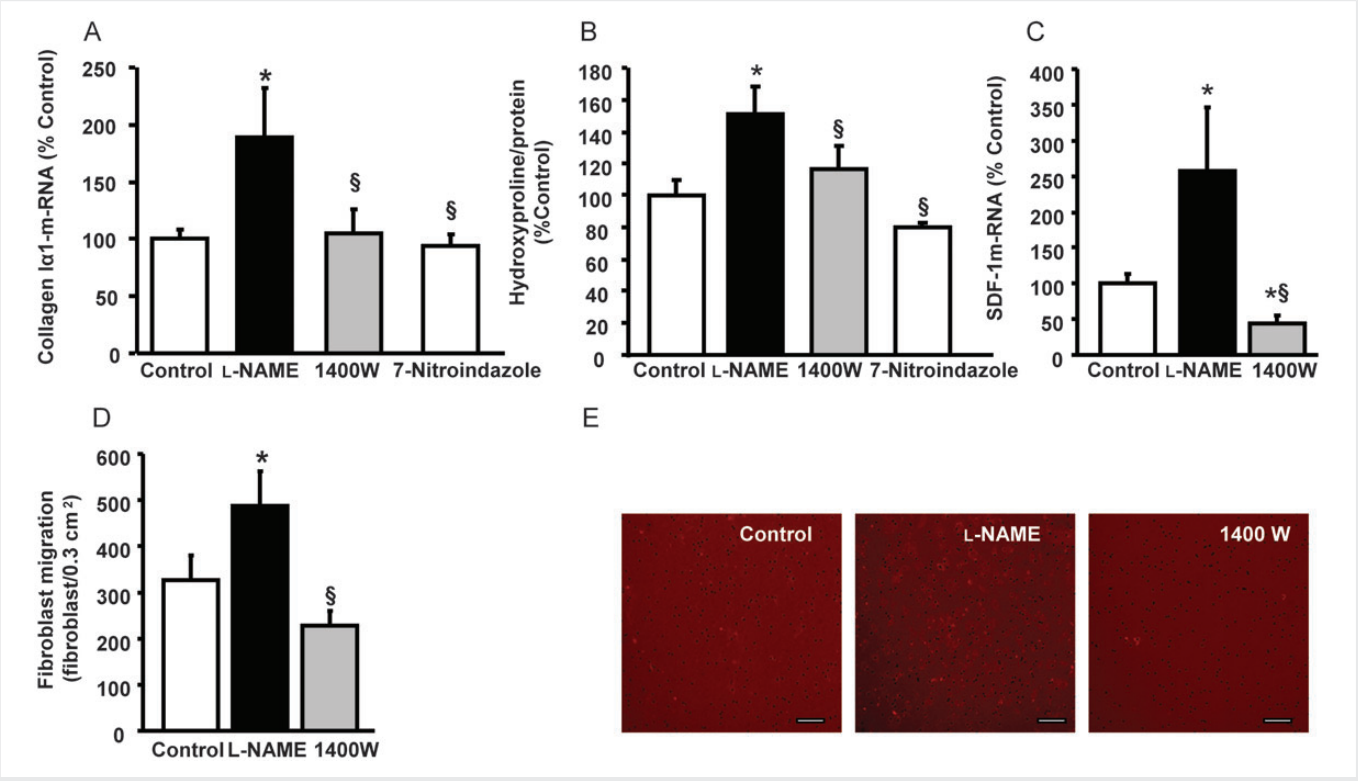

Figure 6 Inhibition of eNOS but not of iNOS and nNOS induces fibrotic response in cardiac fibroblasts and increases their migration. Treatment of cultured neonatal cardiac fibroblasts of rats with L-NAME (30 μM) but not with the selective inhibitor of iNOS 1400 W or nNOS 7-nitroindazole (30 μM) for 72 h increases mRNA expression of collagen Iα (*A*) and hydroxyproline content in the cell culture medium (*B*). L-NAME increases mRNA expression of SDF-1 in cardiac fibroblasts (*C*). L-NAME but not 1400 W increases the number of migrating fibroblasts in a Boyden chamber with an insert size of 0.3 cm^2 (*D*). (*A* and *B*) $n = 19–26$ per group; (*C* and *D*) $n = 5–7$ per group. $^*P < 0.05$ compared with control, $^§P < 0.05$ compared with L-NAME-treated fibroblasts. (*E*) Representative migration assays. Migrating fibroblasts are immunostained for intracellular fibronectin (red). Bars = 100 μm.

known to bind to CXCR4 and to attract fibrocytes to the damaged tissue.[17,20] L-NAME up-regulated and TAC down-regulated SDF-1 expression in the myocardium. Moreover, immunostaining revealed attenuation of SDF-1 production in cardiomyocytes that might be caused by their hypertrophy and its enhancement in fibroblasts in all experimental groups.[16,24] TAC decreased the SDF-1 level in the bone marrow. Therefore, our findings underline the involvement of fibrocytes in cardiac fibrogenesis and demonstrate their pivotal role in cardiac fibrosis induced or enhanced by eNOS inhibition and deficiency.

Cultured neonatal rat cardiac fibroblasts were treated with L-NAME or with the selective inhibitor of iNOS 1400 W and nNOS 7-nitroindazole to investigate whether the effects of L-NAME treatment observed *in vivo* were related to inhibition of eNOS in cardiac fibroblasts. Inhibition of eNOS increased collagen and SDF-1 production and enhanced migratory capacity. The fibrotic response was not caused by myofibroblast transformation of cultured cells. The reduction of cardiac fibrosis in TAC mice treated with 1400 W was observed by Zhang *et al.*[25] Thus, systemic inhibition of eNOS induces and enhances cardiac fibrosis through several cellular mechanisms which can be viewed as parts of a vicious circle: cardiac fibroblast produces extracellular matrix proteins and chemokines, in particular SDF-1, attracting fibrocytes into the fibrotic areas of the myocardium, which in turn further enhance cardiac fibrosis. Moreover, SDF-1 recruits different types of progenitor cells from the bone marrow and accessory myeloid cells participating in cardiac neoangiogenesis and regeneration.[26] However, in our previous experiments on GFP^{pos} BM-transplanted TAC mice, many endothelial and myocyte progenitor cells from the BM were observed within fibrotic areas of the myocardium.[27] These cells might be 'attracted' by fibroblast chemokines and their ability to further differentiation may be suppressed by neighbouring fibroblasts resulting in impaired myocardial capillarization and regeneration observed in L-NAME and TAC induced pressure overload.[27,28] These findings are consistent with previous experimental work, which has shown that both antagonism of the SDF-1/CXCR4 axis and up-regulation of eNOS expression in fibrocytes reduce their numbers in the peripheral blood and damaged tissue diminishing fibrosis.[20,21]

Our study has limitations: we did not study the contribution of other types of cardiac cells (resident stem cells, endothelial cells, mast cells) and extracardiac cells (inflammatory cells) for the fibrogenesis. Moreover, an impact of different types of fibroblasts and their proliferation on cardiac fibrogenesis may depend on the stage of heart failure. Our data set the stage for future studies to address these issues.

In summary, the data show the pivotal role of the systemic activity of eNOS in the prevention of cardiac fibrosis. Systemic eNOS inhibition and deficiency induce and enhance cardiac fibrosis independently of the changes in afterload by activating the SDF-1/CXCR4 axis leading to trafficking of fibroblasts into the fibrotic areas, by increasing the recruitment of fibrocytes from the bone marrow and by increasing

fibroblast migratory capacity and stimulation of extracellular matrix production. These findings may provide interesting perspectives to create novel strategies for the prevention and treatment of myocardial fibrosis.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Acknowledgements

We thank C. Pitke, E. Becker, A. Bohner, and S. Jäger for their excellent technical assistance.

Conflict of interest: none declared.

Funding

This work was supported by the Deutsche Forschungsgemeinschaft, (KFO 196) to A.K., R.H., F.L. (LA997/6-1), M.B. and U.L.

References

1. Förstermann U, Sessa WC. Nitric oxide synthases: regulation and function. *Eur Heart J* 2012;**33**:829–837.
2. Umar S, van der Laarse A. Nitric oxide and nitric oxide synthase isoforms in the normal, hypertrophic, and failing heart. *Mol Cell Biochem* 2010;**333**:191–201.
3. Takimoto E, Champion HC, Li M, Ren S, Rodriguez ER, Tavazzi B *et al*. Oxidant stress from nitric oxide synthase-3 uncoupling stimulates cardiac pathologic remodeling from chronic pressure load. *J Clin Invest* 2005;**115**:1221–1231.
4. Moens AL, Takimoto E, Tocchetti CG, Chakir K, Bedja D, Cormaci G *et al*. Reversal of cardiac hypertrophy and fibrosis from pressure overload by tetrahydrobiopterin: efficacy of recoupling nitric oxide synthase as a therapeutic strategy. *Circulation* 2008;**117**:2626–2636.
5. Ichinose F, Bloch KD, Wu JC, Hataishi R, Aretz HT, Picard MH *et al*. Pressure overload-induced LV hypertrophy and dysfunction in mice are exacerbated by congenital NOS3 deficiency. *Am J Physiol Heart Circ Physiol* 2004;**286**:H1070–H1075.
6. Ruetten H, Dimmeler S, Gehring D, Ihling C, Zeiher AM. Concentric left ventricular remodeling in endothelial nitric oxide synthase knockout mice by chronic pressure overload. *Cardiovasc Res* 2005;**66**:444–453.
7. Buys ES, Raher MJ, Blake SL, Neilan TG, Graveline AR, Passeri JJ *et al*. Cardiomyocyte-restricted restoration of nitric oxide synthase 3 attenuates left ventricular remodeling after chronic pressure overload. *Am J Physiol Heart Circ Physiol* 2007;**293**:H620–H627.
8. Kazakov A, Müller P, Jagoda P, Semenov A, Böhm M, Laufs U. Endothelial nitric oxide synthase of the bone marrow regulates myocardial hypertrophy, fibrosis, and angiogenesis. *Cardiovasc Res* 2012;**93**:397–405.
9. Kim NN, Villegas S, Summerour SR, Villarreal FJ. Regulation of cardiac fibroblast extracellular matrix production by bradykinin and nitric oxide. *J Mol Cell Cardiol* 1999;**31**:457–466.
10. Liou JY, Hong HJ, Sung LC, Chao HH, Chen PY, Cheng TH *et al*. Nicorandil inhibits angiotensin-II-induced proliferation of cultured rat cardiac fibroblasts. *Pharmacology* 2011;**87**:144–151.
11. Wang K, Hu X, Du C, Tu S, Zhang F, Xie X. Angiotensin-(1–7) suppresses the number and function of the circulating fibrocytes by upregulating endothelial nitric oxide synthase expression. *Mol Cell Biochem* 2012;**365**:19–27.
12. Krenning G, Zeisberg EM, Kalluri R. The origin of fibroblasts and mechanism of cardiac fibrosis. *J Cell Physiol* 2010;**225**:631–637.
13. Haudek SB, Xia Y, Huebener P, Lee JM, Carlson S, Crawford JR *et al*. Bone marrow-derived fibroblast precursors mediate ischemic cardiomyopathy in mice. *Proc Natl Acad Sci USA* 2006;**103**:18284–18289.
14. Zeisberg EM, Tarnavski O, Zeisberg M, Dorfman AL, McMullen JR, Gustafsson E *et al*. Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. *Nat Med* 2007;**13**:952–961.
15. Chu PY, Mariani J, Finch S, McMullen JR, Sadoshima J, Marshall T *et al*. Bone marrow-derived cells contribute to fibrosis in the chronically failing heart. *Am J Pathol* 2010;**176**:1735–1742.
16. Sopel MJ, Rosin NL, Lee TDG, Legare JF. Myocardial fibrosis in response to angiotensin II is preceded by the recruitment of mesenchymal progenitor cells. *Lab Invest* 2011;**91**:565–578.
17. Phillips RJ, Burdick MD, Hong K, Lutz MA, Murray LA, Xue YY *et al*. Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. *J Clin Invest* 2004;**114**:438–446.
18. Wang CH, Huang CD, Lin HC, Lee KY, Lin SM, Liu CY *et al*. Increased circulating fibrocytes in asthma with chronic airflow obstruction. *Am J Respir Crit Care Med* 2008;**178**:583–591.
19. Moeller A, Gilpin SE, Ask K, Cox G, Cook D, Gauldie J *et al*. Circulating fibrocytes are an indicator of poor prognosis in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2009;**179**:588–594.
20. Mehrad B, Burdick MD, Strieter RM. Fibrocyte CXCR4 regulation as a therapeutic target in pulmonary fibrosis. *Int J Biochem Cell Biol* 2009;**41**:1708–1718.
21. Chu PY, Zatta A, Kiriazis H, Chin-Dusting J, Du XJ, Marshall T *et al*. CXCR4 Antagonism attenuates the cardiorenal consequences of mineralocorticoid excess. *Circ Heart Fail* 2011;**4**:651–658.
22. Southan GJ, Szabó C. Selective pharmacological inhibition of distinct nitric oxide synthase isoforms. *Biochem Pharm* 1996;**51**:383–394.
23. Garvey EP, Oplinger JA, Furfine ES, Kiff RJ, Laszlo F, Whittle BJ *et al*. 1400W Is a slow, tight binding, and highly selective inhibitor of inducible nitric-oxide synthase *in vitro* and *in vivo*. *J Biol Chem* 1997;**272**:4959–4963.
24. Proulx C, El-Helou V, Gosselin H, Clement R, Gillis MA, Villeneuve L *et al*. Antagonism of stromal cell-derived factor-1α reduces infarct size and improves ventricular function after myocardial infarction. *Pflugers Arch-Eur J Physiol* 2007;**455**:241–250.
25. Zhang P, Xu X, Hu X, van Deel ED, Zhu G, Chen Y. Inducible nitric oxide synthase deficiency protects the heart from systolic overload-induced ventricular hypertrophy and congestive heart failure. *Circ Res* 2007;**100**:1089–1098.
26. Grunewald M, Avraham I, Dor Y, Bachar-Lustig E, Itin A, Jung S *et al*. Cell. VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. *Cell* 2006;**124**:175–189.
27. Müller P, Kazakov A, Semenov A, Böhm M, Laufs U. Pressure-induced cardiac overload induces upregulation of endothelial and myocardial progenitor cells. *Cardiovasc Res* 2008;**77**:151–159.
28. Meirelles Pereira LM, Mandarim-de-Lacerda CA. Effect of antihypertensive drugs on the myocardial microvessels in rats with nitric oxide blockade. *Pathol Res Pract* 2000;**196**:305–311.