Gene Expression, Function and Ischemia Tolerance in Male and Female Rat Hearts After Sub-Toxic Levels of Angiotensin II

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Abstract   To examine the response to chronic high-dose angiotensin II (Ang II) and a proposed milder response in female hearts with respect to gene expression and ischemic injury. Female and male litter–matched rats were treated with 400 ng kg^{-1} min^{-1} Ang II for 14 days. Hearts were isolated, subjected to 30-min ischemia and 30-min reperfusion in combination with functional monitoring and thereafter harvested for gene expression, WB and histology. Ang II-treated hearts showed signs of non-hypertrophic remodeling and had significantly higher end diastolic pressure after reperfusion, but no significant gender difference was detected. Ang II increased expression of genes related to heart function (ANF, β-MCH, Ankrd-1, PKC-α, PKC-δ TNF-α); fibrosis (Col I-α1, Col III-α1, Fn-1, Timp1) and apoptosis (P53, Casp-3) without changing heart weight but with 68% increase in collagen content. High (sub-toxic) dose of Ang II resulted in marked heart remodeling and diastolic dysfunction after ischemia without significant myocyte hypertrophy or ventricular chamber dilatation. Although there were some gender-dependent differences in gene expression, female gender did not protect against the overall response.

Keywords   Angiotensin II · Fibrosis · Gender · Heart function · Ischemia–reperfusion · PKC · Heart remodeling

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Introduction

An increase in circulating angiotensin II (Ang II) is part of the proposed mechanism behind pathological heart hypertrophy, heart failure and hypertension. Therefore, inhibition of the actions of Ang II, whether by reducing formation through inhibiting the angiotensin-converting enzyme (ACE) or by blocking its receptors, has become one of the main strategies in the treatment of these conditions. Ang II production is also increased locally in myocardial infarction [1]. Ang II acts through specific membrane-bound Ang II type-1 (AT1) and type-2 (AT2) G-protein-coupled receptors (GPCRs) [2]. Ang II-induced hypertrophy and apoptosis in cardiomyocytes as well as fibroblast proliferation and collagen synthesis are mediated by the AT1 receptor [3, 4]. Ang II has a very short half-life and is quickly degraded to active Ang III, Ang (1–7) and inactive fragments. Ang III has similar actions to those of Ang II but Ang (1–7) exerts hypotensive action through the release of bradykinin, prostaglandins and endothelial nitric oxide [5].

It has been demonstrated in both clinical [6] and experimental studies [7, 8] that gender influences the cardiac response to prolonged increases in hemodynamic work load. Gender-specific influences have been proposed in patients with aortic stenosis, as female patients show increased hypertrophy, greater concentric remodeling and better preservation of the left ventricular (LV) function compared to male patients, as reviewed by Douglas et al. [9]. The majority of the experimental studies examining the influence of gender have so far evaluated pressure overload, aortic stenosis or post-infarct ventricular remodeling. To our knowledge, the role of Ang II has not been directly addressed in this perspective. Most studies conclude that female gender represents a beneficial effect as reviewed in [10]. On the other hand, some studies indicate that once

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ischemic heart disease is present, female hearts are more vulnerable compared to male hearts [8].

In the present study, we sought to determine how prior chronic high-dose Ang II exposure influenced acute response to ischemia–reperfusion and the corresponding changes in expression of genes related to heart function, fibrosis and apoptosis. In addition, we aimed to examine the role of gender in an experimental model of Ang II exposure.

Materials and Methods

Animal experiments conform to the *Guidelines on Accommodation and Care of Laboratory Animals* (by the European Convention for the protection of vertebrate animals), and all procedures were approved by the Norwegian Committee on Ethics in Animal Experimentation.

Experimental Protocol

The rats were Fischer 344 × Brown Norway F1 hybrid rats of inbred strains (FBN) [11, 12]; siblings in each litter were therefore heterozygote but had the same genetic background. Rats were treated at the age of 12 ± 1 week when average weight was 155 and 280 g in females and males, respectively. Two male and two female groups ($n = 4$–6 rats in each group) received either 400 ng kg^{-1} min^{-1} Ang II (H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-OH, Calbiochem®, Darmstadt, Germany) or sham treatment for 14 days delivered by subcutaneously implanted miniosmotic pumps. Animals were heparinized (200 IU intraperitoneally) and anaesthetized (pentobarbitone sodium, 50 mg kg^{-1} intraperitoneally). The hearts were quickly excised, placed in ice-cold Krebs Henseleit buffer (NaCl 118.5 mM, KCl 4.7 mM, MgSO$_4$ 1.2 mM, NaHCO$_3$ 25.0 mM, CaCl$_2$ 2.4 mM, KH$_2$PO$_4$ 1.2 mM, and glucose 11.1 mM), rapidly mounted on a Langendorff system [13, 14] and perfused with Krebs Henseleit buffer (pH 7.35–7.45, oxygenated with 95% O$_2$, 5% CO$_2$ at 37°C). A water-filled latex balloon, connected to a pressure transducer, was inserted into the left ventricle via the left auricle and thereafter maintained unchanged. The same balloon volume was used for all hearts. The hearts were exposed to a stabilization period of 25 min before global ischemia for 30 min followed by 30-min reperfusion as described previously [13, 14]. Left ventricular pressure, coronary flow and heart rate were monitored. At the end of experiment, hearts were sampled for gene expression, histology and protein expression. With exception of protein expression data, gender grouping were balanced with respect to litter.

Hearts from 4 male and 4 female control rats were subjected to gene expression analysis without prior Langendorff perfusion. Another set of animals was used to test protein expression in sham or Ang II-treated non-perfused hearts (four groups ($n = 2$–6) each) corresponding to those used in the main protocol. A limited number of male Wistar rats were used to validate the function of the miniosmotic pumps, Ang II bioavailability and receptor dependency. The response to three different Ang II doses (150, 300 and 400 ng kg^{-1} min^{-1}) with or without combined angiotensin receptor blocker (ARB) treatment was tested in 16 animals. Angiotensin receptor antagonist Losartan calcium (MSD, Netherlands) was given in drinking water (1 g/l) 24 h before implanting miniosmotic pumps. After 14 days, echo analysis (Vevo 770, Visualsonics Inc., Toronto, Canada) of the heart was performed on isoflurane anesthetized animals to estimate relative wall thickness of the left ventricle.

Gene Expression

At the end of the experiment, apices were excised and stored in RNA later (Qiagen, Hilden, Germany) for expression analysis of 19 genes related to heart function, interstitial fibrosis and apoptosis using quantitative RT–PCR. Samples were homogenized and lysed. Total RNA was isolated according to the RNeasyFibrous Tissue protocol (Qiagen). RNA concentration was measured spectroscopically (NanoDrop, Witec, Switzerland) and stored at −80°C before use.

Reverse transcription of RNA was carried out according to High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA).

The qRT-PCR was performed in an ABI PRISM 7900 HT Fast real-time thermal cycler using the SYBR green or TaqMan Fast Universal PCR master mix (Applied Biosystems). Primers and TaqMan probes were obtained from Eurogentec (Seraing, Belgium) or from Roche Universal Probe Library (Roche diagnostics, Mannheim, Germany). For sequences, please contact corresponding author.

The relative expression ratio of the target gene was calculated based on its real-time efficiency and the Ct differences (Δ) between the different treatment groups. The expression of the target genes was normalized to the stable expressed reference gene based on testing by Normfinder of possible reference genes: GADPH, B2M, Cyclophilin, HPRT and LDHA as described by others [15].

Western Blot

Middle parts of the ventricles were snap-frozen in liquid nitrogen and stored at −80°C until protein extraction was performed. To ensure homogeneity, samples were pulverized under liquid nitrogen prior to extraction of protein; 25–35 mg of powder was weighed out and homogenized

on ice in 600 µl of lysis buffer containing EDTA 2.5, EGTA 0.5, β-glycerophosphate 40, sodium pyrophosphate 10, sodium orthovanadate 4, NaF 30, β-mercaptoethanol 10 (all mM), PBS 1 ml, Igepal CA630 1% (Sigma), sodium dode-cylsulphate (SDS) 0.1%, sodium deoxycholate 0.5% and complete, EDTA-free protease inhibitor cocktail (Roche) 1 ml. The homogenized samples were then centrifuged at $14,000 \times g$ for 10 min at 4°C. The pellet was discarded, and protein quantification of the supernatant from each protein sample was performed using the Bradford method (Bio-Rad). The protein samples were then combined with $2\times$ reducing sample buffer and boiled for 4 min. Samples (30 µg per lane) were then subjected to electrophoresis on a 10% SDS–polyacrylamide gel and transferred onto nitrocellulose membranes. The membranes were blocked for 1 h in phosphate buffer saline (PBS, pH 7.6), containing Tween-20 0.1% and non-fat dry milk 5%, and thereafter incubated overnight at 4°C with antibodies for P53, PKC-α, PKC-δ, PKC-ε (1:1,000 dilution) from (Santa Cruz Biotechnology, USA) and β-actin (1:5,000 dilution) from (Sigma–Aldrich, St. Louis, USA). The membranes were washed and treated for 1 h with anti-rabbit IgG, Horseradish peroxidase–linked whole antibody (Cell Signaling Technology, Danvers, USA). The immunopositive bands were developed with Immobilon chemoluminescent reagent (Millipore, MA, USA) and visualized using a Kodak Image Station 1000 (PerkinElmer, USA). Ponceau S staining (Sigma, St. Louis, USA) confirmed equal loading.

Histology: Toluidine Blue and Sirius Red Staining

For toluidine staining, heart samples from the upper part of the left ventricular free wall were cut in small cubes and fixed in McDowells fixative [16], then washed in Soerensens PBS, post-fixated in 1% OsO_4 in water for 1.5 h and washed in Soerensens PBS before dehydration in a graded series of ethanol. Samples were infiltrated in an Epon/Araldit equivalent (AGAR 100, DDSA, MNA and DMP-30) with propylenoxide as an intermediate step and subjected to polymerization at 60°C over night. Semithin sections (1 µm) were made on a Leica Ultracut S (Vienna, Austria) ultra microtome with glass knives and stained for 20 s with Toluidine blue (1 part 1% aq. Toluidine blue, 9 parts 2.5% Na_2CO_3 washed in double-distilled water and differentiated in 96% ethanol). Pictures were taken using a Leitz Aristoplan microscope with a Leica DFC320 digital camera. By the aid of computer-based morphometry (Leica CTR 600 & Leica Qwin V3), the toluidine sections were used to determine myocyte diameter based on a minimum of forty cells selected from an area of minimal tissue distortion. Cells with visible nucleus were used for quantification, and minimum diameter at the level of the nucleus was measured.

Sirius red staining of collagen fibers (Direct Red 80, Sigma–Aldrich, Germany) was performed as described before [17]. Formalin-fixed transverse sections of the ventricle were paraffin embedded and sliced. Stained tissue was subjected to both quantitative as well as semi-quantitative evaluation. A minimum of 20 sampled images (200×) from the transverse sections from each heart were analyzed for % tissue area occupied by extracellular Sirius red–positive fibers using ImageJ software for direct quantification of the staining using standardized threshold technique. In addition, transverse ventricular sections were examined under microscope using conventional and polarized light at magnification 50× and 200×, and the level of staining as well as tissue changes and injury was evaluated and scored by an experienced pathologist, who was blinded to information about pretreatment or gender.

Statistics

Data are expressed as mean $\pm$ standard error of the mean (SEM). Statistical analysis was done by using Sigma Plot 11.0. Two-way ANOVA was used to investigate the influence of gender and Ang II treatment. The Student–Newman–Keuls method was used as a post hoc test when applicable. $P < 0.05$ was considered statistically significant. For comparison of two groups, T test was used.

Results

Heart weight did not increase in either female or male hearts exposed to 400 ng kg^{-1} min^{-1} of Ang II (Table 1a) in contrast to the use of lower concentrations of Ang II [18]. Body weight of Ang II-treated rats was significantly reduced compared to sham rats as shown in Table 1. We obtained similar results when testing another rat strain (Wistar) with different Ang II doses. Increase in heart weight was present with lower concentrations of Ang II, but with higher concentrations of Ang II heart weight was unchanged from sham. The effects could be blocked with Ang II receptor antagonist Losartan (Fig. 1).

Ang II treatment 400 ng kg^{-1} min^{-1} did not change cardiomyocyte diameter. There was a trend ($P < 0.08$) toward a larger diameter in myocytes from male hearts (21.6 $\pm$ 1.0 µm in male sham and 22.8 $\pm$ 1.6 µm in Ang II-treated males vs. 19.6 $\pm$ 1.3 µm and 19.7 $\pm$ 1.8 µm in female sham and treated females, respectively).

Functional parameters of isolated hearts at 25′stabilization, prior to global ischemia, are shown in Table 1b. Independent of gender hearts treated with Ang II tended to have higher LVDP prior to ischemia compared to sham (sham 124 $\pm$ 32 vs. Ang II 170 $\pm$ 36 mmHg). The volume of the intraventricular balloon was the same for all hearts.

Table 1 (a) Heart weight (HW), body weight (BW) and heart weight to body weight ratio (b) isolated heart function at 25′ stabilization

	HW (g)	BW (g)	HW/BW
(a)			
Female Sham ($n = 15$)	0.9 ± 0.04	166.7 ± 7.2	5.0 ± 0.3
Female Ang II ($n = 14$)	0.9 ± 0.04	135.2 ± 7.7[abc]	6.8 ± 0.3[abc]
Male Sham ($n = 7$)	1.3 ± 0.09[c]	302.3 ± 5.3[c]	4.4 ± 0.3
Male Ang II ($n = 9$)	1.2 ± 0.03[c]	212.9 ± 15.5[abc]	5.9 ± 0.3[abc]
	CF (ml/min/g)	LVDP (mmHg)	LVEDP (mmHg)
(b)			
Female Sham ($n = 4$)	9.2 ± 1.2	121.2 ± 6.5	9.8 ± 3.3
Female Ang II ($n = 4$)	7.8 ± 1.3	158 ± 20.3[a]	8.4 ± 1.4
Male Sham ($n = 4$)	8.5 ± 0.9	142.5 ± 6.2	4.6 ± 1.5
Male Ang II ($n = 4$)	8.3 ± 0.7	182.0 ± 15.8[a]	11.9 ± 1.5

[a] $P < 0.05$ Ang II treatment versus sham overall

[b] $P < 0.05$ Ang II versus sham within the same gender

[c] $P < 0.05$ male versus female within the same treatment category

The smaller female hearts tended to have higher diastolic pressure prior to ischemia. Coronary flow corrected for heart weight (Table 1b) and heart rate (not shown) did not differ between groups.

When subjected to 30-min global ischemia followed by 30-min reperfusion, there was a marked and significant exaggeration of the increase in left ventricular end diastolic pressure in the Ang II-treated hearts reflecting significant deterioration of diastolic function (Fig. 2b).

Ang II Markedly Changed Expression of Several Genes

As shown in Table 2, Ang II significantly increased expression of genes related to heart function: ANF, β-MHC, Ankrd1, TNF-α (α-MHC, Agtr1α, BNP unchanged); fibrosis: Col I-α1, Col III-α1, Fn-1, Timp1; apoptosis: p53 and casp-3 whereas bcl-2 remained unchanged. Three PKC isoenzymes were also tested, and PKC-α and PKC-δ were found to be significantly upregulated after Ang II treatment whereas expression of PKC-ε showed a slight trend to decrease. Subgroup analysis revealed slight but significant increase in Col I-α1, Col III-α1, Fn-1, Timp1, β-MHC and TNF-α expression response to Ang II in female hearts compared to male hearts, whereas the Casp-3 and PKC-α response was more marked in male hearts. Expression of all tested genes normalized to both sham females and males is presented in Table 2. Gene expression analysis of non-perfused untreated control hearts showed no significant gender-dependent variability (data not shown). Expression of the tested genes normalized to house-keeping genes varied considerably with α-MHC having the highest expression ($44,900 \pm 9,000$ in females and $38,120 \pm 12,900$ in males)

and BNP having the lowest expression (0.4 ± 0.1 in females and 0.5 ± 0.2 in males).

P53, PKC-α, PKC-δ, PKC-ε Immunoreactivity

Proteins P53, PKC-α, PKC-δ, PKC-ε were selected to confirm expression at the protein level corresponding to change at the gene expression level. β-Actin was markedly upregulated by Ang II (1.00 ± 0.27 in sham vs. 6.93 ± 1.42 in Ang II), and all data presented are therefore normalized to total protein load by Ponceau S staining. Heart samples from each gender were run on different gels. There was significant difference between Sham and Ang II with respect to PKC-α ($P = 0.006$) in female hearts (trend but n.s. in male hearts) and in PKC-δ in male hearts ($P < 0.001$) (trend but n.s. in female hearts). P53 also demonstrated a trend toward increase in both male and female Ang II-treated hearts ($P = 0.076$ and $P = 0.094$, respectively). PKC-ε decreased significantly in males ($P = 0.006$) and showed a trend to decrease in females (Fig. 3). Similar results were found in non-perfused treated hearts. Expression of p53 and PKC-δ was significantly increased in Ang II-treated females compared to sham ($P = 0.028$ and $P < 0.001$ for p53 and PKC-δ, respectively). Treated males showed the same pattern namely a trend to increased expression of p53, PKC-α and PKC-δ and decrease in PKC-ε following Ang II treatment.

Collagen Content

Collagen content was $2.9 \pm 0.2\%$ of transverse sections in sham hearts and $4.3 \pm 0.5\%$ in Ang II-treated hearts ($P < 0.05$) (Fig. 4a). No difference was detected between
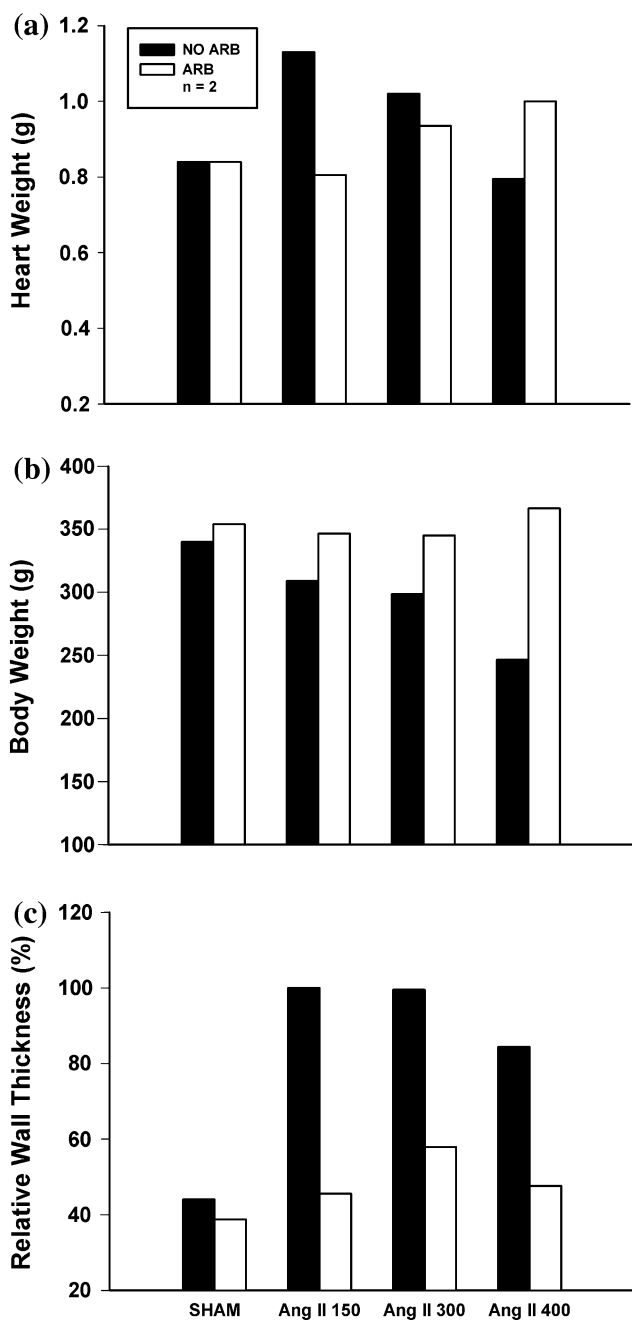
Fig. 1 Response to 14-day treatment with different doses of Ang II with or without Ang II receptor blocker (ARB) losartan per os, 1 g/l in drinking water (n = 2) **a** heart weight, **b** body weight and **c** relative wall thickness (RWT) estimated by echocardiography

Fig. 2 Ventricular pressure (mmHg) in isolated hearts under isovolumetric conditions Left ventricular developed pressure (LVDP) (**a**) and ventricular end diastolic pressure (LVEDP) (**b**) changes during 30-min ischemia (Isc.) followed by 30-min reperfusion (Rep.) *P < 0.05 Ang II versus sham

male and female hearts. Examining heart sections stained with Sirius red staining under conventional and polarized light microscopy also revealed a clear difference between Ang II-treated and sham animals (Fig. 4 b, c, d, e). Samples from sham hearts appeared normal, while samples from Ang II animals showed presence of collagen bundles (focal, diffuse thin or thick bundles) as shown in Table 3. There were 3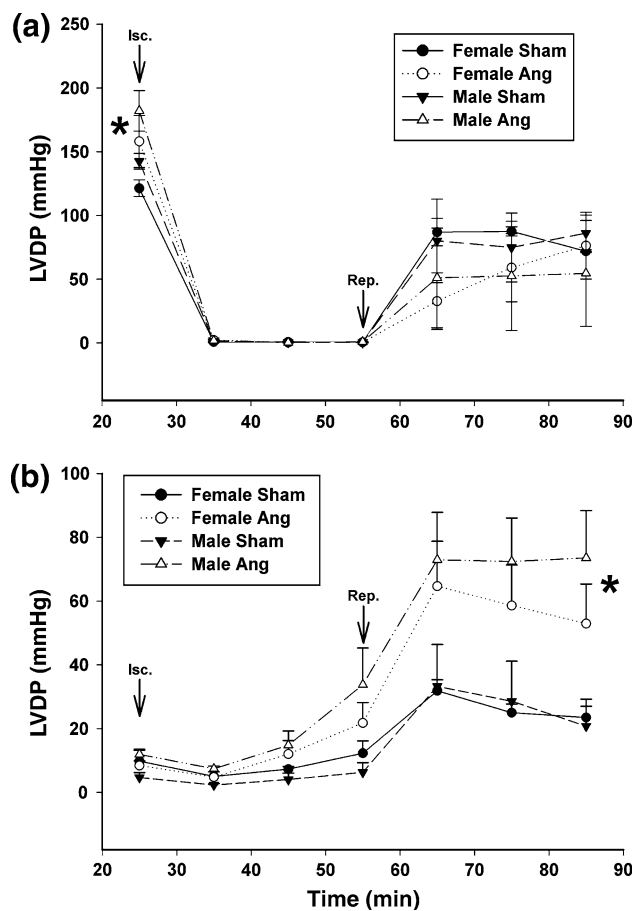 male hearts with markedly increased amounts of thick fibrin bundles and only 1 female heart with such changes. Three female hearts but only one male heart showed some accompanying necrosis.

Discussion

The present study shows, as expected, that chronic infusion of high-dose Ang II significantly changed gene and protein expression in the heart, as well as heart function. Also, post-ischemic recovery of function was significantly impaired with a marked diastolic dysfunction. In agreement with a proposed increase in wall stiffness, we found significant increase in collagen deposition. However, with the high dose used in this study, there was no increase in heart weight or cardiomyocyte diameter following treatment with Ang II.

Body weight was significantly reduced compared to age- and litter-matched sham in correspondence with earlier reports investigating skeletal muscle wasting [19, 20] and a proposed role of Ang II as a candidate for cardiac cachexia

Table 2 Relative gene expression of selected genes normalized to mean values in female and male (in parenthesis) sham hearts

Gene	Female Sham ($n = 4$)	Female Ang II ($n = 4$)	Male Sham ($n = 4$)	Male AngII ($n = 4$)
ANF	1.00 ± 0.31	32.44 ± 15.77[ab]	0.73 ± 0.26	21.04 ± 7.78[ab]
	(1.36 ± 0.43)	(44.28 ± 21.53)[ab]	(1.00 ± 0.36)	(28.72 ± 10.62)[ab]
BNP	1.00 ± 0.29	1.73 ± 0.29	0.90 ± 0.18	1.25 ± 0.24
	(1.10 ± 0.32)	(1.91 ± 0.33)	(1.00 ± 0.20)	(1.38 ± 0.27)
α-MHC	1.00 ± 0.21	0.73 ± 0.17	0.60 ± 0.09	0.54 ± 0.12
	(1.66 ± 0.36)	(1.20 ± 0.29)	(1.00 ± 0.16)	(0.90 ± 0.22)
β-MHC	1.00 ± 0.21	6.48 ± 2.25[ab]	1.98 ± 0.87	4.84 ± 1.39[a]
	(0.50 ± 0.11)	(3.27 ± 1.14)[ab]	(1.00 ± 0.44)	(2.44 ± 0.70)[a]
Ankrd-1	1.00 ± 0.13	1.69 ± 0.27[a]	1.25 ± 0.22	1.86 ± 0.46[a]
	(0.80 ± 0.11)	(1.35 ± 0.22)[a]	(1.00 ± 0.18)	(1.48 ± 0.37)[a]
Agtr1-α	1.00 ± 0.19	2.46 ± 1.32	0.69 ± 0.16	1.10 ± 0.31
	(1.43 ± 0.29)	(3.52 ± 1.90)	(1.00 ± 0.25)	(1.56 ± 0.45)
TNF-α	1.00 ± 0.11	1.86 ± 0.31[ab]	0.84 ± 0.14	1.18 ± 0.22[a]
	(1.18 ± 0.13)	(2.20 ± 0.37)[ab]	(1.00 ± 0.17)	(1.40 ± 0.26)[a]
PKC-α	1.00 ± 0.11	1.38 ± 0.14[a]	1.00 ± 0.02	1.68 ± 0.35[ab]
	(1.00 ± 0.11)	(1.38 ± 0.14)[a]	(1.00 ± 0.02)	(1.67 ± 0.36)[ab]
PKC-δ	1.00 ± 0.09	2.16 ± 0.48[ab]	0.93 ± 0.06	2.09 ± 0.44[ab]
	(1.07 ± 0.10)	(2.33 ± 0.52)[ab]	(1.00 ± 0.07)	(2.26 ± 0.48)[ab]
PKC-ε	1.00 ± 0.07	0.77 ± 0.05	0.93 ± 0.08	0.75 ± 0.20
	(1.06 ± 0.08)	(0.82 ± 0.06)	(1.00 ± 0.09)	(0.80 ± 0.22)
Col I-α1	1.00 ± 0.27	16.36 ± 13.33[ab]	0.87 ± 0.19	4.33 ± 1.55[a]
	(1.14 ± 0.32)	(18.69 ± 15.25)[ab]	(1.00 ± 0.22)	(4.91 ± 1.79)[a]
Col III-α1	1.00 ± 0.33	12.38 ± 9.19[ab]	1.10 ± 0.28	4.62 ± 2.12[a]
	(0.91 ± 0.30)	(11.22 ± 8.33)[ab]	(1.00 ± 0.26)	(4.19 ± 1.92)[a]
Fn-1	1.00 ± 0.09	19.53 ± 14.01[ab]	1.03 ± 0.17	6.51 ± 1.74[ab]
	(0.97 ± 0.09)	(18.91 ± 13.60)[ab]	(1.00 ± 0.17)	(6.24 ± 1.67)[ab]
Timp1	1.00 ± 0.31	4.86 ± 2.57[ab]	0.97 ± 0.14	4.13 ± 1.00[a]
	(1.02 ± 0.32)	(4.96 ± 2.63)[ab]	(1.00 ± 0.15)	(4.23 ± 1.03)[a]
P53	1.00 ± 0.24	1.68 ± 0.33[a]	0.82 ± 0.14	1.36 ± 0.29[a]
	(1.21 ± 0.30)	(2.02 ± 0.42)[a]	(1.00 ± 0.17)	(1.63 ± 0.36)[a]
Casp3	1.00 ± 0.09	1.96 ± 0.56[a]	0.54 ± 0.20[c]	1.57 ± 0.26[ab]
	(1.85 ± 0.19)	(3.62 ± 1.07)[ab]	(1.00 ± 0.37)[c]	(2.89 ± 0.50)[ab]
Bcl-2	1.00 ± 0.046	0.99 ± 0.189	0.87 ± 0.06	0.78 ± 0.078
	(1.14 ± 0.06)	(1.12 ± 0.19)	(1.00 ± 0.08)	(0.89 ± 0.09)
iNOS	1.00 ± 0.16	1.64 ± 0.82	0.87 ± 0.15	0.70 ± 0.26
	(1.15 ± 0.18)	(1.88 ± 0.95)	(1.00 ± 0.18)	(0.81 ± 0.31)
eNOS	1.00 ± 0.12	0.89 ± 0.14	1.17 ± 0.15	0.94 ± 0.14
	(0.85 ± 0.11)	(0.76 ± 0.12)	(1.00 ± 0.13)	(0.80 ± 0.12)

[a] $P < 0.05$ Ang II versus sham overall

[b] $P < 0.05$ Ang II versus sham within the same gender

[c] $P < 0.05$ male versus female within the same treatment category

[21]. Gene expression analysis of the traditional failing heart biomarkers TNF-α and ANF showed significant increase after treatment with Ang II in both genders. Thus, the present study shows that Ang II at high dose promotes marked remodeling with no hypertrophy. In this study, BNP was not significantly increased after 2 weeks of Ang II treatment and this is in agreement with the findings of Suo et al. [22] who reported that left ventricular BNP mRNA levels increased significantly after 2- and 6-h Ang II treatment and peaked after 12 h before it started to decrease and return to control levels one week after treatment.

One aim of this study was to examine if chronic Ang II overexposure leads to a milder response in female compared to male hearts with respect to gene expression changes and ischemic injury; however, a milder response could not be confirmed. Studies have shown an impact of gender on hypertrophy induced by pressure overload [8], left ventricular hypertrophy induced by hypertension after myocardial infarction [7] and in ischemic injury in diabetic rat hearts [23]. Podesser et al. [8] showed that hypertrophy is significantly higher in female than male hypertensive hearts and that female hypertrophied hearts showed more
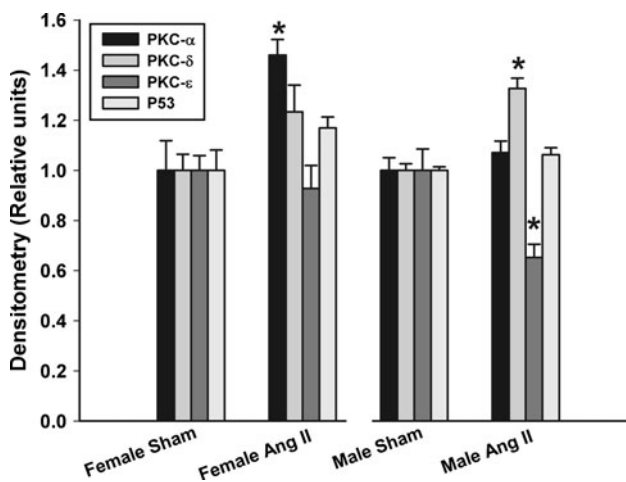
Fig. 3 PKC α, δ and ε and P53 in hearts. Western Blot results presented as units normalized to protein load and to mean values in sham hearts within the same gender. *$P < 0.05$ compared to sham in the same gender group

diastolic dysfunction compared to male and non-hypertrophied female hearts. However, Jain et al. [7] showed that female gender influenced favorably the remodeling and physiological response to hemodynamic overload after myocardial infarction where the concentric hypertrophy in females resulted in elevated contractile function, whereas this elevation was absent in counterparts males. These differences are not only related to geometric remodeling but also to other consequences of differences in gene expression [24]. Gonadal hormones testosterone and estrogen have both been found to modulate ventricular hypertrophy [25, 26]. On the other hand, other studies reported no difference between males and females in different models and using other parameters. Saeedi et al. [27] report no influence of gender on post-ischemic recovery of function in hearts with aorta constriction-induced hypertrophy. The present results are in accordance with the last mentioned finding but this time in an Ang II overexposure model. Although it has been reported that estrogen affects the renin angiotensin system by, for example, modulating AT1 receptors [28], we found no difference in the expression of the corresponding gene (Agtr1-α) between males and females as shown in Table 2. Thus, we conclude that female gender does not seem to improve favorably the outcome with respect to remodeling induced by high-dose Ang II.

In the present experimental model, the specific role of high-dose Ang II was tested. This is an important distinction compared to models using post-ischemic heart failure, isolated volume overload or afterload increase. With increasing levels of Ang II, pressure-independent effects become more evident and in this respect it is possible that the response to Ang II could be gender independent in contrast to the more complex response to increase in

afterload. As mentioned, subgroup analysis revealed slight gender difference in the expression of some genes in response to Ang II (collagen I and III). However, these changes were not translated to differences in function neither prior to global ischemia nor after global ischemia. There was no gender difference in pre-ischemic contractile force elevation in Ang II-treated hearts, in post-ischemic diastolic dysfunction or in the tolerance to ischemia.

Gene expression analysis revealed significant increases in genes that are closely related to heart contractility after Ang II treatment. The α-isoform of myosin heavy chain (α-MHC) which has higher shortening velocity has been shown to be downregulated in rodents exposed to pressure overload associated with upregulation of β-MHC expression [29]. In the present study, there was a significant increase in the expression of β-MHC gene and a tendency to a decrease (n.s.) in the expression of α-MHC in response to Ang II treatment indicating most likely a shift in the isoform from α- to β-MHC.

Ankrd-1 (Ankyrin repeat domain 1) has been characterized as an inducible gene that is over-expressed in fetal, early-postnatal and adult heart in response to multiple forms of cardiovascular stress, including pressure overload, chronic ischemia, infarction-reperfusion injury and at heart failure [30]. The present results show significant increases in the expression of the Ankrd-1 gene in the Ang II-treated animals supporting other reports that the augmented expression of Ankrd-1 can represent an adaptive response of the myocardium to stress both during development and various heart insults [30].

At the cardiomyocyte level protein kinase C (PKC), isoenzymes are key regulators of cytosolic [Ca^{2+}], contractility and ischemic cell death [31]. With respect to ischemic injury, it has been proposed that PKC-δ promotes cell injury whereas PKC-ε delays injury [32]. In agreement with this, expression of isoenzymes PKC-α and PKC-δ in the present study increased significantly with high concentrations of Ang II. In addition PKC-α is involved in the development of contractile dysfunction and thus heart failure susceptibility [33]. PKC-α and PKC-δ were markedly upregulated at the protein level. These changes occurred with cardiomyocyte diameter remaining constant. At the same time, we found increased fibrosis in sections from treated hearts. It has been shown earlier that stimulation with Ang II results in cardiac fibroblast proliferation and a net accumulation of fibrillar collagen in vitro and cardiac fibrosis in vivo. The upregulation of PKCs could support the hypothesis that Ang II induces fibrosis and probably heart failure in a PKC-dependant way (particularly via α- and δ isozymes) and future tests of the use of isoenzyme-specific PKC inhibitors are warranted [34, 35].

Disproportionate accumulation of fibrous tissue mainly collagen types I and III is one of the important contributing

Fig. 4 Collagen fibers stained with Sirius Red **a** Percent of collagen content in heart sections, *$P = 0.035$ Ang II treated versus sham. Pictures are histological sections (100×) with normal structure in sham hearts under conventional light **b** and polarized light **c** and areas of thick bundles of collagen (*arrows*) under conventional and polarized light, (**d**) and (**e**) respectively
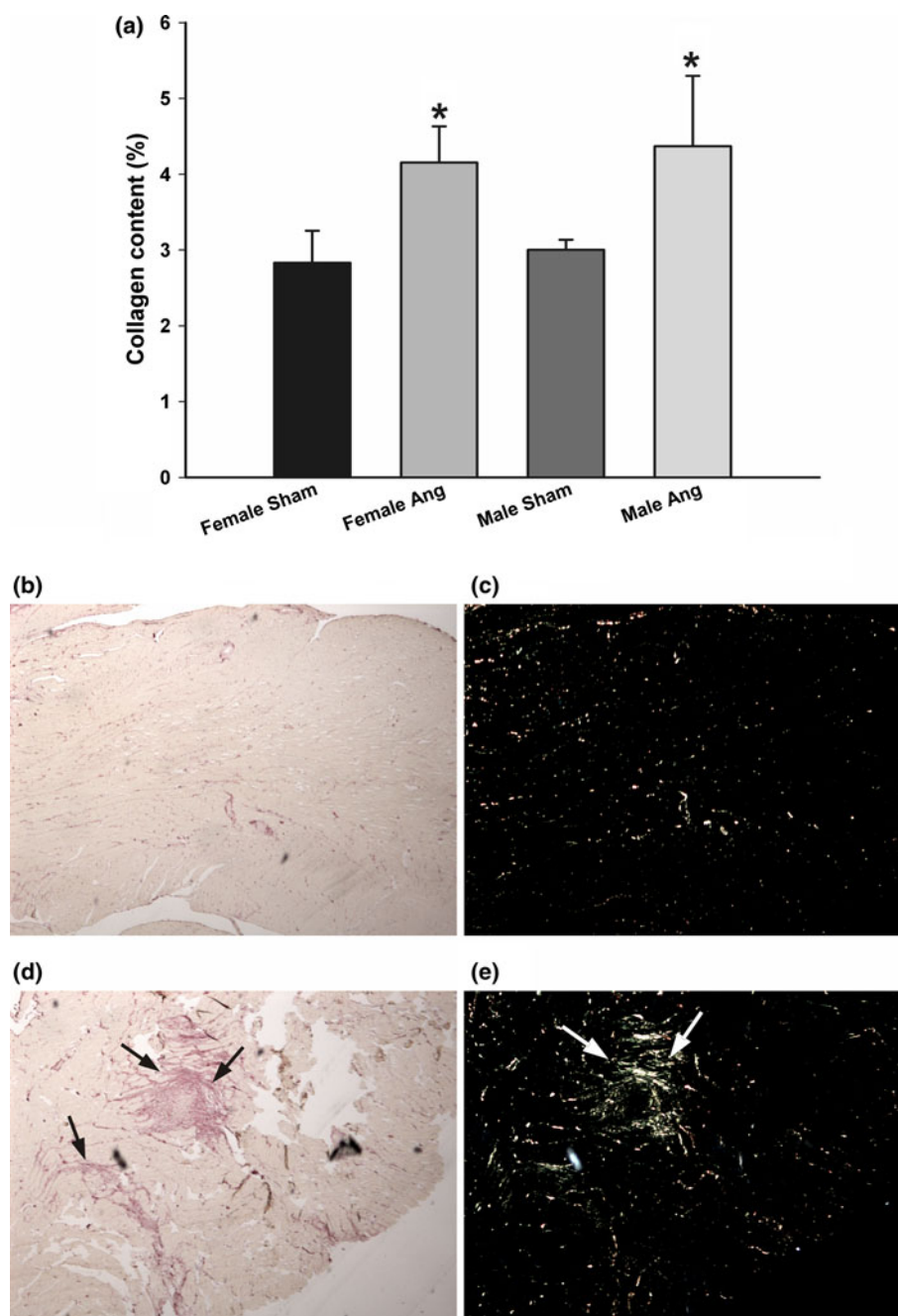
factors to heart failure [1]. Fibrosis of various tissues, including the heart, is regulated by Ang II and transforming growth factor (TGF)-β [36]. The present study demonstrates a significant increase in fibrosis on gene expression and phenotype levels which supports earlier findings. Treated hearts quickly developed marked diastolic dysfunction upon reperfusion although recovery of heart function evaluated by LVDP seemed to be maintained and at the same level as the sham-treated hearts subjected to ischemia–reperfusion. To explain the lack of further deterioration of LVDP in spite of elevated diastolic pressure

after ischemia, one could speculate that functional changes in cardiomyocyte excitation–contraction coupling has also taken place due to Ang II exposure. The lack of diastolic relaxation reflects diastolic dysfunction. The increase in expression of fibrosis-related genes and the amount and thickness of collagen fibers in Ang II-treated hearts supports that Ang II is important in regulating fibrotic changes.

RAS might play a role in cardiac apoptosis [4]. We showed slight but significant increase in expression of apoptosis-related genes coding for proteins p53 and caspase 3. The p53 transcription factor promotes apoptosis via

Table 3 Fibrosis evaluated by collagen staining of heart sections

Group	Collagen (%)	Normal structure	I	II	III	Necrosis
Female Sham ($n = 4$)	2.83 ± 0.42	4	0	0	0	0
Female Ang II ($n = 4$)	4.15 ± 0.47^a	0	2	1	1	3
Male Sham ($n = 4$)	3.00 ± 0.13	4	0	0	0	0
Male Ang II ($n = 4$)	4.36 ± 0.92^a	0	0	1	3	1

Quantification of collagen content in heart sections expressed in % of cross-section area. Number of hearts allocated to in each scoring category by semi-quantification

^{a} $P < 0.05$ Ang II versus sham

Normal Normal structure, *I* Focal thin collagen bundles only, *II* Increased amounts of diffuse thin collagen bundles, *III* Areas of thick bundles of collagen in addition to increased thin bundles

elevation of caspases and Bax and reduction of bcl-2 [37]. Caspase is activated by ischemia–reperfusion, and inhibition of caspases reduces myocyte cell death induced by myocardial ischemia and reperfusion in vivo [38]. In a study on porcine hearts, Kossmehl et al. showed that the Ang II potentiated the ischemia-induced increase in Fas, Bax, bcl-2 and p53-proteins [4]. Interestingly, we showed that the caspase response to Ang II was more marked in male hearts. Ang II is an activator of NADPH oxidase, and via increasing ROS production and cellular oxidative stress the signaling pathway leading to increased levels of p53 would be triggered [39, 40]. In the present study, infarct size after reperfusion was not investigated. An increase in cell death would lead to increase in diastolic pressure, but would also have an impact on LVDP at reperfusion together with the post-ischemic stunning. We did not, however, find a significant reduction in LVDP at reperfusion in Ang II-treated hearts compared to sham hearts.

The present study was based on a balanced 2×2 design using litter- and age-matched animals to assure an identical genetic background. Thus, in each litter, excess animals without siblings of the opposite gender were excluded from the study, and the number of animals that could be included was therefore limited. The standard deviations in heart function measurements indicate that small gender-dependent differences in post-ischemic recovery of function would not be detected (risk for type II statistical error). Neither blood pressure nor heart morphology was monitored during the 2-week long exposure period. The study model was based on experimental results reported by others [18] and previously reported blood pressure changes [18, 41]. Changes in gene expression do not necessarily translate into change at the functional level, and therefore, further proteomic and functional analysis could have been

performed in addition to those selected in this study. This would be particularly interesting when large amplification of gene expression occurred, such as with fibronectin.

In conclusion, we have shown that chronic sub-toxic Ang II exposure leads to changes in the contractile apparatus and reduced tolerance to ischemia in the absence of cellular hypertrophy but with significant upregulation of genes related to heart function, fibrosis and apoptosis as well. Increase in the expression of key genes involved in fibrosis is followed by increase in interstitial fibrosis in heart tissue which partly explains the post-ischemic diastolic dysfunction. An important finding of the study was that although there were differences in magnitude of gene expression response between the genders, female gender did not protect against these changes.

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