

ANGIOTENSIN II AND HEART REMODELLING

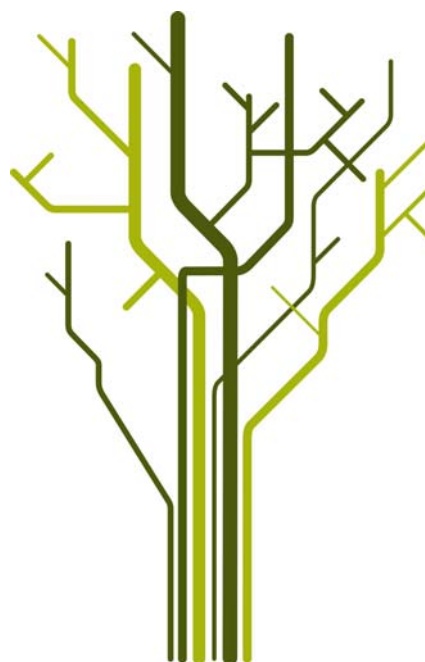
ROLE OF ISCHEMIA, GENDER AND PREGNANCY



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List of papers

- PAPER I** M. B. Aljabri , T. Lund, T. V. Andreassen and K. Ytrehus
Angiotensin II abates the beneficial effects of postconditioning in ERK and GSK-3 β dependent manner. Submitted.
- PAPER II** M. B. Aljabri, T. Lund, A. C. Höper, T. V. Andreassen, S. Al-Saad, S. Lindal and K. Ytrehus
Gene expression, function and ischemia tolerance in male and female rat hearts after sub-toxic levels of angiotensin II. *Cardiovascular Toxicology*. 2011 Mar;11(1):38-47.
- PAPER III** M. B. Aljabri, N. T. Songstad, T. Lund, M. C. Serrano, T. V. Andreassen, S. Al-Saad, S. Lindal, V. Sitras, G. Acharya, K. Ytrehus
Pregnancy protects against antiangiogenic and fibrogenic effects of angiotensin II in rat hearts. *Acta Physiologica (Oxf)*. 2011 Apr;201(4):445-56.
- PAPER IV** M. B. Aljabri , T. Lund, S. Al-Saad, J. Benjaminsen, T. V. Andreassen and K. Ytrehus
Chronic isoproterenol treatment enhances extracellular fibrosis and postischemic diastolic dysfunction in male but not female rat heart. Manuscript.

Abbreviations

α -MHC	α -Myosin heavy chain
β -MHC	β -Myosin heavy chain
ACE	Angiotensin converting enzyme
AMI	Acute myocardial infarction
ANF	Atrial natriuretic factor
Ang II	Angiotensin II
Ankrd-1	Ankyrin repeat domain-containing protein 1
Agtr 1 α	Angiotensin II receptor 1 α (gene)
ARB	Angiotensin receptor blocker
AT1	Angiotensin II receptor 1
AT2	Angiotensin II receptor 2
BNP	B-type natriuretic peptide
Bcl-2	B-cell leukemia/lymphoma 2
Casp-3	Apoptosis related cysteine protease; caspase 3
cDNA	Complementary deoxyribonucleic acid
CF	Coronary flow
Col I- α 1	Collagen type I- α 1
Col III- α 1	Collagen type III- α 1
CVD	Cardiovascular disease
DAG	Diacylglycerol
eNOS	Endothelial Nitric Oxide Synthase
GI	Global ischemia
GSK-3 β	Glycogen synthase kinase-3 β
Fn-1	Fibronectin
IP3	Inositol triphosphate
Iso	Isoproterenol/Isoprenaline
iNOS	Inducible Nitric Oxide Synthase
LVDP	Left ventricular developed pressure
LVEDP	Left ventricular end diastolic pressure
mPTP	Mitochondrial permeability transition pore

mRNA	Messenger ribonucleic acid
NO	Nitric oxide
NOS	Nitric oxide synthase
(n.s.)	Non significant
P53	Tumour suppressor gene (TP53)
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PKC- α	Protein kinase C- α
PKC- δ	Protein kinase C- δ
PKC- ϵ	Protein kinase C- ϵ
PGF	Placental growth factor
RAS	Renin-angiotensin system
ROS	Reactive oxygen species
STAT	Signal transducer and activator of transcription
Timp1	Tissue inhibitor of metallopeptidase
TNF- α	Tumour necrosis factor- α
VEGF	Vascular endothelial growth factor
WB	Western blot
WHO	World health organization

1. Introduction

1.1 Background

Cardiovascular diseases (CVDs) are the number one cause of death globally claiming 17.1 million lives a year according to World Health Organization (WHO). More people die annually from CVDs than from any other cause. In Norway, according to the Norwegian Institute of Public Health, CVDs cause 35% of all deaths every year. It has been registered 13489 deaths from CVDs in 2009 and 40 % of these were attributed to ischemic heart diseases (<http://www.norgesshelsa.no>). According to the WHO, almost 23.6 million people will die from CVDs by 2030, mainly from heart disease and stroke. These are projected to remain the single leading causes of death. The largest percentage increase will occur in the Eastern Mediterranean Region according to WHO. The largest increase in number of deaths will occur in the South-East Asia Region. Cardiovascular deaths occur almost equally in men and women but cardiovascular risk of women is particularly high after menopause (<http://www.who.int/mediacentre/factsheets/fs317/en/>). A cardioprotective effect of sex hormones, especially oestrogen has been suggested, and a large number of articles in the literature describe this effect.

Ischemic heart diseases include acute myocardial infarct (AMI) and stable and unstable angina. AMI occurs when the coronary flow is no longer sufficient to meet the oxygen demand of the heart. Impairment of coronary blood supply to the myocardium is often caused by thrombosis, embolism or other alterations of coronary atherosclerotic plaques. The heart can survive a short period of ischemia and exhibit recovery upon reperfusion, but if the ischemic challenge sustains tissue injury, cell death will occur. There are species-dependant differences with respect to cell survival time. These differences are mainly due to the different degrees of collateral flow.

1.2 Angiotensin II

Angiotensin II (Ang II), a main product of renin-angiotensin system (RAS), is an active vasoconstrictor peptide composed of eight amino acids (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe). Ang II affects virtually all organs including the heart and blood vessels. It has both physiological and pathological effects, and its effects are dependent on the type of cells which are affected and on the time course of exposure - acute or chronic. Pathological effects appear when the balance of RAS is disturbed and the exposure to elevated Ang II lasts longer than it should. Heart consists of different cell types, cardiomyocytes, smooth muscle cells, endothelial cells and fibroblasts. In addition, macrophages could be present in the interstitium and cells in the circulation (monocytes, leucocytes) are also present. All these cells have been shown to harbour Ang II receptors.

Heart consists of different cell types, cardiomyocytes, smooth muscle cells, endothelial cells and fibroblasts.

Ang II triggers its various pathophysiological effects by activating a series of signaling pathways. The main effect of acute Ang II on vasculature is vasoconstriction, which is mediated by G-protein-dependent signaling pathways. Ang II acts through specific membrane-bound Ang II type-1 (AT1) and type-2 (AT2) G-protein coupled receptors, and most of the known Ang II effects are mediated by AT1 receptors. When AT1 receptor is activated by an agonist it binds to $G\alpha_{q/11}$, $G\alpha_{12/13}$, and $G\beta\gamma$ complexes (1) which activate downstream effectors like phospholipase C (PLC), phospholipase A₂ and phospholipase D (2). PLC activation produces inositol triphosphate (IP₃) and diacylglycerol (DAG). IP₃ binds to receptors on sarcoplasmic reticulum, allowing calcium (Ca²⁺) efflux into the cytoplasm. Calcium binds to calmodulin and activates myosin light chain kinase which phosphorylates the myosin light chain and enhances the interaction between actin and myosin light chain, causing smooth muscle cell contraction (3). DAG activates PKC

which increases intracellular pH during cell contraction by phosphorylating the Na⁺/H⁺ pump with intrusion of Na⁺ (4).

Ang II has also direct effects on myocardial cells (5). Cardiac myocyte hypertrophy induced by Ang II is mediated by AT-1 (6). When Gq is activated by neurohormonal mediator such as Ang II, it activates PLCβ, which leads to an IP3-mediated increase in cytosolic [Ca²⁺] and generation of diacylglycerols. Sustained increase in [Ca²⁺] activates the protein phosphatase calcineurin and its target, the NFAT (nuclear factor of activated T cells) family of transcription factors, which are critical mediators of pathologic, but not physiologic, hypertrophy (5). When Ang II binds to its cell surface receptors and activates G-protein, it triggers a cascade of multiple second messenger systems (7). This second messenger cascade causes the activation of angiotensinogen gene (6) and thereby more production of Ang II and activation of RAS (8). Ang II activates mitogen-activated protein kinases (MAPK) via several cascades including extracellular signal-regulated kinase (ERK 1/2), JNK and p38MAPK (9;10). It has also been reported that Ang II promotes myocardial hypertrophy by activating the JAK/STAT signal transduction pathway (8;11;12).

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) seems to work as an indigenous inhibitor of Ang II by exerting hypotensive action through the synthesis and release of vasodilator prostaglandins, augmentation of the metabolic actions of bradykinin and increasing the release of endothelial nitric oxide (13;14).

Important downstream pathways for Ang II's pathological effects are the NADPH-oxidase and ROS signalling (15;16). Ang II leads to increased ROS production and cellular oxidative stress. PKC is an important kinase linked to the control of NADPH activity. ANG II also inhibits the Na⁺-K⁺ pump via PKC-dependent NADPH oxidase activation (17). The generation of ROS induced by Ang II in cardiomyocytes has also recently shown to be mediated by PI3-kinase (18).

In hypertrophic cardiomyocytes, Ang II enhances the synthesis and secretion of TNF- α and IL-1 β , which is mediated by AT2, but not AT1 receptors (19).

Ang II has also multiple effects on endothelial cells such as producing ROS, activating apoptotic signalling pathways and promoting thrombosis (20). The increase in oxidative stress caused by Ang II leads to impaired endothelial relaxation and endothelial dysfunction (21). ROS activates the transcription factor NF- κ B, which results in increased levels of VCAM-1 (vascular cell adhesion molecule 1) which is an important factor in endothelial cell adhesion (22). Ang II modulates also the secretion of the inflammatory cytokine TNF- α and matrix metalloproteinase (MMP)-2 from endothelial cells (23). It has also been shown that Ang II stimulates the production of TNF- α in a PKC-dependent pathway in adult mammalian heart (24).

Ang II stimulates fibroblast proliferation and collagen synthesis. Disproportionate accumulation of extracellular matrix including fibrous tissue mainly collagen types I and III is one of the important contributing factors to heart failure and hypertrophy after an ischemic insult (20;25). Fibrosis of various tissues, including the heart, is regulated by Ang II and transforming growth factor (TGF)- β (26). It has been reported that fibroblast proliferation and collagen synthesis are mediated by the AT1 receptor (27). In addition, activation of EGFR (epidermal growth factor receptor) and MAPK- dependent pathways induced by Ang II may participate in matrix formation and regulation (28;29).

1.3 Gender

It has been demonstrated in both clinical (30) and experimental studies (31;32) that gender influences the cardiac response to prolonged increase in hemodynamic work load. Gender-specific differences have been proposed in patients with aortic stenosis, with female patients showing increased hypertrophy, greater concentric remodelling, and better preservation of the left

ventricular function compared to male patients, (reviewed by Douglas *et al.*) (33). The majority of the experimental studies examining the influence of gender have so far evaluated pressure overload, aortic stenosis or post infarct ventricular remodelling. Most studies conclude that female gender represents a beneficial effect, as reviewed in (34) by Murphy *et al.*, while some studies indicate that once ischemic heart disease is present, female hearts are more vulnerable compared to male hearts (32).

1.4 Pregnancy

Pregnancy together with exercise is the main cause of physiological cardiac remodelling. During pregnancy, the heart develops a reversible physiological hypertrophy and diastolic dysfunction in response to the mechanical stress of increased volume load and wall stretch, as well as changes in myocardial gene expression (35;36). Since 1961 it has been observed that there is up-regulation of the renin-angiotensin system in pregnancy, and circulating levels of renin and angiotensinogen are increased which could lead to systemic or tissue specific increase in Ang II (37;38).

1.5 Adrenergic activity

Signs of increased activity in the sympathetic nervous system and the adrenal glands characterize cardiovascular failure, and are part of the neuroendocrine activation accompanying decreased cardiac output. Isoproterenol (Iso) is a well characterized adrenergic agonist with selectivity towards the β -adrenergic receptor. The compound has been used experimentally to study the hypertrophic and remodelling response to adrenergic activation (39).

1.6 Ischemia – reperfusion injury and postconditioning

Early restoration of blood flow after ischemia is crucial in order to salvage the ischemic myocardium. However, reperfusion itself may trigger deleterious effects and exacerbate the

damage occurring during the ischemic period. This injury is referred to as reperfusion injury (40), and clinical manifestations of this injury can be of multifactoral nature and include arrhythmias, myocardial stunning, microvascular dysfunction, myocardial apoptosis and other forms of irreversible cell damage (41;42).

Postconditioning is defined as repeated brief cycles of ischemia interrupted by reperfusion applied at the onset of reperfusion after prolonged ischemia (43). In addition to the important reduction in extent of necrosis and apoptosis following reperfusion, postconditioning reduces endothelial activation, dysfunction and the inflammatory response to reperfusion (44). Clinical trials have shown that application of postconditioning during coronary angioplasty (cycles of inflation and deflation of angioplasty balloon) protects human hearts after acute myocardial infarction and this protection with postconditioning is still apparent 1 week following reperfusion, suggesting long-term protection (45-47). Reports suggest that blockade of opening of the mitochondrial permeability transition pore (mPTP), which play a crucial role in reperfusion injury, is involved in the cardioprotective effect of postconditioning (48;49).

2. Aims of study

2.1 General aims

The aim of this work was 1) to test the response of acute ischemic stress in *ex vivo* perfused rat hearts with or without established cardiac hypertrophy and 2) to compare the response to stress of hearts from animals of both genders and in case of pregnancy.

2.2 Specific aims

- To study ischemia-reperfusion in isolated hearts, in particular the impact of Ang II on the anticipated cardioprotective effect of postconditioning, including the signalling pathways involved.
- To compare the response to chronic high dose Ang II in male *vs* female rats with respect to gene expression and ischemic injury.
- To examine potential differences between physiological and pathological cardiac remodelling induced by pregnancy and Ang II, respectively, and test the hypothesis that pregnancy protects against the effects of Ang II.
- To examine the responses to chronic adrenergic stimulation with isoproterenol with respect to gene expression and ischemic injury in male *vs* female rats.
- To compare the response to Ang II and Iso.

3. Methods and methodological considerations

3.1 Animal preparation

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

In paper II the rats used were Fischer 344 x Brown Norway F1 hybrid rats of inbred strains (FBN) (50;51). Wistar rats were used in the rest of the studies. The animals were housed in cages in pairs under controlled conditions of temperature, light-dark periods of 12 h, and with free access to water and standard laboratory diet. Chronic treatments with Ang II or Iso were given through osmotic mini-pumps implanted subcutaneously on the back of the neck by conventional surgical technique. Surgery was performed under semi sterile conditions. General anesthesia was induced by isoflurane gas, and postoperative analgesia with buprenorphine (0.05 mg/kg given subcutaneously). After surgery the animals were kept in separate cages and allowed free access to water and standard laboratory diet.

(In paper II rats were bred) and in paper III the rats were mated in the Department of Comparative Medicine at the University of Tromsø. After mating, pregnancy was identified next morning by the presence of a vaginal plug, and this day was considered gestational day 0.5. On gestational day 8.5-9.5 micro-osmotic pumps releasing Ang II or saline were implanted as described above.

3.2 Langendorff perfusions

The Langendorff perfusion technique, first described in 1895, is a well established model for *ex vivo* perfusion of isolated hearts (52). Hearts were perfused with constant pressure (100 cm H₂O) under iso-volumetric conditions to study the response to ischemia-reperfusion in hearts treated either acutely or chronically with Ang II or Iso (Papers I, II and IV). Ventricular functional data were obtained using an intraventricular balloon, while coronary flow was determined by timed collections of venous effluent. In addition, to constitute a low-cost, technically reproducible method, isolated Langendorff perfused hearts have the advantage that external variables may be readily standardized with respect to temperature, pH, pressure, ion concentrations, energy substrates and administration of drugs. Also neuro-hormonal interference is eliminated.

3.2.1 Experimental protocols

Several protocols have been used in this work. The common procedure for perfused hearts was 25 minutes stabilization and global ischemia for 30 minutes, followed by 30 minutes reperfusion (53;54). In paper I postconditioning consisted of four cycles of 30 seconds ischemia/reperfusion, which were applied from 1 min reperfusion. Depending on the study-protocol, pharmacological or chemical agents (Ang II or GSK-inhibitor) were added to the buffer. A scheme of the current protocols is shown in appendix 1. We reperfused hearts for only 30 minutes after global ischemia. Therefore, we do not know if a longer reperfusion period would have given different results. Sampling for western blot was done at 30 minutes of reperfusion

3.3 Echocardiography

Echocardiography is a non-invasive and useful method to get hemodynamics measurements *in vivo*. We used echocardiography chiefly in paper III (and in a small group of rats in paper II to test the dose-response to Ang II). Echocardiography was performed on gestational day 18.5-20.5

in the pregnant animals, and 9-10 days after implantation of mini-pumps in the non-pregnant rats. Isoflurane inhalation anesthesia was provided. The rats were placed supine on a warm pad and heart rate and rectal temperature was monitored continuously while performing echocardiography. The temperature was kept stable by regulating the temperature on the board and, when necessary, by using a heating lamp. Transthoracic echocardiography was performed using a high frequency ultrasound imaging system (Vevo 770, Visualsonics Inc., Toronto, Canada) equipped with an RMV-710B transducer with a frequency of 25 Hz. A fixed focal length of 15 mm was used to obtain parasternal long-axis and short-axis views, as well as apical four-chamber views. M-mode recordings were obtained from the parasternal long-axis or short-axis views for measurement of left ventricle internal diameters and anterior and posterior wall thickness at diastole and systole. Anatomical parameters were calculated using mathematical formulas to estimate relative wall thickness and left ventricular mass according to the following formulas $RWT = (LVPWd + LVAWd) / LVIDd$ where RWT: Relative wall thickness, LVPWd: LV posterior wall thickness, LVAWd: LV anterior wall thickness and LVIDd: LV internal diameter in diastole. Left ventricular mass was calculated using the formula: $LV\ mass = 1.04 \times (LVIDd + LVPWd + LVAWd)^3 - LVIDd^3$ (55;56). After echocardiography a 2F micro-tip pressure transducer (connected to an amplifier) was inserted into the ascending aorta via the right carotid artery to measure aortic blood pressure.

3.4 Validation of response to different doses of Ang II

In order to validate delivery of Ang II by miniosmotic pumps and the response to the different doses of Ang II, we did a supplementary experiment including sixteen Wistar male rats (10 weeks old, 300-350 g), testing three different doses of Ang II (150, 300 and 400 ng·kg⁻¹·min⁻¹) with or without the angiotensin receptor blocker (ARB) Losartan. Heart anatomy and ventricular

function was assessed by echocardiography twelve to fourteen days after implantation of the Ang II pumps or sham operation. Table 1 and figures 1(a) to (f) show the response to different Ang II doses in sixteen Wistar male rats. In the same way we tested the response to the different Ang II doses in sixteen Wistar female rats (table 2 and figure 2(a) to (f)). In male hearts we found increased heart weight at the lower concentrations of Ang II (150 and $300 \text{ ng}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$), which could be blocked with Losartan. At the highest Ang II concentration ($400 \text{ ng}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) the increase in heart weight was lost, and heart weight was similar to sham and in addition body weight decreased. In females Wistar rats in contrast to the Fisher rats in paper II the increase in heart weight remained even with the highest dose though accompanied by reduction in relative wall thickness and ejection fraction. Importantly, all changes seemed to be fully or partly blocked by the AT1 receptor blocker thus confirming that the response was angiotensin II receptor dependent.

Table 1 Heart and body weight and echocardiographic measurements in male Wistar rats treated with different Ang II doses.

(n=2)	Sham	Sham + ARB	Ang II (150)	Ang II (150) + ARB	Ang II (300)	Ang II (300) + ARB	Ang II (400)	Ang II (400) + ARB
HW (g)	0.84	0.84	1.13	0.80	1.02	0.93	0.79	1.00
BW (g)	340	354	309	346	298	345	246	366
HW/BW	2.5	2.8	3.6	2.3	3.4	2.7	3.2	2.7
RWT (%)	44	39	99	45.6	99.5	58	84	48
LV mass (g)	616	486	832	568	923	632	689	775
EF (%)	65	71	56	77	71	74	78	82

ARB: Angiotensin Receptor Blocker, HW: Heart Weight, BW: Body Weight, RWT: Left Ventricular Relative Wall Thickness, EF: Ejection Fraction.

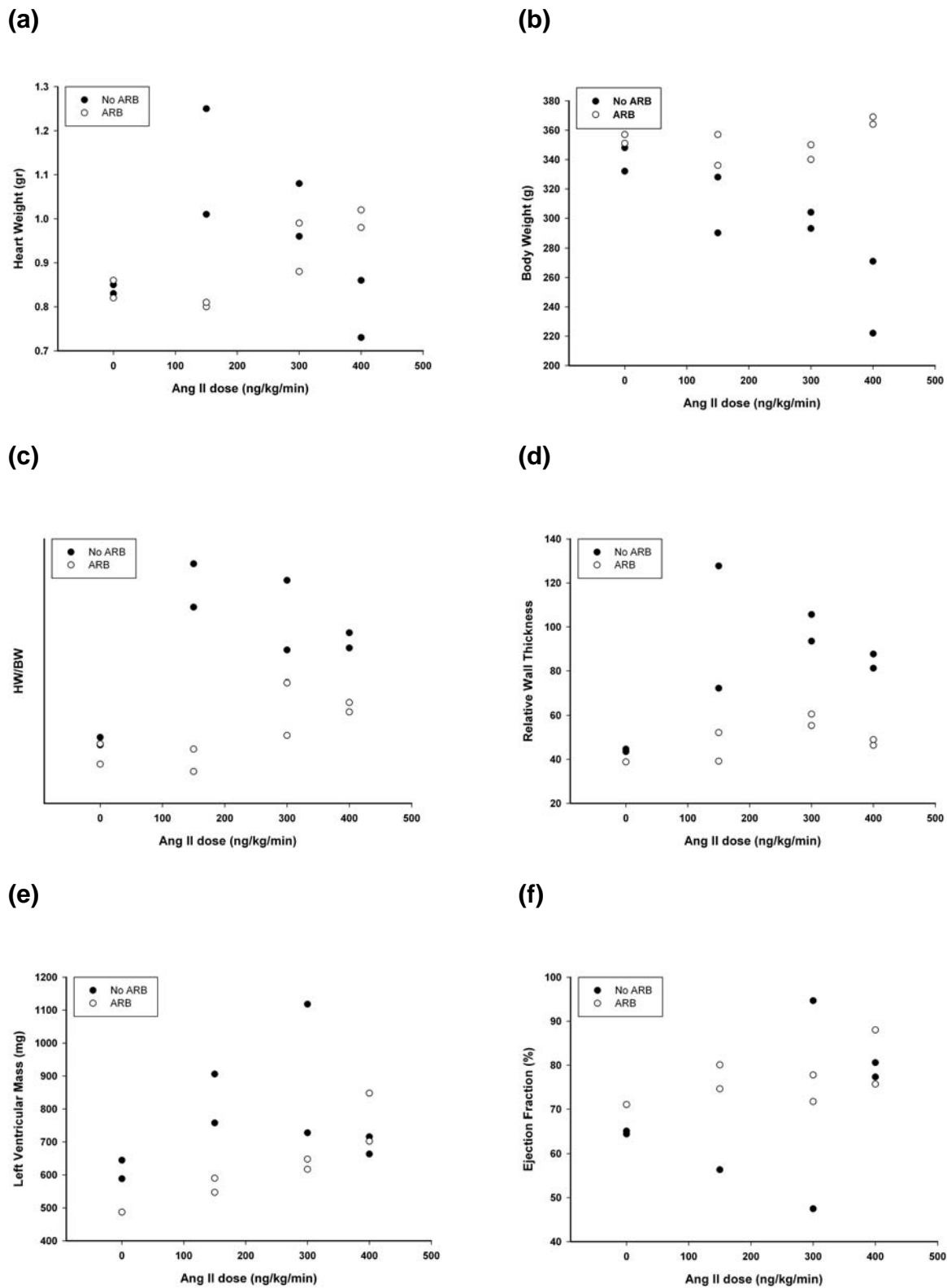


Figure 1 Response to different Ang II doses in male Wistar rats (a) heart weight, (b) body weight, (c) HW/BW, (d) relative wall thickness, (e) left ventricular mass and (f) ejection fraction.

Table 2 Weight and echocardiographic measurements in female rats treated with different Ang II doses.

(n=2)	Sham	Sham + ARB	Ang II (150)	Ang II (150) + ARB	Ang II (300)	Ang II (300) + ARB	Ang II (400)	Ang II (400) + ARB
HW (g)	0.64	0.62	0.65	0.56	0.78	0.63	0.90	0.53
BW (g)	197	209	197	202	186	215	187	194
HW/BW	3.2	3.0	3.2	2.8	4.2	2.9	4.8	2.7
RWT (%)	47	43	55	40	97	50	73	42
LV mass (g)	0.5	0.5	0.6	0.5	0.7	0.6	1.0	0.4
EF (%)	71	74	78	74	90	74	59	84

ARB: Angiotensin Receptor Blocker, HW: Heart Weight, BW: Body Weight, RWT: Left Ventricular Relative Wall Thickness, EF: Ejection Fraction.

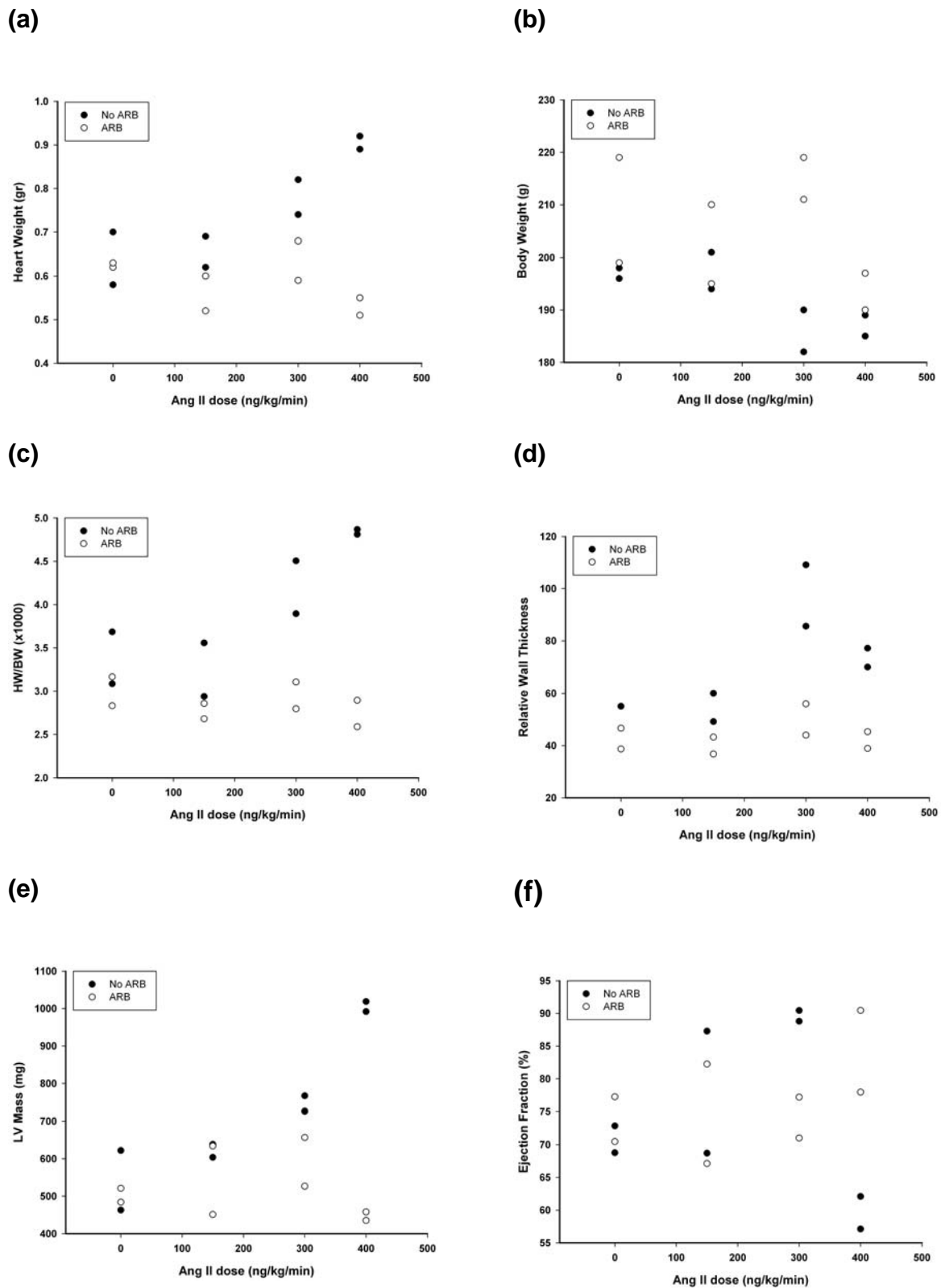


Figure 2 Response to different Ang II doses in female Wistar rats (a) heart weight, (b) body weight, (c) HW/BW, (d) relative wall thickness, (e) left ventricular mass and (f) ejection fraction

These experiments confirm that the response to Ang II in our rat model is dose-dependent and receptor-mediated. From these small experiment series we can also find a gender difference in response to the different doses of Ang II. Female hearts seem to tolerate Ang II better than males as HW kept increasing with the highest studied dose of Ang II in order to compensate and overcome the increasing afterload while male hearts started to become cachexic at earlier point already at the dose of $300 \text{ ng}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$. So the initiation of cardiac cachexia in female hearts requires higher Ang II dose in comparison with male counterparts.

3.5 Western Blot

Western Blot technique was used to study protein expression of selected proteins and activation by phosphorylation of kinases known to regulate cell survival. At the end of Langendorff-perfusion, ventricles were snap-frozen in liquid nitrogen and stored at -80 C . To ensure homogeneity, samples were pulverized under liquid nitrogen prior to extraction of protein. 25–35 mg of powder was homogenized on ice in 600 μl of lysis buffer as described in detail in paper I and II. In the present studies we have used Ponceau S staining (Sigma, St. Louis, USA) to confirm equal loading of protein in each lane. The immuno-positive bands were developed with Immobilon chemoluminescent reagent (Millipore, MA, USA) and visualized using a Kodak Image Station 1000 (PerkinElmer, USA).

Changes in the phosphorylation of a specific protein can be evaluated by Western Blot technique by studying the ratio between the densitometric values of bands containing the phosphorylated form of a specific protein and the total amount of the same protein. The method is semi-quantitative since it is based on a chemiluminescence reaction coupled to binding of an antibody. Provided that the used antibodies are of good quality it is assumed that WB provides a good indication of phosphorylation status of a specific protein. Phosphorylation levels do not

necessarily reflect the activity of an enzyme; an inhibitor or an activator may change the activity even though it may or may not change phosphorylation. In the present study we have tested phosphorylation status of kinases known to be regulated by phosphorylation and used commercially available polyclonal antibodies for the detection of both the phospho-form and the total amount of the selected kinase. We have also examined the total amount of protein kinases C- α , - δ , - ϵ and P53 in chronically treated hearts. Secondary antibody used was Anti-rabbit IgG, Horseradish-peroxidase (Cell Signaling Technology, Danvers, USA). A list of antibodies used with suppliers is shown in table 3.

Table 3 Antibodies used in this thesis.

Antibody	Supplier
P-Akt (Ser ⁴⁷³) and total Akt (Paper I)	Cell Signalling Technology , USA
P-GSK3 β (Ser ⁹) and total GSK3 β (Paper I)	
P-p44/42 ERK (Thr ²⁰² /Tyr ²⁰⁴) and total ERK (Paper I)	
P-STAT3 (Tyr ⁷⁰⁵) and total STAT3 (Paper I)	
P-PKC- δ (Thr ⁵⁰⁵) and total PKC- δ (Paper I)	Santa Cruz Biotechnology, USA
P-PKC- ϵ (Ser ⁷²⁹) and total PKC- ϵ (Paper I)	
P53 (Paper II)	
PKC- α , - δ and - ϵ (Paper II)	
β -actin (Paper II)	

3.6 Gene Expression

Gene expression analysis was performed to study changes induced as a result of chronic treatment and to investigate differences between genders. Real-time quantitative PCR (qPCR) is rapid and sensitive method to study gene expression. qPCR can be applied for both absolute and relative quantification of gene expression. In all studies included in this thesis, relative quantification has been used. In relative mRNA quantification data is normalized against

reference genes (housekeeping genes) and to a control sample (57). This is a common method for normalizing qRT-PCR.

Initially, complimentary DNA, synthesized from isolated mRNA by reverse transcription is channelled through different phases of real-time q-PCR namely; denaturation, annealing and extension along with primers and probes of the genes of interest. The fluorescence signal in the exponential phase is then captured by the qPCR detector and displayed by the software as an amplification plot. When performing the relative quantification of the expression of a target gene it is important to choose a suitable gene as a reference or housekeeping gene. Ideally, reference genes should not vary in response to the experimental intervention. Despite being occasionally constant in a given cell type or experimental condition, these genes still can vary considerably between the different cell types and experimental conditions (58). Therefore, instead of using one reference gene we have used the geometric mean of best three out of 5-6 housekeeping genes as recommended by Vandesompele *et al.* (58). 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 compared to a control group. In our case the expression of the target genes were normalized to the stable expressed reference gene evaluated by Normfinder (59) of possible reference genes: GAPDH, B2M, Cyclophilin, HPRT and LDHA as described by others (57). Primers and probes of reference and tested genes used in the present thesis are shown in appendix 2.

3.7 Histology: Toluidine blue staining, Sirius Red staining and non-muscular β -actin immunohistochemistry

Simple Toluidine blue stained sections were used to measure cell diameter or cross section area (Papers II, III and IV). Pictures were taken using a Leitz Aristoplan microscope with a Leica

DFC320 digital camera or Nikon Invert Microscope Eclipse TE300 camera (paper IV). Cells with visible nucleus were used for quantification and minimum diameter or cross section area at the level of the nucleus was measured. The Toulidin sections were used to determine myocyte diameter or cross section area based on a minimum of twenty to thirty cells selected from an area of minimal tissue distortion. ImageJ (National Institutes of Health, Bethesda, MD, USA) was used for calculation of cross section area whereas software connected to the microscope was used for calculation of diameter (Leica CTR 600 & Leica Qwin V3).

For collagen fibre staining, Sirius Red staining (Direct Red 80, Sigma-Aldrich, Germany) was used (Papers II, III and IV). Sirius Red is a strong anionic dye which stains collagen by reacting with basic groups present in the collagen molecule. The elongated dye molecules are attached to the collagen fibre in such a way that their long axes are parallel (60). The collagen content was estimated in two different ways; semi-quantitative and quantitative. Transverse ventricular sections were examined under the microscope using conventional and polarized light at magnification 50x and 200x. 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, gender or pregnancy status. Each heart was finally assigned to one category with respect to collagen content and also scored according to presence of necrosis or not. In addition, a minimum of twenty sampled images (200x) from each heart were analyzed for % tissue area occupied by extracellular Sirius Red positive fibres avoiding perivascular collagen using Image J for quantification of staining.

Vessel density in hearts was estimated (paper III) by immunohistochemistry using non-muscular β -actin to identify endothelial cells (55;61). The non-muscular β -actin isotype is present in rat cardiac endothelial cells but not in cardiac myocytes and cardiac smooth muscle cells (61). Identification of endothelial cells in this way allowed us to compare vessel density in the studied

groups. Sections were examined under light microscope at (200x) and twelve microscopic fields from each heart section were photographed. All pictures were taken systematically randomly. Sections were taken from the left ventricle wall only and pictured from four different pre-selected areas of the sections under the microscope (three pictures from each position 3, 6, 9 and 12 o'clock). Using ImageJ software, a grid of 80 points (area per point was 3.22 cm²) was applied on each picture and relative vessel density was expressed as the number of points crossing a blood vessel identified by the staining.

3.8 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 pregnancy and Ang II treatment, gender and Ang II or gender and Iso treatment. The Holm-Sidak or Student-Newman-Keuls (paper II) 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.

In paper II the size of the groups was limited due to imbalance in birth rates between males and females rats. This was compensated for by the use of a balanced design and identical group size with individuals with identical genetic background.

4. Summary of results

4.1 Paper I

The aim of this paper was to study the impact of Ang II on the anticipated cardioprotective effect of postconditioning. Thirty minutes of global ischemia followed by thirty minutes of reperfusion resulted in decreased recovery of LVDP, diastolic dysfunction and reduction in coronary flow in the isolated perfused rat heart. Soon after global ischemia, LVDP fell to near zero and remained near zero during the whole 30 minutes ischemia. Once perfusion was initiated, LVDP started to increase. There was usually arrhythmia at the onset of reperfusion but this was spontaneously converted to normal rhythm in most hearts.

Recovery of LVDP was significantly ($p < 0.05$) improved by postconditioning when compared to control, but this improvement was not present in hearts perfused with Ang II-containing buffer. Similarly, recovery of coronary flow (CF) was impaired in hearts perfused with Ang II. However, when adding the GSK3 β -inhibitor (SB216763) for 15 minutes at the onset of reperfusion, the detrimental effects of Ang II disappeared, suggesting that inhibiting GSK3 β could be part of the mechanism behind the cardioprotection induced by postconditioning.

Western blot results showed that postconditioning increased P-ERK1, but also this response was lost in hearts perfused with Ang II-containing buffer. On the other hand, Ang II increased P-ERK2 and this increase was lost when the hearts were postconditioned. Surprisingly, both Ang II and postconditioning decreased P-STAT3.

There was no increase in the immunoreactivity of P-Akt in the postconditioning group which can indicate that Akt may not be involved in the cardioprotective mechanism of postconditioning or that the increase in phosphorylation was already returned to normal levels when the tissue

sampling was done. At 30 minutes of reperfusion no significant changes in immunoreactivity of phosphorylated GSK3 β , PKC- δ or - ϵ were observed.

The conclusions obtained from paper I were that postconditioning protects the heart from ischemia-reperfusion insults, shown by improved heart function (increased CF and LVDP recovery). The cardioprotective effect of postconditioning is probably mediated via the ERK1/2 signalling pathways and GSK3 β . Ang II and postconditioning counteract each others effects, most probably at the level of ERK, which could result in the lack of phosphorylation of GSK-3 β . Direct inhibition of GSK-3 β by SB216763 turned on the beneficial effect of postconditioning in the presence of Ang II. Thus enhanced levels of Ang II seen in some diseases in humans may potentially interfere with the beneficial effect of postconditioning. This has to be considered if postconditioning is to be used in clinical practice.

4.2 Paper II

The aim of this study was to examine the response to chronic high dose Ang II in male *vs.* female rats with respect to gene expression and ischemic injury. We found that exposure to Ang II had pathophysiological effects like cachexia (loss of muscular mass) and diastolic dysfunction, even if it did not induce heart hypertrophy (heart weight and cardiomyocyte diameter did not increase in neither males nor females). Independent of gender, hearts treated with Ang II had significantly higher LVDP prior to ischemia compared to sham. Coronary flow corrected for heart weight did not differ between groups. When subjected to 30 minutes global ischemia followed by 30 minutes reperfusion there was a marked and significant aggravation of the increase in left ventricular end diastolic pressure in the Ang II treated hearts, reflecting significant diastolic dysfunction and contracture development.

Ang II significantly increased expression of genes related to heart function: ANF, β -MHC, Ankrd1, TNF- α (α -MHC, Agtr1 α , BNP unchanged); fibrosis: Col I- α 1, Col III- α 1, Fn-1, Timp1; apoptosis: p53 and casp-3, whereas bcl-2 remained unchanged. Three PKC isozymes were also tested, and PKC- α and PKC- δ were found to be significantly up-regulated after Ang II treatment whereas expression of PKC- ϵ showed a slight trend to decrease.

Protein expression of P53, PKC- α , PKC- δ tended to increase by Ang II treatment while PKC- ϵ tended to decrease.

Collagen content was 2.9 % in sham hearts and 4.3% in Ang II treated hearts. Samples from sham hearts appeared normal while samples from Ang II animals showed presence of collagen bundles (focal, diffuse thin or thick bundles).

In order to test if there is a difference in gene expression of the selected genes between untreated male and female hearts (before interaction), two groups of animals which received no specific treatment were included. Hearts from 4 male and 4 female control rats were subjected to gene expression analysis without prior Langendorff-perfusion. Gene expression analysis of these hearts showed no gender dependent variability (Table 4).

The conclusion from paper II was that chronic sub-toxic Ang II exposure leads to changes in the contractile apparatus and to reduced tolerance to ischemia in the absence of cellular hypertrophy but with significant change in genes related to heart function, fibrosis as well as apoptosis. Increased expression of key genes involved in fibrosis was followed by an 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 the magnitude of gene expression response between the genders, female gender did not protect against the changes in function or the increase in interstitial fibrosis.

Table 4 Gene expression of selected genes from non-treated unperfused hearts. Expression is normalized to the house keeping genes.

Gene	Female (n=4)	Male (n=4)
ANF	40±1	70±3
BNP	0.4±0.1	0.5±0.2
α-MHC	44900±9000	38120±12900
β-MHC	110±8	290±130
Ankrd-1	5320±290	7300±1190
Agtr1-α	1.2±1	1.1±1
TNF-α	0.9±0.2	0.7±0.1
PKC-α	20±2	20±1
PKC-δ	20±3	20±3
PKC-ε	230±20	240±20
Col I-α1	90±20	100±20
Col III-α1	220±30	290±50
Fn-1	8.5±0.6	9.7±1.3
Timp1	9.0±1.1	14±0.8
P53	15.6±0.8	15.5±1.8
Casp3	10±1	10±0.8
Bcl-2	40±7	40±1
iNOS	1.3±0.2	1.4±0.2
eNOS	21.4±2.2	22.9±1.3

4.3 Paper III

The aim of this study was to examine potential differences between physiological and pathological cardiac remodelling induced by pregnancy and Ang II, respectively, and test the hypothesis that pregnancy protects against the effects of Ang II.

Pregnancy represents a physiological condition with increased production of female sex hormones. Pregnancy is associated with physiological cardiac hypertrophy induced by mechanical stress (wall stretch) due to increased volume load, as well as by changes in myocardial gene expression (35;36). We sought to investigate the difference between physiological and pathological cardiac remodelling induced by pregnancy and Ang II, respectively, and to test the hypothesis that pregnancy protects against deleterious effects of Ang II.

There were significant interactions between the response to Ang II treatment and pregnancy with respect to heart weight, which was significantly higher in Ang II treated pregnant rats compared to both untreated pregnant rats and to Ang II treated non-pregnant rats. Furthermore, relative wall thickness was significantly increased in Ang II-treated pregnant rats compared to untreated pregnant rats and to Ang II-treated non-pregnant rats. Ejection fraction was significantly higher in Ang II-treated pregnant rats compared to non-pregnant and pregnant sham. However, measurement of cardiomyocyte diameter showed no significant differences between the experimental groups.

Aortic systolic and diastolic pressures were significantly increased in Ang II-treated rats in both pregnant and non-pregnant groups. However, there were no differences between pregnant and non-pregnant groups in the same treatment category.

Quantification of % tissue area occupied by extracellular Sirius Red positive fibres using ImageJ showed that Ang II treatment resulted in a significant increase in collagen content, and that pregnancy opposed this effect.

Histological evaluation of blood vessel density in the left ventricle using non-muscular β -actin revealed a significant decrease (47.8%) in vessel density in Ang II treated hearts compared to sham. Pregnancy alone tended to increase vessel density by almost 25% of sham value but this was not statistically significant compared to control. However, reduction of vessel density after Ang II treatment in the pregnant group was only 13.9% (n.s.).

With respect to gene expression level there were significant changes in genes related to heart function, fibrosis and apoptosis. Ang II treatment resulted in a significant *increase* in the expression of the following genes regardless of pregnancy status: ANF, BNP, Ankrd-1, PKC- δ and P53. Col III- α 1 and Timp-1 were significantly increased only in Ang II treated pregnant rat hearts compared to sham while PKC- α was increased only in non-pregnant rats after Ang II treatment. Pregnancy, however, did not induce expression changes of known markers of pathological remodelling, but rather resulted in a significant *decrease* in the expression of the following genes: α -MHC, TNF- α , eNOS, iNOS, P53 and BCL-2, regardless of Ang II treatment status.

The conclusion from paper III was that pregnancy and chronic exposure to a moderate dose of Ang II has contrasting effects on fibrosis and angiogenesis in the heart. Pregnancy seemed to inhibit the detrimental effects of Ang II on cardiac remodelling despite synergistic effects with respect to heart hypertrophy. Pregnancy and Ang II lead to opposite changes in the expression of some of the genes related to heart function and apoptosis.

4.4 Paper IV

The aim of this study was to examine the responses to chronic adrenergic stimulation with isoproterenol with respect to gene expression and ischemic injury in male vs female rats.

A recent study by Heather *et al.* (39) showed that isoproterenol caused a 73% increase in end-systolic pressure, which was associated with a marked hypertrophy, fibrosis and a 33% increase in end-diastolic pressure. Our last study therefore investigated changes in cardiac function and gene expression in response to isoproterenol, and in particular whether the response differed between genders. Heart weight and heart weight to body weight ratio (HW/BW), increased significantly after Iso treatment in both genders. Body weight did not differ between treatment groups of same gender. Baseline values of LVDP, LVSP and LVEDP (25 min stabilization) did not differ between Iso-treated and non-treated rats, nor did we find any differences in these parameters between males and females. Hearts from male rats showed a significantly higher ischemic contracture as compared to those from females, and male Iso-treated hearts showed a more marked diastolic dysfunction at the end of reperfusion. Recovery of CF and LVDP after global ischemia did not differ between the two gender groups.

Expression of the following genes significantly changed after Iso-treatment both in male and female rat hearts: ANF, α -MHC, β -MHC, Agtr-1 α , Col I- α 1, Col III- α 1, Fn-1, Timp-1, PKC- α , PKC- δ , PKC- ϵ , Bcl-2 and Casp3. α -MHC, PKC- δ and Bcl-2 were down-regulated – the other up-regulated.

Genes related to fibrosis (Col I, Col III, Fn-1 and Timp1) were over-expressed in hearts from Iso-treated males compared to females. In accordance with this result we found a significant increase in collagen content in histological sections of heart muscle from Iso-treated males. Collagen content increased from 1.87% \pm 0.14 in male sham to 3.63% \pm 0.54 in the male Iso group, while no

significant changes were detected between female sham ($2.05\% \pm 0.39$) and the female Iso (group, $2.13\% \pm 0.24$).

We conclude from this study that that the pathophysiological response of the heart to sub-chronic combined β_1 - β_2 -adrenergic stimulation is gender dependent and that female gender represents an advantage. Interstitial remodelling with increase in collagen content is a main factor behind this difference.

5. Discussion

5.1 Effects on heart function and hemodynamics

The present thesis shows as expected that Ang II-induced heart remodelling is dose-dependent.

In the experimental model presented in paper II, the specific role of high dose Ang II was tested. This model differs from models of post-infarct heart failure, isolated volume overload or afterload increase. Not only cardiac cachexia was induced by high dose Ang II, but there was also cachexia of skeletal muscles. Body weight was significantly reduced in both genders with the highest dose of Ang II compared to counterpart sham. These findings correspond to earlier reports investigating skeletal muscle wasting (62;63) and are in line with the proposed role of Ang II as a candidate for induction of cardiac cachexia (64). Ang II has the potential to cause muscle atrophy through an increased muscle protein catabolism through the ubiquitin-proteasome proteolytic pathway (65). This reduction in body mass has been shown to be AT1 receptor-mediated and suppression of hepatic growth hormone receptor and insulin-like growth factor has been suggested (66).

Treatment with a lower dose of Ang II ($150 \text{ ng}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) increased aortic blood pressure and resulted in cardiac hypertrophy in pregnant rats. All hypertrophy parameters were highest in Ang II treated pregnant hearts. Seven days of β -adrenergic stimulation resulted as expected in a remarkable increase in heart weight and HW/BW ratio. Interestingly, pre-ischemic contractile function measured under iso-volumetric conditions using perfusion buffer without isoproterenol was not influenced when the Iso-treated hearts were compared with sham hearts. Further studies of pressure-volume-relationship will be needed to reveal whether change in contractility has taken place. Also, it is known that prolonged exposure to β -adrenergic agonists might down-regulate the receptor, but the potential consequence of this was not tested. Heather *et al.* showed

that Iso-treatment resulted in impaired cardiac function with 33% increase in end-diastolic and 73% increase in end-systolic pressure (39).

The role of gender

The dose-response pattern seemed to be gender-dependent. Heart weight tended to increase at a lower concentration in male hearts compared to female hearts. Relative wall thickness and ejection fraction increased and peaked around the dose of $300 \text{ ng}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$. With higher dose, Ang II appeared sub-toxic and induced cachexia of the heart muscle. Apparently, induction of this response requires higher doses in females than in males.

5.2 Response to ischemia-reperfusion

In paper I, the beneficial effects of postconditioning were not expressed in the presence of Ang II. This could be due to interference with the activity of cardioprotective cell signalling pathways. Adding GSK-inhibitor at the onset of reperfusion, however, restored the cardioprotective potential of postconditioning (improved CF and LVDP recovery). However, the western blot results did not show significant changes in the phosphorylation of GSK-3 β in the postconditioned hearts. This could be due to relatively late sampling of hearts after onset of reperfusion (30 minutes), while the inhibition of GSK-3 β , and thus the protective effect, took place earlier in reperfusion. Akt seemed not to be involved in the interaction between Ang II and postconditioning. Darling *et al.* found that ERK1/2 rather than PI3-kinase/Akt is involved in the protection achieved by postconditioning (67), which is supported by the present findings. Phosphorylation of ERK 1 and ERK 2 was quantified separately in this study. Few studies report differential regulation of ERK1 and 2, but Indrigo *et al.* recently proposed that although they are activated by similar stimuli in many situations, they may have different roles (68). A schematic

drawing of the proposed mechanism in which postconditioning and Ang II interact is shown in figure 3.

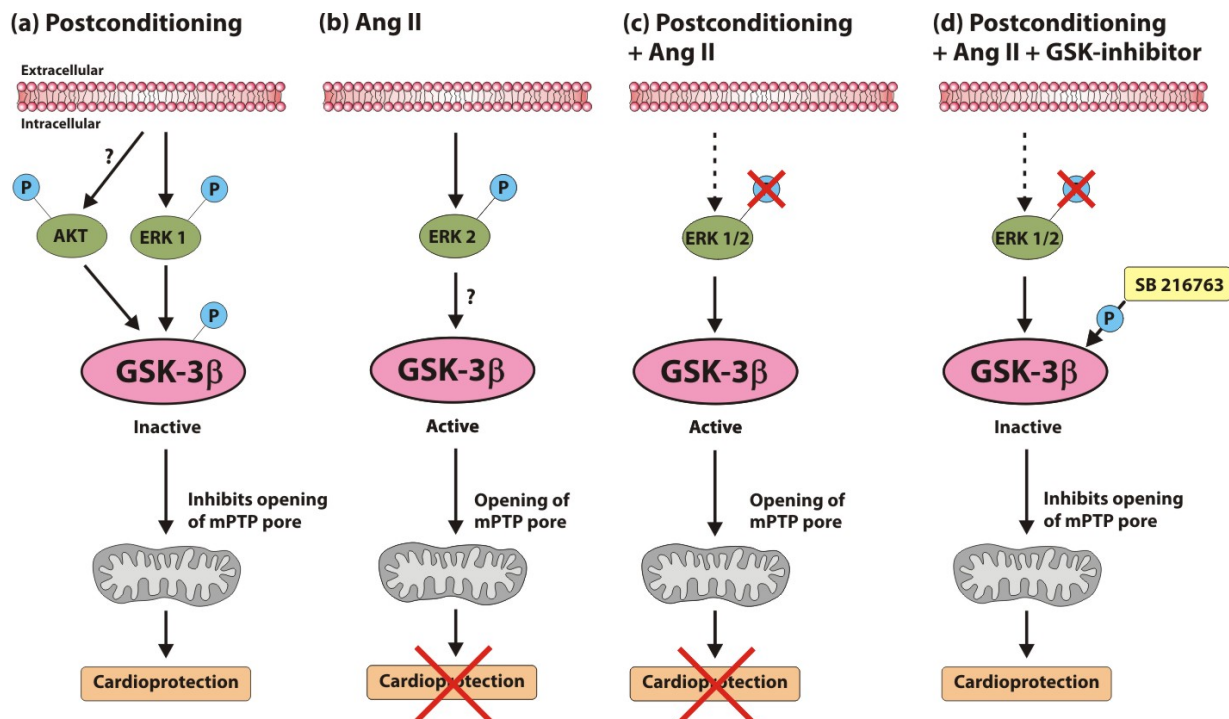


Figure 3 Proposed signal pathway for cardioprotection induced by postconditioning (a), effect of Ang II alone (b), cardioprotection blocked when Ang II is present (c) and restored when GSK-inhibitor (SB 216763) was added (d). ERK: extracellular signal-regulated kinase, GSK: glycogen synthase kinase, Ang II: angiotensin II.

Studies of time-dependent phosphorylation pattern of ERK 1 and 2 during Ang II exposure and postconditioning might give more information about the differential regulation. In hearts exposed chronically to Ang II or Iso the overall trend was that sham hearts were more protected against post-ischemic diastolic dysfunction. Post-ischemic recovery of function was significantly impaired with a marked diastolic dysfunction after chronic high-dose Ang II in combination with marked remodelling and no hypertrophy.

The role of gender

Two protocols in the present thesis involved comparison of the response to ischemia-reperfusion between male and female hearts. Interestingly, there was a significant difference in ischemic contracture development between male and female hearts in general in the protocol using Wistar

rats (Paper IV). This could probably be explained by differences in myocardial calcium handling between males and females under the prevailing conditions (69).

5.3 Effects on extracellular collagen

Fibrosis in the extracellular matrix is an important part of the pathology of chronic heart failure. A considerable part of this thesis therefore focuses on fibrosis in the extracellular matrix (papers II, III and IV). In papers III and IV we found that pregnancy and female gender protected against fibrosis induced by Ang II and Iso, respectively. In fact, stimulation of oestrogen receptors- β has been reported to prevent cardiac fibrosis (70). The post-ischemic diastolic dysfunction in animals treated with Ang II or β -adrenergic stimulation was associated with increased deposition of collagen fibres (paper II, III and IV). Fibrosis of various tissues, including the heart, is regulated by Ang II and transforming growth factor (TGF)- β (26). The increase in expression of fibrosis related genes and the amount and thickness of collagen fibres in Ang II treated hearts in paper II support the notion that Ang II is important in regulating fibrotic changes. Further proteomic analysis would be necessary to determine the changes at the protein level associated with the highly significant alterations in gene expression and the corresponding phenotype, such as fibronectin content (paper II and III).

The role of gender

It has been shown that cardiomyocytes and fibroblasts express functional oestrogen receptors (71) giving rise to oestrogen as a potential contributor to the gender-based difference in susceptibility to heart diseases. In agreement with this, the low dose Ang II-induced fibrosis seemed to be blunted during pregnancy. In parallel, the collagen content and expression of fibrosis-related genes were markedly more enhanced in males than females after Iso treatment, followed by more elevated diastolic pressure in response to ischemic insult, both during and after

ischemia. Although it has been reported that oestrogen affects the renin-angiotensin system by modulating AT1 receptors (72), we found no difference in the expression of the corresponding gene of this receptor (Agtr1- α) between males and females. However, subgroup analysis revealed enhanced expression of fibrosis-related genes in females (Col I, Col III, Fn-1 and Timp-1) in response to high dose Ang II which was not translated into a difference in heart histology. In paper III pregnancy reduced the fibrogenic effects of Ang II in heart tissue, which was also reflected in the mRNA expression of Col I, Col III, Fn-1 and Timp-1. Voloshenyk *et al.* showed that oestrogen treatment attenuated interstitial collagen disorganization and prevented the accumulation of perivascular fibrosis (73). Elevated oestrogen levels during pregnancy might therefore be a part of the mechanism behind the down-regulation of fibrosis (and the altered gene expression) in pregnant hearts treated with Ang II. The most distinct gender difference was, however, observed after hypertrophy induced by β -adrenergic stimulation. In this part of the study fibrosis-related genes and collagen content in heart tissue were significantly higher in males than females. Figure 4 illustrates the hypothesis about how Ang II and Iso induce cardiac fibrosis.

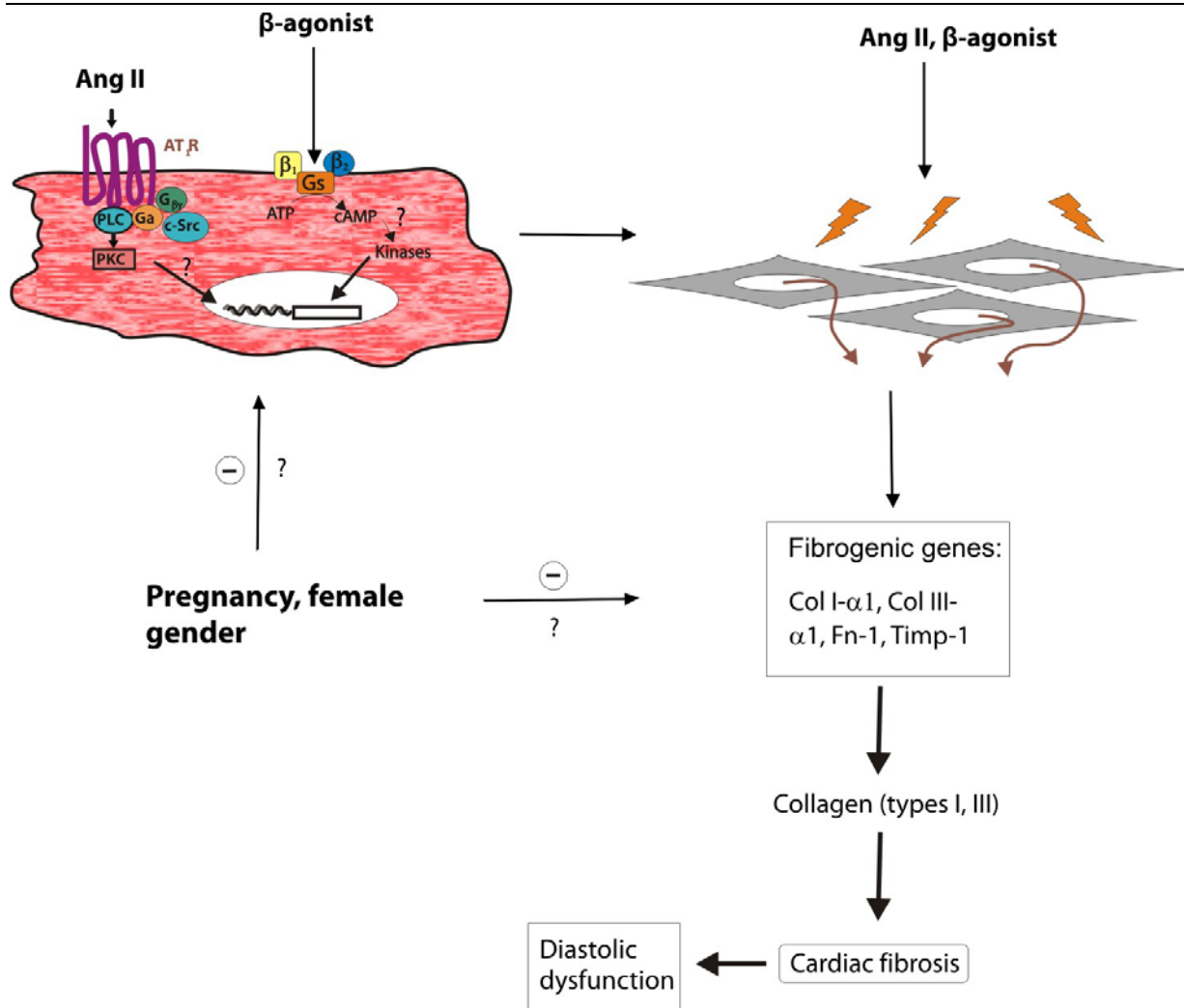


Figure 4 Proposed effects of angiotensin II (Ang II) and isoproterenol (Iso) on cardiac fibrosis. Modified from Nishida *et al.* The EMBO journal (2008) 27, 3104 – 3115.

5.4 Effects on vessel density in ventricular tissue

An additional phenomenon that was assessed in paper III was the change in vessel density in the heart muscle. Angiogenesis is defined as the budding of capillaries that leads to the formation of new micro vessels from pre-existing vascular structures (74). Reduced vessel density in Ang II-treated rats was blunted by pregnancy. This could be due to either an hormonal effect or to augmented blood flow during pregnancy (35), as it has been shown that augmentation of blood flow for example during exercise and in hyperthyroidism stimulates vascular sprouting (74;75).

Several factors have been recognized to play a role in angiogenesis including: fibroblast growth factor, vascular endothelial growth factor (VEGF), platelet-derived growth factor, insulin-like growth factor, transforming growth factor (α and β), tumour necrosis factor α , placental growth factor (PGF) and several others as reviewed by Tabibiazar (74). The mRNA expression for VEGF-A and -B and for PGF was examined but found unchanged, and thus change in the expression of these factors could not be responsible for neither the reduced vessel density after Ang II treatment nor the restoration of this density in pregnant individuals.

5.5 Changes in expression of key genes

Analysis of the mRNA expression of the traditional biomarkers of the failing heart ANF, BNP and TNF- α was performed. After treatment with Ang II, expression of ANF and TNF- α was increased while only ANF expression increased after β -adrenergic stimulation. BNP was not significantly increased after two weeks of high dose Ang II treatment, but it did increase following two weeks treatment with the lower dose. This shows that BNP expression, just as hypertrophy, is dose-dependent with respect to Ang II stimulation.

Both Ang II and Iso treatment tended to down-regulate α -MHC and up-regulate β -MHC. The α -isoform of myosin heavy chain (α -MHC), which has higher shortening velocity, has been shown to be down-regulated (combined with up-regulation of β -MHC) in rodents exposed to pressure overload (76). This shift in myosin heavy chain isoforms could contribute to the cardiac dysfunction observed in Ang II- and Iso-treated animals.

Ankrd-1 has been characterized as an inducible gene that is over-expressed in foetal, early-postnatal and adult heart in response to multiple forms of cardiovascular stress, including pressure overload, chronic ischemia, ischemia-reperfusion injury and heart failure (77). Ankrd-1 increased in the Ang II but not in the Iso-treated animals. This partly supports 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 (77).

We found a slight but significant increase in the expression of the apoptosis-controlling genes p53 and caspase 3 in most of the treated groups, including both Ang II and β -adrenergic stimulation. The p53 transcription factor promotes apoptosis via elevation of caspases and Bax and reduction of bcl-2 (78). Caspase 3 was not increased in Ang II-treated groups in paper III. These hearts were not exposed to ischemia-reperfusion injury. It is known that caspase is activated by ischemia-reperfusion, and inhibition of caspases reduces myocyte cell death induced by myocardial ischemia and reperfusion *in vivo* (79). Thus, an increased level of caspase which could be the case after high dose Ang II and β -adrenergic stimulation may be responsible for increase in ischemia-reperfusion injury.

At the cardiomyocyte level protein kinase C (PKC) isoenzymes are key regulators of cytosolic $[Ca^{2+}]$, contractility and ischemic cell death (80). With respect to ischemic injury it has been proposed that PKC- δ promotes cell injury whereas PKC- ϵ delays injury (81). Therefore, we also used PKC expression as an index to evaluate the susceptibility to cell injury. In agreement with this, expression of isoenzymes PKC- α and PKC- δ in the treated animals increased and PKC- ϵ decreased or tended to decrease. These changes occurred with cardiomyocyte diameter remaining unchanged in Ang II treated animals (paper II and III). At the same time we found increased fibrosis in sections from those hearts. The up-regulation 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). The use of isoenzyme specific PKC inhibitors in the tested models of heart remodelling is warranted to fully understand the role of these PKC isoenzymes (82;83).

The role of gender

In paper III and IV female gender seemed to have beneficial effect on heart which was also visible in the mRNA expression. The enhanced expression of mRNA of fibrosis related genes in males after Iso treatment and the tendency to reduced expression of these genes in pregnant rats contributed to less deposition of collagen fibres in heart tissue of the corresponding groups. When Ang II was given in higher dose the differences between males and females became minimal and even reversed for the some genes as subgroup analysis revealed increase in Col I- α 1, Col III- α 1, 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.

6. Main conclusions

In this thesis different factors that promote or inhibit heart remodelling induced by angiotensin II were studied. The following conclusions can be drawn:

1. The presence of elevated levels of Ang II in the perfusion buffer did not increase myocardial injury during ischemia-reperfusion. However, the ability to protect the heart by ischemic postconditioning was lost. This seemed to be due to blockade of ERK1/2 signalling and GSK3- β inhibition induced by postconditioning. Elevated levels of Ang II, which is seen in untreated cardiovascular disease and in kidney disease, could therefore contribute to limit the outcome of the potential clinical use of postconditioning.
2. Non-compensatory (non-hypertrophic) heart remodelling was triggered by high-dose Ang II. This leads to changes in the contractile apparatus and reduced tolerance to ischemia in the absence of cellular hypertrophy. Increased expression of key genes involved in fibrosis was followed by an increase in interstitial fibrosis in heart tissue, which partly explains the post ischemic diastolic dysfunction.
3. Pregnancy did protect against the detrimental effects of Ang II since cardiac remodelling, fibrosis and reduced vessel density were less prominent in pregnant rats.
4. Iso treatment induced heart hypertrophy in both genders, but increased fibrosis was found only in males. Unlike the situation with exposure to high concentrations of Ang II, female gender did protect against the increase in fibrosis after Iso treatment.

In summary, we can conclude that female gender and the unique female physiological status of pregnancy protect the heart, and provide a better handling of various stress stimuli after moderate Ang II and β -adrenergic influence. When the angiotensin II exposure reaches sub-toxic levels the protection afforded by female gender is lost.

7. Errata

In paper II, figure 2 b there is a misprint in Y-axis text. It should be LVEDP and not LVDP.

In paper II, figure 4 there is a misprint in the legend. Pictures are histological sections (200x) and not (100x).

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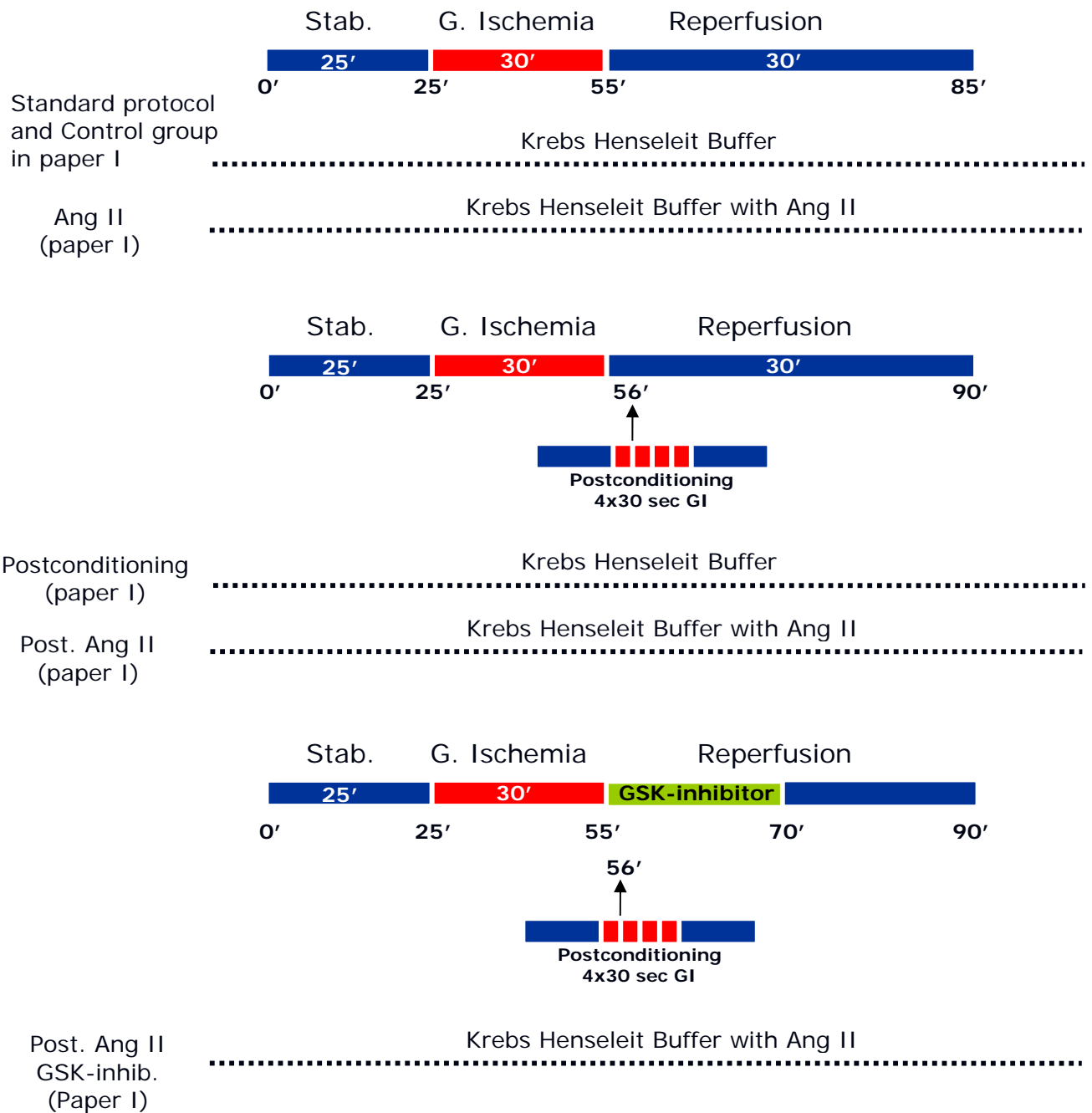
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Appendices

Appendix 1: A schematic illustration of the protocols used in this study. Stab.: Stabilization, Post.: Postconditioning, GSK-inhib.: GSK-inhibitor.



Appendix 2: Primers used for RT-PCR analysis. (Probe from Cyber Green unless otherwise stated)

RAT mRNA	Primer/probe sequence (5'-3')
Housekeeping genes	
<i>GADPH: Glyceraldehyde-3phosphate dehydrogenase</i>	
Forward primer	CTG-CAC-CAC-CAA-CTG-CTT-AC
Reverse primer	CAG-AGG-TGC-CAT-CCA-GAG-TT
Probe	Roche Probe Library #9
<i>B2M: β-2 microglobulin</i>	
Forward primer	TGC-CAT-TCA-GAA-AAC-TCC-CC
Reverse primer	GAG-GAA-GTT-GGG-CTT-CCC-ATT
Probe	AAT-TCA-AGT-GTA-CTC-TCG-CCA-TCC-ACC-G
<i>Cyclo: Cyclophilin</i>	
Forward primer	CTG-ATG-GCG-AGC-CCT-TG
Reverse primer	TCT-GCT-GTC-TTT-GGA-ACT-TTG-TC
Probe	CGC-GTC-TGC-TTC-GAG-CTG-TTT-GCA
<i>HPRT: Hypoxanthine phosphoribosyltransferase</i>	
Forward primer	GAC-CGG-TTC-TGT-CAT-GTC-G
Reverse primer	ACC-TGG-TTC-ATC-ATC-ACT-AAT-CAC
Probe	Roche Probe Library #95
<i>LDHA: Lactate dehydrogenase</i>	
Forward primer	GAT-CTC-GCG-CAC-GCT-ACT
Reverse primer	CAC-AAT-CAG-CTG-GTC-CTT-GAG
Heart function related genes	
<i>ANF: Atrial Natriuretic Factor</i>	
Forward primer	CAA-CAC-AGA-TCT-GAT-GGA-TTT-CA
Reverse primer	CGC-TTC-ATC-GGT-CTG-CTC
Probe	Roche Probe Library #25
<i>BNP: B-type natriuretic peptide</i>	
Forward primer	GTC-AGT-CGC-TTG-GGC-TGT
Reverse primer	CAG-AGC-TGG-GGA-AAG-AAG-AG
Probe	Roche Probe Library #13
<i>α-MHC: α-Myosin Heavy Chain</i>	
Forward primer	CAA-GGC-AAA-CCT-GGA-GAA-AG
Reverse primer	GGG-TAT-AGG-AGA-GCT-TGC-CC
<i>β-MHC: β-Myosin Heavy Chain</i>	
Forward primer	GAG-GAG-AGG-GCG-GAC-ATT
Reverse primer	ACT-CTT-CAT-TCA-GGC-CCT-TG
Probe	Roche Probe Library #95

Ankrd-1: Ankyrin repeat domain-containing protein 1

Forward primer GCTGGAGCCCAGATTGAA
 Reverse primer CTCCACGACATGCCAGT
 Probe Roche Probe Library #76

Agtr 1a: Ang II receptor 1a

Forward primer CAC-CCG-ATC-ACC-GAT-CAC
 Reverse primer CAG-CCA-TTT-TAT-ACC-AAT-CTC-TCA
 Probe Roche Probe Library #53

TNF- α : Tumor Necrosis Factor- α

Forward primer GCC-CAG-ACC-CTC-ACA-CTC
 Reverse primer CCA-CTC-CAG-CTG-CTC-CTC-T
 Probe Roche Probe Library #119

eNOS: Endothelial Nitric Oxide Synthase

Forward primer TGA-CCC-TCA-CCG-ATA-CAA-CA
 Reverse primer CGG-GTG-TCT-AGA-TCC-ATG-C
 Probe Roche Probe Library #5

iNOS: Inducible Nitric Oxide Synthase

Forward primer ACCATGGAGCATCCCAAGTA
 Reverse primer CAGCGCATAACCACTTCAGC

PKC- α : Protein Kinase C- α

Forward primer CAA-GCA-GTG-CGT-GAT-CAA-TGT
 Reverse primer GGT-GAC-GTG-CAG-CTT-TTC-ATC

PKC- δ : Protein Kinase C- δ

Forward primer TCA-AGA-ACC-ACG-AGT-TCA-TCG
 Reverse primer GCA-TTG-CCT-GCA-TTT-GTA-GC

PKC- ϵ : Protein Kinase C- ϵ

Forward primer CGT-CAC-TGA-TGT-GTG-CAA-TG
 Reverse primer TCG-AAC-TGG-ATG-GTG-CAG-TTG

Fibrosis related genes**Col I- α 1: Collagen type I- α 1**

Forward primer CAT-GTT-CAG-CTT-TGT-GGA-CCT
 Reverse primer GCA-GCT-GAC-TTC-AGG-GAT-GT
 Probe Roche Probe Library #92

Col III- α 1: Collagen type III- α 1

Forward primer TCC-CCT-GGA-ATC-TGT-GAA-TC
 Reverse primer TGA-GTC-GAA-TTG-GGG-AGA-AT
 Probe Roche Probe Library #49

Fn-1: Fibronectin

Forward primer	CAG-CCC-CTG-ATT-GGA-GTC
Reverse primer	TGG-GTG-ACA-CCT-GAG-TGA-AC
Probe	Roche Probe Library #76
<i>Timp1: Tissue inhibitor of metallopeptidase</i>	
Forward primer	CAG-CAA-AAG-GCC-TTC-GTA-AA
Reverse primer	TGG-CTG-AAC-AGG-GAA-ACA-CT
Probe	Roche Probe Library #76
Apoptosis related genes	
<i>P53: Tumor suppressor gene(TP53)</i>	
Forward primer	GTT-AGG-GGG-TAC-CTG-GCA-TC
Reverse primer	CGA-CTG-TGA-ATC-CTC-CAT-GA
Probe	Roche Probe Library #3
<i>Casp-3: Apoptosis related cysteine protease; caspase 3</i>	
Forward primer	CCG-ACT-TCC-TGT-ATG-CTT-ACT-CTA
Reverse primer	CAT-GAC-CCG-TCC-CTT-GAA
Probe	Roche Probe Library #80
<i>Bcl-2: B-cell leukemia/lymphoma 2</i>	
Forward primer	GTA-CCT-GAA-CCG-GCA-TCT-G
Reverse primer	GGG-GCC-ATA-TAG-TTC-CAC-AA
Probe	Roche Probe Library #75
Angiogenesis related genes	
<i>VEGF-A: Vascular Endothelial Growth Factor-A</i>	
Forward primer	CAA-GCC-AAG-GCG-GTG-AGC-CA
Reverse primer	TCT-GCC-GGA-GTC-TCG-CCC-TC
<i>VEGF-B: Vascular Endothelial Growth Factor-B</i>	
Forward primer	ACC-AGA-AGA-AAG-TGG-TGT-CAT-G
Reverse primer	TGA-GGA-TCT-GCA-TTC-GGA-CTT-G
<i>PGF: Placental Growth Factor</i>	
Forward primer	GTG-TGG-GGC-CGC-AGC-TAC-TG
Reverse primer	AGC-GCC-ACA-CAG-TGC-AGA-CC

Paper I

Paper II

Paper III

Paper IV



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