Hormone Replacement Therapy and cardiovascular disease: Differential effects of the regimes Medroxyprogesterone Acetate plus 17β- estradiol and unopposed 17β- estradiol.

Dissertation zur Erlangung des
Naturwissenschaftlichen Doktorgrades
Der Bayerischen Julius-Maximilians-Universität Würzburg

vorgelegt von

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aus La Paz, Bolivia

Würzburg 2008

eingereicht am: …….....

Mitglieder der Promotionskommission:

Vorsitzender: .....

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Tag des Promotionskolloquiums: .....

Doktorurkunde ausgehändigt am: .....

To my family.

The present study was performed under the supervision of Dr. Theo Pelzer in the laboratory of Molecular Cardiology, Medicine Clinic and Policlinic I (Director Prof Dr. G. Ertl) at the Julius-Maximilians-University of Würzburg - Germany. The financial support for my PhD studies was provided by the German Academic Exchange Service ("DAAD", Promotionstipendium) and by the University Clinic and Policlinics in Würzburg (Ausbildungsstipendium).

Eidesstattliche Erklärungen

Hiermit erkläre ich ehrenwörtlich, dass ich die vorliegende Dissertation in allen Teilen selbständig angefertigt und keine anderen als die von mir genannten Quellen und Hilfsmittel verwendet habe.

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Hiermit bewerbe ich mich erstmals um den akademischen Grad *„Doctor rerum naturalium"* (Dr. rer. nat.) durch ordentliche Promotion an der Bayerischen Julius-Maximilians-Universität Würzburg.

Würzburg, den .. 2008

Anahi-Paula Arias-Loza

Acknowledgements.

I would like to address my deep gratitude to my supervisor Dr. Theo Pelzer, I cannot begin to adequately thank him for all his motivating discussions, scientific advices and moral support. He was uniformly generous and kind to show the most heroic reserves of patience, in teaching this biologist cardiology.
I would like to thank Professor Ertl for giving me the opportunity to do my Ph.D. work in the "Universtity Klinik Wuerzburg", where I enjoyed a nice and motivating enviroment.
I have received valuable help from numerous collegues that provided me with time, experience and personal support for the conclussion of this work. Therefore I take this oportunity to thank all of those who helped me. I am specially indebted to Dr. Kai Hu for being extremely patient with the unexperienced student that I was, without his surgical experience, enthusiasm and cooperation the conclussion of this work would not have been possible. I am also gratefull to Charlotte Dienesch, Barbara Bayer, Helga Wagner, Natalie Burkard, Dr. Matthias Hallhuber, Jenny Mück, Claudia Gebhardt, Melanie Muehlfelder, Dr. Garnet Bendig, Dr. Kai Schuh and Dr. Oliver Ritter, for sharing a nice and motivating working environment with me, for giving me good advices and a smile when I needed it. A special thank go to Dr. Virginija Jazbutyte who taugh me all the important molecular protocols during my beging in the lab. I was so lucky to have all of them as my collegues. I am also in debt to our animal house keeper Evi Reichert for doing the impossible possible and making some extra space for more animals or more metabolic cages during intense times, for her friendship and all her help when I was thinking that there were to many animals for me thank you. I would also like to thank to the student Vijayakumar Govindaray for showing me that I actually know something to explain to someone else.
I am grateful to the Endocrynology Department of the University Wuerzburg for their patience and technical support for the RIAs, as well as to Dr. Thomas Renné for placing his fluorescence microscope at my disposal. I would also like to thank to the personal from the working groups of Dr. Johann Bauersachs and Dr. Thomas Quaschning for the fruitfull collaborations that resulted in the inclussion of vasomotor studies in the AST cardiovascular effects paper. I am also in debth to Dr. Susan Elmore and Dr. Robert Maronport of the NIH for their collaboration with the kidney histopathology analysis.
I would also like to thank Schering AG who kindly provided with the drospirenone compound for treatment of the AST rats.
My special thanks go to Dr. Rong xue Wu for her friendship and personal support throughout the whole period of this work, we share so many nice moments and tears together, she will allways be in my heart. For their priceless friendship I would like to thank Dr. Laura Calvillo, Dr. Carmem Sartorio, Dr. Guadalupe Camarero, Dr. Maria Stoimenova, Dr. Sarah Zonnur, Agnieska Stefanska and Dr. Catia Andreassi for being my family away from home.
I am also grateful to Dr. Adriana Rico, Dr. Aneth Vasquez, Ingrid Preciado, Joselin Vana and Carlos Valderrama for their lifetime friendship, for dealing with fustrating times, for understanding my silences and my unstoppable mounth during my crisis, even away from me, thank you for just being there.
Above all my profoundest thanks to my parents Martha and Freddy and my sister Karina for their constant, inestimable understanding and emotional support despite the distance, you made me the person who I am.

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TABLE OF CONTENTS

FIGURES AND TABLES.

Summary.

A rising percentage of women with risk factors for cardiovascular disease (CVD) reach menopause and experience postmenopausal symptoms. In consequence they require assessment concerning the appropriate combination and safety of a hormone replacement therapy. Clinical trials using the combination of equine estrogens and medroxyprogesterone acetate (MPA) reported an increased risk of thromboembolic events and no cardiovascular protective effects in women receiving this type of hormone replacement therapy. However unopposed estradiol and different regimes estrogens/progestins in vitro and in animal studies have proved to be beneficial for the cardiovascular system. Thus it is possible that the negative outcomes of the clinical trials are an exclusiv feature of the regime equine estrogens plus MPA. The present study was initiated to evaluate the cardiovascular effects and possible mechanism of damage of the regime MPA plus 17β-estradiol in comparison to unopposed 17β-estradiol during cardiac disease. The role of 17β-estradiol and MPA during left ventricular dysfunction and chronic heart failure was studied in female Wistar rats that received myocardial infarction. After 8 weeks of treatment the combination of MPA plus estradiol aggravated left ventricular remodelling and dysfunction as judged by increased heart weight, elevated left ventricular end diastolic pressure and decreased left ventricular fractional shortening, effects that were accompanied by increase left ventricular oxidative stress and expression of rac 1 and p67phox regulatory subunits of the NADPH oxidase. In contrast ovariectomy as well as 17β- estradiol supplementation conferred neutral effects on cardiac function and remodelling post myocardial infarction. Suggesting that the aggravating symptoms of the regime MPA plus 17β –estradiol are inherent to this pharmacological regime and are not a class effect of the progesterone receptor ligands and are neither due to inhibition of estradiol beneficial effects.

Considering that aldosterone plays an important role in the development and aggravation of cardiovascular disease the cardiovascular effects of MPA plus 17β –estradiol was studied in a model of mineralocorticoid receptor activation and compared to the effects of regimes based in drospirenone, a new progestin with antimineralocorticoid properties. The complex pattern of cardiovascular injury in ovariectomized Wistar rats induced by 8 weeks of continuous chronic aldosterone infusion and high-salt diet was significantly attenuated in sham-ovariectomized rats and by coadministration of 17 β-estradiol in ovariectomized animals. The beneficial role of 17 β-estradiol on blood pressure, cardiac hypertrophy, vascular osteopontin expression and perivascular fibrosis was completely abrogated by coadministration of MPA. In contrast, drospirenone was either neutral or additive to 17 β-estradiol in protecting against aldosterone salt-induced cardiovascular injury and inflammation. Taking into account that the kidney plays a major role for the development and aggravation of hypertension a further characterization of fluid balance, renal morphology and renal gene expression in the aldosterone salt treated rats was conducted. Aldo-salt treatment resulted in remnant kidney hypertrophy without structural damage, effects that were not modified by 17 β-estradiol. However combination of MPA with 17 β-estradiol enhanced kidney hypertrophy, fluid turnover, renal sodium retention and potassium excretion and was associated with increased renal ENaC expression, extensive renal lesions, tubular damage and enhanced p67phox expression and protein tyrosin nitrosylation. Different to the protective effects of drospirenone that included a complete blockade of kidney hypertrophy and sodium retention and enhanced renal expression of angiotensin II type-2 receptors. Therefore the loss of 17 β-estradiol cardiovascular beneficial effects and the renal harmful effects in the aldosterone salt treated rats receiving MPA can not be extrapolated to other progestins. Indeed drospirenone conferred protective effects due to its antimineralocorticoid properties.

In conclusion, the choice of specific synthetic progestins has profound implications on the development of cardiovascular and renal injury; MPA aggravated cardiac disease, which contributes to explain the adverse outcomes of clinical trials on the prevention of cardiovascular disease by combined estrogen and MPA treatment.

Zusammenfassung.

Eine zunehmende Zahl postmenopausaler Frauen mit kardiovaskulären Risikofaktoren leidet unter menopausalen Beschwerden. Diese Patientinnen benötigen daher eine Beratung hinsichtlich der Sicherheit einer post-menopausalen Hormonersatz-THerapie da klinische Studien ein gehäuftes Auftreten thromboembolischer Ereignisse unter einer kombinierten Gabe von Östrogenen und Medroxyprogesteron Acetat (MPA) nachgewiesen haben. Zudem war eine Protektion gegen kardiovaskuläre Erkrankungen, die nach der Menopause gehäuft auftreten, nicht nachweisbar. Im Gegensatz hierzu belegt eine Vielzahl von experimentellen Studien eine günstige Wirkung einer alleinigen Östrogensubstitution. Es erscheint daher möglich, dass die ungünstigen Wirkungen einer Hormonersatz-Therapie im Wesentlichen auf die Progesteron Komponente zurückzuführen ist, welche bei Frauen mit intaktem Uterus jedoch erforderlich ist um einer Endometriumhyperplasie vorzubeugen. Wenige experimentelle Studien haben bislang die Wirkungen unterschiedlicher, synthetischer Progestine im Herz- Kreislaufsystem untersucht. In der vorliegenden Studie war es daher erstmalig die Wirkung einer alleinigen Gabe von Östradiol mit einer kombinierten Applikation von Östradiol und MPA nach experimentellem Myokardinfarkt bei weiblichen Ratten verglichen. Die Kombination von Östradiol und MPA, nicht jedoch Östradiol allein oder natives Progesteron, führte zu einer Verschlechterung myokardialer Umbauprozesse (remodeling) welches in einer weiteren Verschlechterung der linksventrikulären Pumpfunktion resultierte. Diese sehr ungünstigen funktionellen Effekte waren mit einer vermehrten Generierung freier Sauerstoffradikale durch NADPH Oxidasen verbunden. Diese Beobachtungen unterstützen die Hypothese, dass MPA möglicherweise einen wesentlichen Anteil an den ungünstigen Wirkungen einer Hormonersatz-Therapie hat. Hierbei handelt es sich nicht um einen Klassen-Effekt aller Progestine sondern um eine spezifische Eigenschaft von MPA.

Mineralokortikoid-Rezeptoren, welche durch Aldosteron und durch MPA aktiviert werden, besitzen auch eine wesentliche Funktion für pathologische Umbauprozesse im Myokard und im Gefäßsystem. Daher wurde in einem weiteren Ansatz die Frage untersucht, ob Östrogene und unterschiedliche synthetische Progestine (MPA, Drospirenon) aldosteron-gesteuerte, pathologische Umbauprozesse im Herzkreislaufsystem möglicherweise gegensinnig beeinflussen. Nach 8-wöchiger Aldosteron-Salz Behandlung zeigten zuvor normotensive Wistar Ratten eine arterielle Hypertonie, eine Myokardhypertrophie sowie ausgeprägte, perivaskuläre inflammatorisch-fibrosierende Veränderungen im Myokard und der Aorta. Diese wurden durch eine Ovarektomie verstärkt und durch die Substitution von Östradiol gemindert. Die Kombination von Östradiol und MPA, nicht jedoch von Östradiol und Drospirenon führte zu einer massiven Verstärkung des kardiovaskulären remoidelings. Gleichsinnige Beobachtungen wurden auch an den Nieren der Tiere gemacht; MPA, nicht jedoch DRSP, induzierte eine massive Nephropathie mit extensiver Glomerulosklerose, inflammatorischen Infiltraten und einer stark ausgeprägten Tubulo- und Vaskulopathie. Die ungünstigen Effekte von MPA waren auch hier wiederum mit einer verstärkten Expression und Aktivität der NADPH Oxidase verbunden. An der Niere MPA behandelter Tiere wurde zudem eine verstärkte Expression des endothelialen Natrium Kanals (ENaC) nachgewiesen, welche als kausaler Mechanismus der unter MPA exzessiv gesteigerten Natriumresorption in Betracht kommt. Drospirenon, welches neben seiner Wirkung als Progestin auch eine starke anti-mineralokortikoide Wirkung besitzt, führte in Kombination mit Östradiol zu einer kompletten Normalisierung des kardiovaskulären und renalen Phänotyps.

Zusammenfassend besitzt die Wahl eines spezifischen, synthetischen Progestins (MPA, DRSP) einen hohen Stellenwert für die Sicherheit und Effizienz einer Hormonersatz-Therapie

bei Patientinnen mit bereits bestehenden Herz- Kreislauferkrankungen zu besitzen. Neuere Progestine (DRSP) mit einem genau definierten Wirkungsspektrum könnten auch klinisch zu einer besseren Verträglichkeit einer HRT führen und die protektiven Wirkungen von Östrogenen unterstützen. Hierzu sind weitere klinische und experimentelle Untersuchungen erforderlich.

Chapter 1: General Introduction.

1.1. Prevalence of Cardiovascular disease

Cardiovascular disease (CVD) threatens to eclipse infectious disease as the major cause of death worldwide, with the prevalence rising as population age and western diets are emulated. (Benjamin Ivor 2005). Estimates from the year 2003 are that 71 millon Americans have one or more forms of cardiovascular disease, of them 65 millon have high blood pressure and 13 millon coronary artery disease. In Europe CVD causes 4 million deaths each year being responsible for nearly half of all deaths (48%). CVD is the main cause of death in women in all countries of Europe (55% of deaths) and is the main cause of death in men in most countries (43 % of deaths). Each year CVD kill 430.000 & 910.614 people in Europe and America respectively, being not only a major threat to individual lives and their quality of life but also a major economic burden due to the increased expenses in health care. (AHA 2003; Rayner M & Petersen S- 2000).

1.2. Gender aspects of cardiovascular disease

Women live longer and develop cardiovascular disease at an older age than men. CVD is not common in premenopausal women and raises its incidence sharply after the menopause transition. (Framingham Heart Study ; American National Heart, Lung, and Blood Institute; American National Health and Nutrition Examination Survey & AHA official web page). Blood pressure is lower in premenopausal women compared to age-matched men, and rises following menopause (Dubey 2002; Sader 2002). Cardiac contractility is also higher in healthy women than in age matched men, and hormone replacement withdrawal decreases cardiac performance in women (Levy 1990; Merz 1996). Gender differences are also present in both inherited and acquired heart muscle

disease. Some familial hypertrophic cardiomyopathies are more severe in males than in females. (Geitefer 1996; Stefanelli 2004). Hearts of women with aortic stenosis had better preserved contractile function than those of men with the same disorder (Douglas 1998). In heart failure, female gender is associated with improved cardiac function and survival (Adams 1999; Ghali 2003). Hormone dependent gender differences also exist in vascular function. Estrogens cause vasodilatation through both rapid increases in nitric oxide production and through the induction of endothelial nitric oxide synthase (eNOS) expression. (Mendelsohn, 1999; Edwards 2005; Chambliss 2002). Vasodilatation and blood pressure are both affected by fluctuations in circulating estrogens levels during the menstrual cycle, pregnancy, or 17β -estradiol supplementation. (Dubey, 2002; Reckelhoff 2001). These differences have been attributed to the loss of female sex steroid hormones at the time of menopause, suggesting a possible beneficial effect for hormone replacement therapy against cardiovascular disease. In addition, short-term estrogens administration in men has little effect on vascular relaxation, whereas long term administration improves vasodilatation (Sader 2002) suggesting that both genders respond similarly to female sexual hormones.

1.3. Hormone Replacement therapy.

Ovarian steroid hormones, estrogens and progesterone, maintain reproductive function and modulate target-cell activity via estrogen and progesterone receptors in the cardiovascular, neural, immune, gastrointestinal, and musculoskeletal systems. During pregnancy, serum estrogen and progesterone levels increase not only to provide a suitable uterine environment but also to adapt maternal metabolism and cardiovascular function to the presence of the growing fetus. Natural menopause, which occurs in women at an average age of 51 years, results in diminished ovarian hormone secretion, which results in

decreased of bone density, and increased vasomotor flushes, vaginal dryness and urinary symptoms.

In 1998 there were more than 477 million postmenopausal women in the world, with approximately 9% expected to live to age 80. By 2025, the number of postmenopausal women is expected to rise to 1.1 billion with a life expectancy, in developed countries, of 82 years. (Utian 1999) consequently over one third of a woman's life takes place after menopause. In the modern society the female population is also a carrier of a number of risk factors for heart disease such as overweight, high cholesterol, diabetes, high blood pressure and obesity. Accordingly, increasing numbers of women under risk of cardiovascular disease will face the question of whether or not to initiate hormone replacement therapy (HRT) to treat menopausal sympthoms. Therefore it is necessary to optimize the safety of HRT. Although estrogens and progesterone have been prescribed for postmenopausal women over the last 60 years the cardiovascular effects of different regimes and comparison of unopposed estrogens vs. combination with a progestin are still understudied.

1.4. Hormone Replacement therapy and vascular disease.

In 1985 the Framingham Heart Study reported a nearly 2-fold increased risk of cardiovascular disease to be associated with hormone replacement therapy over an 8 year period for 1234 postmenopausal women aged ≥ 50 years. (Genant HK,1982) This observation appeared short before the first Nurses Health Study reported a 50% lover risk of CAD (Coronary Artery Disease) in ever vs. never-user of estrogens in 32.317 postmenopausal women aged 30 to 55 years. (Stampfer MJ, 1985). Several other cohort studies corroborated the Nurse's Health Study finding of reduced CAD risk, including the Lipid Research Clinics follow-up Study of 2270 women aged 40 to 69 years who were followed for 8,5 years (Bush TL, 1987), the Leisure World Study of 8841 women aged 40

to 101 years who were followed for 5,5 years (Petitti DB,1987) and a Kaiser Permanente program cohort of 6093 women aged 18 to 54 years who were followed for 10 to 13 years (Henderson BE,1988). Accumulating evidence pointed towards a substantial reduction in CVD risk among women using estrogen replacement. However, women who took estrogens after menopause were more likely to be white, educated, upper-middle class, and lean and thereby at lower risk of heart disease than women who did not use hormone replacement. (Barrett-Connor E,1991). In 1992 Grady et al. (Grady D, 1992) reported in a meta-analysis study that postmenopausal hormone use was associated with approximately 33% lower rate of fatal heart disease compared to non-users.

The 1995 PEPI clinical trial findings of favourable lipoprotein changes in women assigned to CEE (Conjugated Equine Estrogens) with or without a progestin reinforced the belief that estrogens reduce the risk of CVD but also showed that the addition of cyclic or daily MPA reduced the beneficial effect of estrogens on HDL cholesterol (The writing group of the PEPI trial, 1995). The first large clinical trial specifically designed to evaluate hormone therapy for the prevention of CAD was the NIH-funded Heart and Estrogens/Progestin Replacement Study (HERS), which randomly assigned 2763 postmenopausal women (mean age 67 years) with documented CAD to daily CEE 0.625 mg plus MPA 2.5 mg or placebo for an average of 4,1 years. The HERS trial found no overall difference in the primary outcome (nonfatal MI and CAD death), despite significant lowering of low density lipoprotein and total cholesterol and an increase of HDL cholesterol. However in the first year there was a statistically significant excess risk of coronary events in the CEE plus MPA arm (Hulley S, 1998). It was proposed that, for women who already had coronary atherosclerosis estrogen substitution was started to late to do any good. The NIH-funded WHI (Women's Health Initiative) trial was designed to evaluate primary prevention in postmenopausal women receiving combined HRT or unopposed estrogen. In 2002 the WHI arm of combined estrogens and progestin (MPA),

stopped 3 years early, because, compared with placebo the risks of CAD, stroke, pulmonary emboli and breast cancer exceeded the benefits (e.g. prevention of hip fracture) in women assigned to CEE 0.625 mg/day plus daily MPA 2.5 mg/day (Roussow JE,2002). A year and a half later, the WHI arm of only CEE was also stopped early due to an increased incidence of stroke in women assigned to CEE compared with placebo. (Anderson GL, 2004). A puzzling aspect of the WHI study was the high drop out rate of 42% in the estrogens plus progestin (MPA) group that, over time, exceeded the design projections (Neves-E-Castro, 2002). In parallel two randomized trials lasting 2.8 and 3.2 years reported neutral effects of estrogens plus progestins on progression rate of atherosclerosis in the coronary arteries. (Herrington DM, 2000; Waters DD, 2002). Importantly, a two year trial of unopposed estradiol in young postmenopausal women reported slower progression of carotid atherosclerosis with estradiol substitution (Hodis HN, 2001). Differences in the design and results in diverse clinical trials and arms of unoppossed vs. combined estrogens suggest that the cardiovascular effects of estrogens also depend on age and on the addition of a progestin.

1.5. Steroidogenesis.

Estrogens and progestins belong to the steroid hormone family. As other members of this family they are derivatives of cholesterol and are synthesized by a variety of tissues, but most importantly by the adrenal glands and gonads. The synthesis of these hormones is under tight control, with short and long negative feedback loops that regulate their synthesis and secretion. The biosynthesis of steroid hormones requires several oxidative enzymes located in both mitochondria and endoplasmic reticulum. The first and rate-limiting step in the synthesis of all steroid hormones is the conversion of cholesterol to pregnenolone. Pregnenolone is generated on the inner membrane of the mitochondria and then shuttled back and forth between mitochondria and endoplasmic reticulum for further

enzymatic transformations that are involved in synthesis of derivative steroid hormones. Pregnenolone itself is not a hormone, but is the immediate precursor for the synthesis of all steroid hormones as illustrated in figure 1. Newly synthesized hormones are rapidly secreted from the cell, with little if any intracellular storage. Following secretion all steroids bind to some extent to plasma proteins. This binding is often of low affinity and non-specific but some steroids are transported by specific binding proteins, which affects their pharmacological half life and rate of elimination. Steroid hormones are typically eliminated by inactivating metabolic transformations and excretion in urine or bile. (Bowen R.A. 2001).

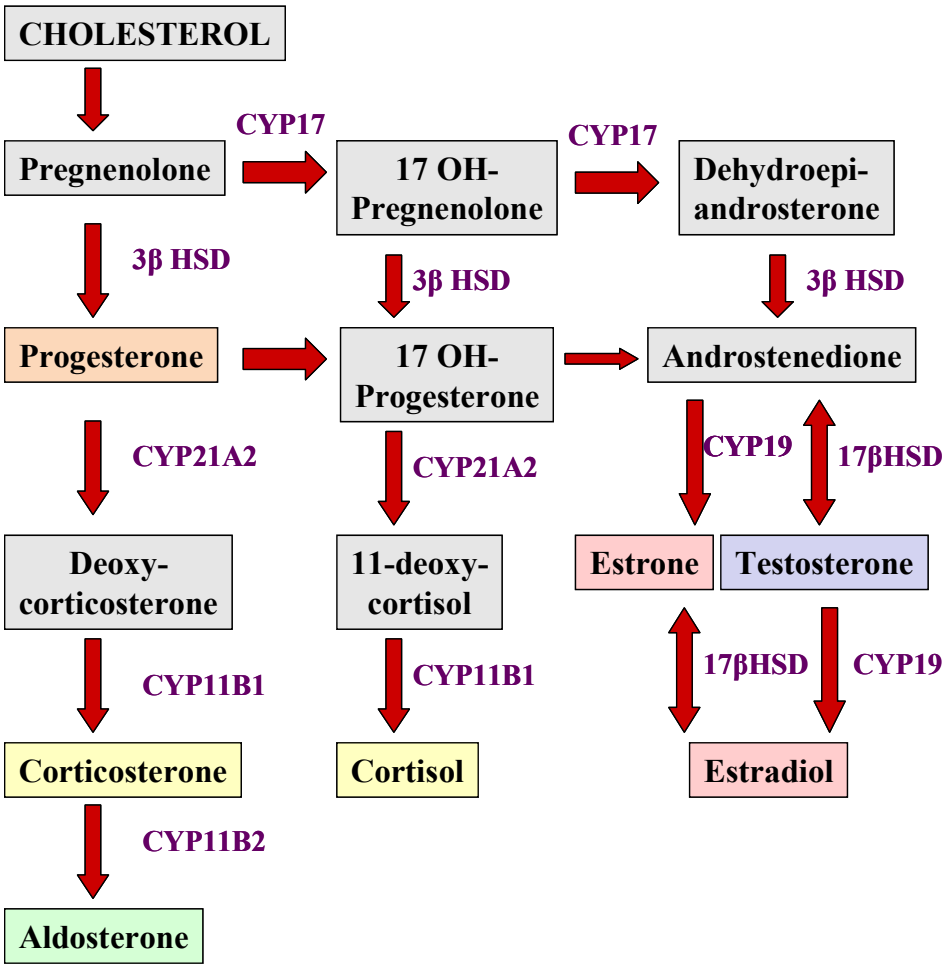

Figure 1. Synthesis of various steroid hormones from cholesterol. In purple Enzymes that catalyze the conversion of one steroid into the next steroid. Structures in grey represent precursors of active hormones. In colour steroid hormones with major physiological relevance, including in orange progestogens; in green mineralocorticoids (aldosterone being the most prominent); in blue androgens; in yellow glucocorticoids and in pink estrogens (Modified from: Bowen R.A. 2001).

1.6. Estrogens.

Estrogens promote mainly proliferation and growth of specific cell types of the body and are responsible of the development of most of the secondary sexual characteristics in women. In non gestating women, only the ovaries secrete relevant amounts of estrogens but the suprarenal cortex also secrets small amounts of estradiol. 17 β–estradiol is the

predominant estrogen secreted by the ovaries. Estrone is mainly formed in peripheral tissues by modification of androgens, while estriol appears as the oxidation product of 17β–estradiol and estrone in the liver and is a very weak estrogen. The pharmacological potency of 17β-estradiol is 12 times higher than the one of estrone and 80 times than the one of estriol. (Guyton 1998). Estrogens used for estrogen replacement therapy can be classified based on their chemical structures into: steroidal and non-steroidal ligands. Steroidal estrogens (such as estrone, estradiol, estrone-sulfate, equilin, equilin-sulfate and ethinyl estradiol) contain the basis 4-ring 17-carbon steroid nucleus (gonane), whereas non steroidal estrogens (such as tamoxifen, raloxifen and tibolone) do not have this common structural feature. In Europe, 17 β–estradiol (E2) is the most commonly prescribed estrogen, whereas in the US conjugated estrogens (CEE) are most frequently used for the treatment of post-menopausal symptoms. CEE was first developed in 1941 by Wyeth-Ayerst (Premarin) and is derived from pregnant mares' urine and contains several different estrogens. (Shoham Z., 2004).

Estrogens mediate gene transcription through the activation of 2 receptors: ER-α and ER-β. Both ERs belong to the nuclear receptor (NR) gene family of transcription factors, which show an evolutionary and functionally conserved structure. The classical action of NR involves binding of the liganded receptor to cis-regulatory DNA elements in the promoter region of target genes to influence transcription rate. Phylogenetic analysis and functional characterization of estrogen receptors permitted its classification into the steroid receptor subgroup of NR (Type 1 receptors). This subgroup consists of the receptors for glucocorticoid, mineralocorticoids, progesterone, androgens and estrogens (GR, MR, PR, AR and ER, respectively). These receptors act as homodimers or heterodimers and rely on ligand binding for efficient DNA-binding and transcriptional activity. (Petterson K, 2001). ER-β is 96% identical to ER-α in its DNA binding domain,

but lacks homology (18%) in the N-terminal transactivation domain. This lack of homology led to speculation that the two ERs may differentially regulate various genes.

In contrast to the findings of randomized controlled trials of HRT in humans, a cardiovascular benefit of estrogens is strongly supported in animal models. Cumulating observations suggest that estrogens are atheroprotective and have effects in vascular cells, as well as in the myocardium, depending in the relative expression of ER-α and ER-β. Further demonstration that estrogen receptors play a role for myocardial health is the observation that ER-α knock out male mice develop severe myocardial damage after ischemia-reperfusion injury. (Zhai 2000). ER-α was also involved in the protective effects of estrogens on injured blood vessels by promoting re-endothelialisation, inhibiting smooth muscle cell proliferation and matrix deposition and attenuating atherosclerotic plaque progression. (Brouchet L., 2001; Pare G., 2002; Hodgin J., 2002; Egan K., 2004). In addition ER-β is required for normal vasodilatation and blood pressure maintenance in both, male and female mice because the loss of ER-β causes substantial hypertension in both genders. In addition, ER-β knock out mice show increased mortality and an aggravation of heart failure after myocardial infarction (Pelzer 2005).

A strong argument for the atheroprotective effect of estrogens is the consistent observation that estrogen treatment inhibits atherosclerosis plaque formation in hypercholesterolemic rabbits and monkeys. The magnitude of protection varies from a 35% to an 80% reduction in lesion size or cholesterol content in aortic and coronary arteries. (Hodgin J, 2002; Hanke H, 1996; Haines CJ, 1999; Bjarnason NH, 1997; Buko VU, 2001; Adams MR, 1990; Williams JK, 1990). Mice with a targeted deletion of the apolipoprotein E gene and the low density lipoprotein (Ldlr) develop atherosclerosis under appropriate conditions mimicking human atherosclerosis lesions. (Hodgin J, 2002). Studies of estrogens in these mice have consistently demonstrated an inhibitory effect of estradiol on plaque initiation and progression demonstrating that estradiol targets

atherosclerosis at early stages of lesion development. (Bourassa P, 1996; Elhage R, 1997; Marsh MM, 1997). To determine the relative contribution of each estrogen receptor in the estrogen mediated atheroprotection ER-α and ER-β knock out mice were crossed with Apoe knock out mice and treated with estrogens for 3 months. (Hodgin JB, 2001). Lesion size in estrogen treated ER-α and Apoe double knock out female mice was only slightly but not significant smaller than in control Apoe knock out mice, whereas the plasma lipid-lowering effects were eliminated in ER-α and Apoe double knock out mice. In contrast, estradiol had similar atheroprotective effects in the ER-β/Apoe double knock out mice as in the Apoe knock out mice, suggesting that the remaining ER-α in the ER-β /Apoe knock out may have mediated the beneficial effects. These results demonstrated that ER-α is the major mediator of the atheroprotective effects of estradiol. (Karas RH, 1999).

1.7. Progestins.

The main role of progestins is to modulate the final changes of the uterus and breast for gestation and lactation. Women who didn't had a hysterectomy receive as prescription a combination of progestins and estrogens to eliminate the risk of endometrial cancer by unopposed estrogen effects. The effects of progesterone are mediated by two progesterone receptor (PR), PRA and PRB that are encoded by a single gene under the control of distinct promoters. Both PR isoforms differ by an extended N-terminal region in the PRB (Mulac-Jericevic B, 2004; Sitruk-Ware 2002). In vitro studies show that PRA and PRB homodimers and heterodimers have distinct transcriptional regulatory activities, including PRB-mediated inhibition of PRA and inhibition of estrogen receptor function. (Giangrande PH,1999). Both PR isoforms are present in varying ratios in different tissues and ablation of both PRs or selective deletion of PRA or PRB yields distinct biological effects in mouse reproductive tissues (Mulac-Jericevic B, 2004). The full PR knockout mice (KO) lack the uterine response of decidualization and progesterone does not inhibit

endometrial hyperplasia in PRKO. Additionally, PRKO mice lack full mammary epithelial development. Progesterone effects in the uterus are preserved in the PRBKO, which express only PRA, indicating that PRA is critical for the uterine response to progesterone. PRB seems to be important for mammary gland development as PRAKO mice maintain mammary growth in response to progesterone. In relation to the cardiovascular system female PRKO mice show greater vascular medial hypertrophy and vascular smooth muscle cell (VSMC) proliferation in response to vascular injury than WT mice (Mulac-Jericevic B, 2004).

Progesterone is the main natural progestin but it is poorly absorbed and its bioavailability after oral administration is only 10%. Therefore, several synthetic progestins with better bioavailability have been developed and are available for HRT (Shoham 2004). The most widely used synthetic progestin is MPA (Medroxyprogesterone acetate), an acetylated pregnane derivative of progesterone. In the circulation, MPA is bound non specifically to albumin and undergoes extensive metabolism by hydroxylation and conjugation. Some of the most prescribed progestins partially oppose the beneficial cardiovascular effects of estrogens. Consistently the estrogen plus MPA arm of the WHI trial resulted in worsening cardiovascular effects compared to the unopposed estrogen-only arm. These observations gave rise to the hypothesis that MPA might have blocked or eventually aggravated pre-existing cardiovascular disease. Progestins down-regulate the estrogens receptors in reproductive organs and may hence interfere with some of the genomic actions of estrogens in other organs as well. This hypothesis is supported by the complex vascular phenotype of the PRKO mice and the fact that progesterone receptors are present and functionally active in vascular cells and in cardiac myocytes. (Karas RH, 2001; White, 1995; Perrot-Applanat, 1988; Sitruk-Ware, 2003, Karas, 2001). Importantly, synthetic progestins such as MPA do not only bind the progesterone receptor (PR) but interact also with other nuclear hormone receptors such as the androgen receptor (AR), the

mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). Therefore, the effects of synthetic progestins are variable, complex and different from those of progesterone. (Sitruk-Ware, 2002). This concept is supported by recent studies indicating that enhanced nitric oxide generation in human umbilical vein endothelial cell (HUVECs) exposed to 17β-estradiol is abrogated by MPA but not by progesterone. (Oishi, 2004; Simoncini, 2004). Similarly, progesterone and some of its derivatives, such as the 19-norprogesterone didn´t have the negative effects of MPA on the serum lipids profile. Whereas the 19-nortestosterone derivatives and even some 17-hydroxyprogesterone molecules exert a partial androgenic effect and reverse the lipid profile improvements induce by estrogens treatment. (Sitruk-Ware,2000). Al-Azzawi reported in a double blinded randomized multicenter study, on a population of 487 postmenopausal women younger as 65 years, that the combination of estradiol plus trimegestone reduced the risk of fatal myocardial infarction by 10 % while the combination of estradiol plus norethisterone acetate had no effect. (Al-Azzawi, 2006). Thus each progestin appears to have a unique profile of cardiovascular effects.

1.8. Cardiac hypertrophy.

The heart is able to adapt to elevated hemodynamic loads by increasing its mass, a characteristic that confers physiological flexibility. The initial step for the development of a cardiac hypertrophy is hemodynamic stress that is compensated by increased thickness of the heart chamber walls, a phenomenon that according to the La place law decreases parietal wall stress. When increased cardiac mass compensates increased work load this is termed “compensatory hypertrophy”, an event that is commonly present in athletes. A non compensated hypertrophy is described as a decreased in the normal heart ability to refill and to develop an adequate contraction force. Compensated hyperthrophy is characterized by cardiomyocytes with increased length and no significant changes in collagen

deposition in the extracellular matrix. In contrast, in the non-compensated and mal-adaptive cardiac hypertrophy the change in cardiac mass is the result of the increase of cardiomyocytes thickness and accumulation of collagen. The increased thickness and volume of cardiomyocytes results in an insufficient number of mitochondria and an increased intra-capillary distance; both events predict insufficient energy utilization and lower tissue oxygenation. Increased collagen content of the extracellular matrix increases the stiffness of the myocardium, which compromises both systolic force development and diastolic relaxation. The later can lead to abnormal diastolic function and diastolic heart failure, despite preserved systolic function. In addition, decreased coronary flow reserve increases the risk of cardiac ischemia, of potentially lethal cardiac arrhythmias and of myocardial infarction. Reduced cardiac contractility finally occurs as the result of increased workload and myocardial ischemia. (Opie L, 1991; Gosse P, 2005).

1.9. Heart Failure.

Congestive heart failure (CHF) occurs when cardiac function is unable to meet the peripheral oxygen and nutrient demand. The major causes of heart failure are either ischemic or non ischemic in their nature.

- Ischemic heart failure develops when a cholesterol plaque builds-up and ruptures which blocks the blood supply to a specific region of the heart (CAD= Coronary Artery Disease). CAD and myocardial infarction are the major causes of CHF in the United States affecting approximately 1 out of 100 people (AHA official web page).
- Non-ischemic causes of CHF include structural and functional changes that affect the heart pumping capacity and include mainly:
    - **Increased afterload** due to increased peripheral resistance that leads to elevation of the arterial blood pressure (hypertension) and exacerbation of the

cardiac mechanics leading to congestion of the left and right ventricles and pulmonary circulation.

- **Valvular disfunction** that affects the amount and the direction of blood flow between different cardiac compartments. It can develope due to a narrowing of the valves (stenosis: impeding the transport of blood from the left ventricle to the aorta) or to a leakage of the valves (regurgitation: impling a backward blood through the leaky valve).
- **Cardiomyophaty** a condition in which cardiac myocytes are directly affected by inflammation or genetic abnormalities and decrease their pumping capacity. The most common form is Dilated Cardiomyopathy (DCM).

The body has several mechanisms to compensate cardiac dysfunction including changes in vascular function, blood volume, and neurohumoral status. These changes serve as compensatory mechanisms to maintain cardiac output (primarily by the ability of the heart to change its force of contraction and stroke volume in response to changes in venous return "Frank-Starling" mechanism) and arterial blood pressure (by systemic vasoconstriction and increased resistance). However, persistance of these compensatory changes over months and years can worsen cardiac function. Overall, the changes in cardiac function associated with heart failure result in a decrease in cardiac output (volume of blood ejected per minute). This results from a decline in stroke volume (volume ejected per beat) that is due to systolic dysfunction, diastolic dysfunction, or a combination of the two. Systolic dysfunction results from a loss of intrinsic inotropy (contractility), most frequently due to alterations in signal transduction mechanims responsible for regulating inotropy. Systolic dysfunction can also result from the loss of viable, contracting muscle as occurs following acute myocardial infarction. Diastolic dysfunction refers to the diastolic properties of the ventricle and occurs when the ventricle becomes less compliant (stiffer),

which impairs diastolic ventricular filling. Both systolic and diastolic dysfunction results in a higher ventricular end-diastolic pressure, which serves as a compensatory mechanism by utilizing the Frank-Starling mechanism to augment stroke volume. The neurohumoral responses include activation of sympathetic nerves and the renin-angiotensin-aldosterone system, and increased release of antidiuretic hormone (vasopressin) and atrial natriuretic peptide. The net effect of these neurohumoral responses are arterial vasoconstriction (to help maintain arterial pressure), venous constriction (to increase venous pressure), and increased blood volume. In general, these neurohumoral responses can be viewed as short term compensatory mechanisms, but their persistance can also aggravate heart failure by increasing ventricular afterload (which depresses stroke volume) and increasing preload to the point where pulmonary or systemic congestion and edema occur.

1.10. Myocardial ischemia.

Myocardial ischemia is the pathological loss or reduction in blood flow (ischemia) to a part of the muscular tissue of the heart (myocardium). Atherosclerosis of the larger coronary arteries is the most common condition to diminish coronary blood flow. Local and prolonged ischemia produces irreversible damage of cardiac myocytes including apoptosis and necrosis that concludes in myocardial infarction. The development of myocardial infarction is complex as it doesn't depends only on the lack of oxygen and lack of elimination of metabolic products, but it also depends on the existence of collateral perfussion. One important factor of myocardial ischemia is the reduction of oxygen supply to the mitochondria resulting in insufficient energy supply to maintain cellular function (reversible) or integrity (cell death) (Steenbergen et al, 1990; Marban et al. 1990).

1.11.Myocardial infarction.

Myocardial infarction (MI) results from the complete stop of blood supply to a specific part of the heart. The resulting ischemia causes cellular damage and death of heart tissue in particullar of cardiac myocytes, if impaired blood flow lasts beyond a critical limit, oxygen demanding cell types (cardiomyocytes) first undergo necrosis and then a rigid collagen scar is formed in place of viable myocytes (Fig. 3). Most myocardial infarctions are caused by the closure of a coronary artery by a platelet-rich blood clot that results from a disruption in the vascular endothelium on top of an unstable atherosclerotic plaque that stimulates the formation of an intracoronary thrombus, which may result in complete coronary artery occlusion. If such an occlusion persists long enough (20 to 40 min), irreversible myocardial cell damage and cell death occurs. (De Wood et al, 1986). The pathogenesis of a myocardial infarction not only includes occlusive intracoronary thrombus (causes 90% of transmural acute myocardial infarctions), but may also arrise from vasospasm and arterial emboli.

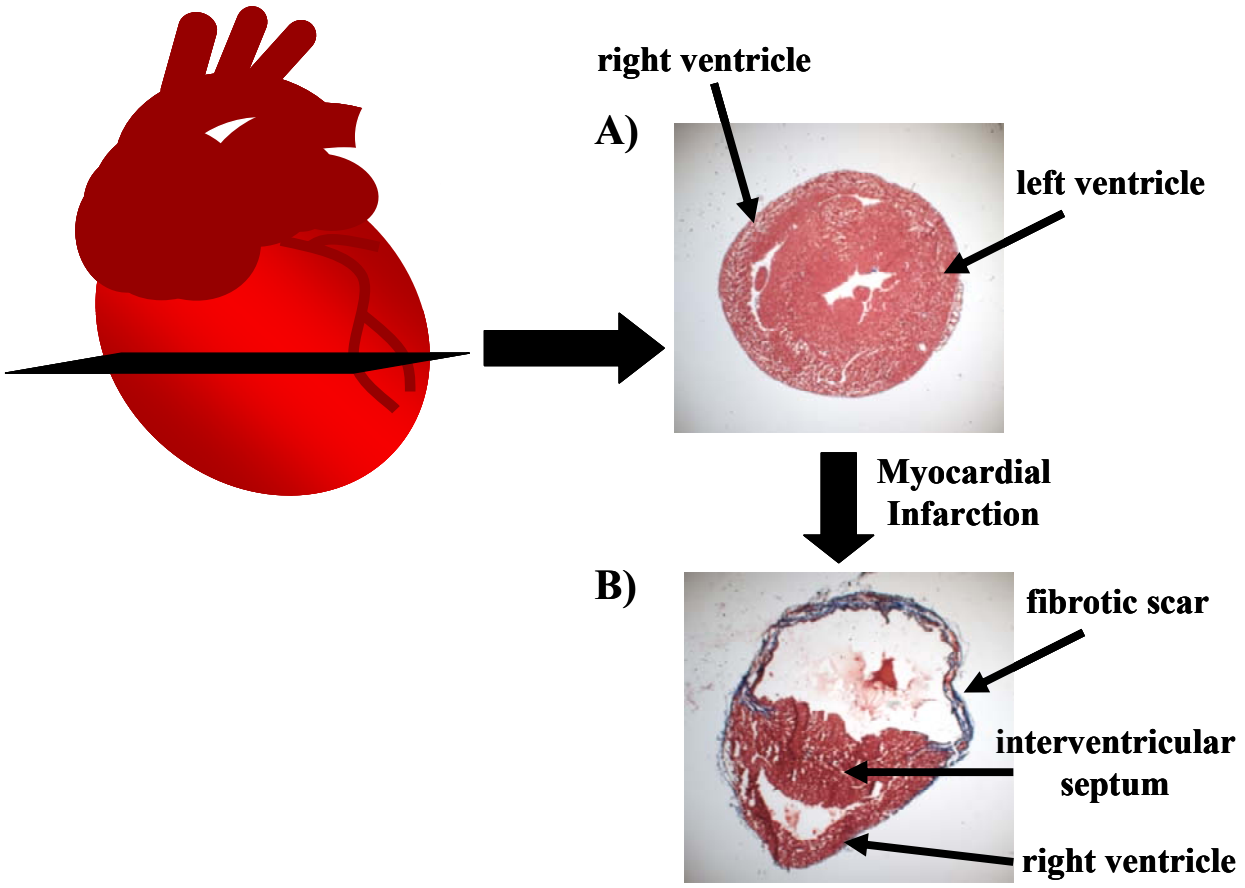

Figure 2. Morphology of the murine heart after myocardial infarction. In the non-diseased heart, the left ventricle is formed of compact and homogeneous muscular tissue as shown in a transversal section of a healty heart A). After myocardial infarction the muscular tissue suffering ischemia is substituted by a stable collagen scar B).

Myocardial infarction presents a circumscribed area of ischemic necrosis (cell death). On gross examination, the infarct is not identifiable within the first 12 hours (Rubin, 2001), afterwards the ischemic area looks pale and after 7 weeks a white scare is bild up. The interstitium at the margin of the infarcted area is initially infiltrated with neutrophil followed by lymphocytes and macrophages, which phagocyte myocyte debris. The necrotic area is surrounded and progressively invaded by granulation tissue, which will replace the necrotic tissue with a fibrous (collagenous) scar (Rubin, 2001) (Fig 3).

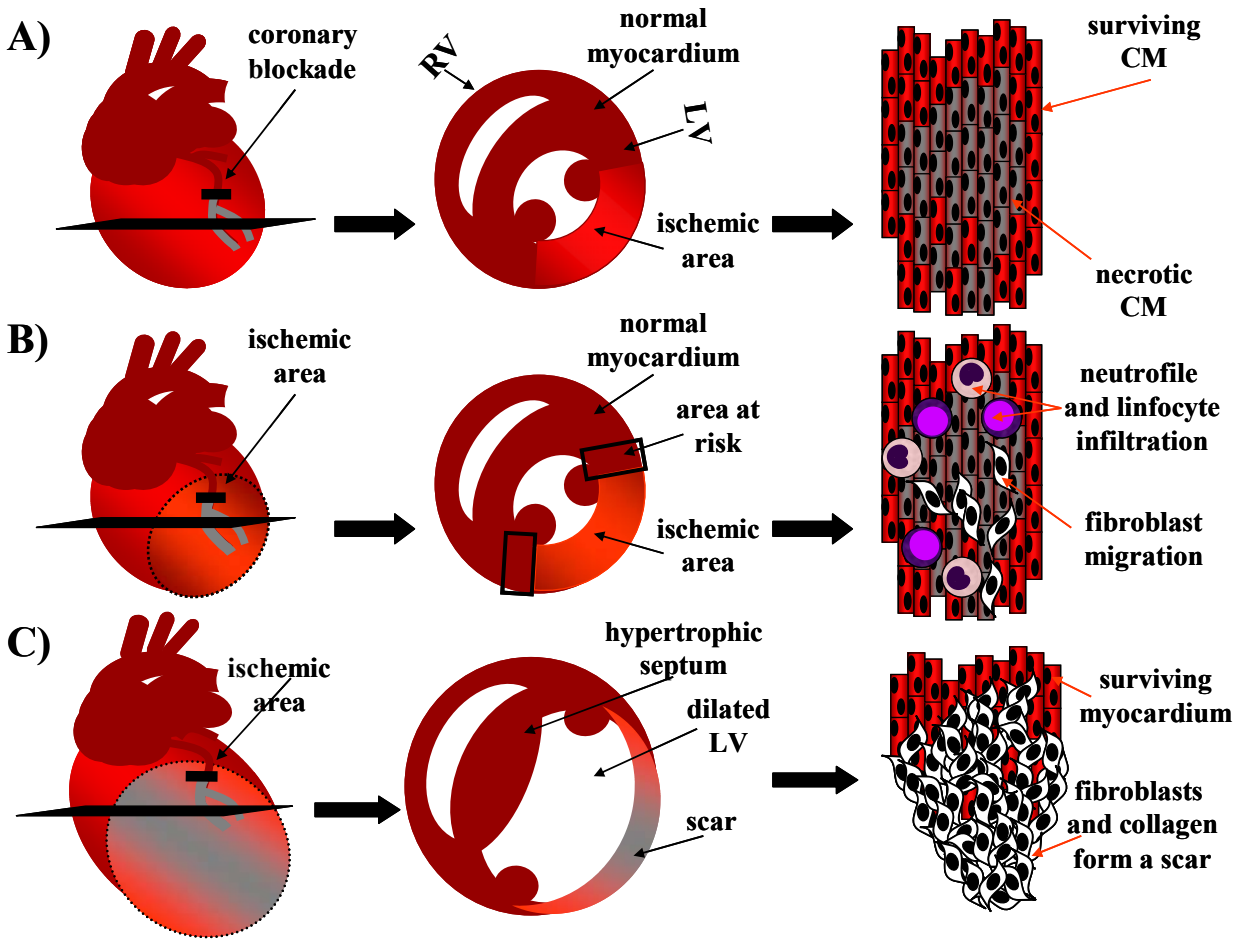

Figure 3. Morphological changes following myocardial infarction. A) Occlusion of a coronary artery results in a complete cessation of blood flow to a specific region of the heart (ischemic area). During the first hours of ischemia the lack of oxygen and nutrients leads to cardiomyocyte necrosis. B) In the healing phase, necrotic cardiomyocytes are englobed by neutrofiles, fibroblast infiltrate that start to secrete collagen to the extracellular matrix. C) In the remodelling phase, necrotic cardiomyocytes are replaced by a stable collagen scar. The lost of contractile force increases physical stress in the surviving cardiomyocytes and results in cardiomycyte hypertrophy. RV=right ventricule; LV=left ventricle; CM=cardiomyocytes.

In an effort to mimic the progression of heart failure in humans with coronary disease, animal models of chronic or intermittent myocardial ischemia have been developed. One of the most common and standardized models is the ligation of the anterior descending coronary artery that causes myocardial infarction. The rat myocardial infarction model mimics accurately the human patophysiology, the infarcted chamber walls are thinned out

with resultant scar formation, while the non-infarcted myocardium hypertrophies in response to increase wall stress. In rats with infarcts with less than 20% of the left ventricle circumference there are minimal, if any changes in hemodynamic. Rats with large infarctions develop increased left ventricular end-diastolic pressure and progressive left ventricle dilatation within 2 to 4 months (Gaballa M, 2002). In this model, the progression of left ventricular dysfunction and myocardial failure is associated with neurohumoral activation that fits precisely with neuro-humoral activation that occurs in patients with chronic heart failure following a large myocardial infarction (Hasenfuss G, 1998).

1.12. Role of SERCA 2a and phospholamban (PLN) in intracellular Ca+2 handling during Heart Failure.

Considerable evidence indicates that abnormalities in the calcium homeostasis of cardiomyocytes represent an important mechanism for heart failure. Mutations in calcium handling proteins like phospholamban, calcineurin and ryanodine receptors have been observed in cardiomyopathies. Protein levels and RNA sequence encoding these proteins are also altered in acquired and inherited forms of heart failure. At low concentrations of intracellular calcium, troponin I and actin interactions block acto-myosin ATPase activity. An increase in calcium levels fosters its binding to troponin C, which releases troponin I inhibition and stimulate contraction. Cardiac relaxation occurs when calcium dissociates from troponin C. Calcium is mobilized into the sarcoplasmic reticulum (SR) through the cardiac sarcoplasmic reticulum Ca+2 ATPase (SERCA2a). Calcium reuptake into sarcoplasmic reticulum via SERCA2a is regulated by phospholamban (PLN) that inhibits SERCA 2a activity in its non-phosphorylated form thus slows ventricular relaxation. (Fig.5) (Morita 2005; Yano M 2005).

Many studies have demonstrated a reduced expression of SERCA2a protein in the failing heart. Consistently, SR calcium uptake by SERCA2a is reduced in the failing animal or human myocardium. The level of PLN phosphorylation has also been report to be reduced in heart failure (Hassenfuss 1998; Yamamoto 1999; Limas 1987; Schwinger 1999; Sande 2002; Yano 2003). In support of this concept SERCA2a overexpression enhances cardiac contractility and has been reported to protect against both heart failure and cardiac hypertrophy. (Miyamoto 2000; del Monte 2001; Muller 2003). On the other side PLN gene ablation has been shown to prevent ventricular dysfunction, fibrosis and development of heart failure both in dilated cardiomyopathy and hypertrophic cardiomyopathy (Minamisawa 1999; Sato 2001; Freeman 2001).

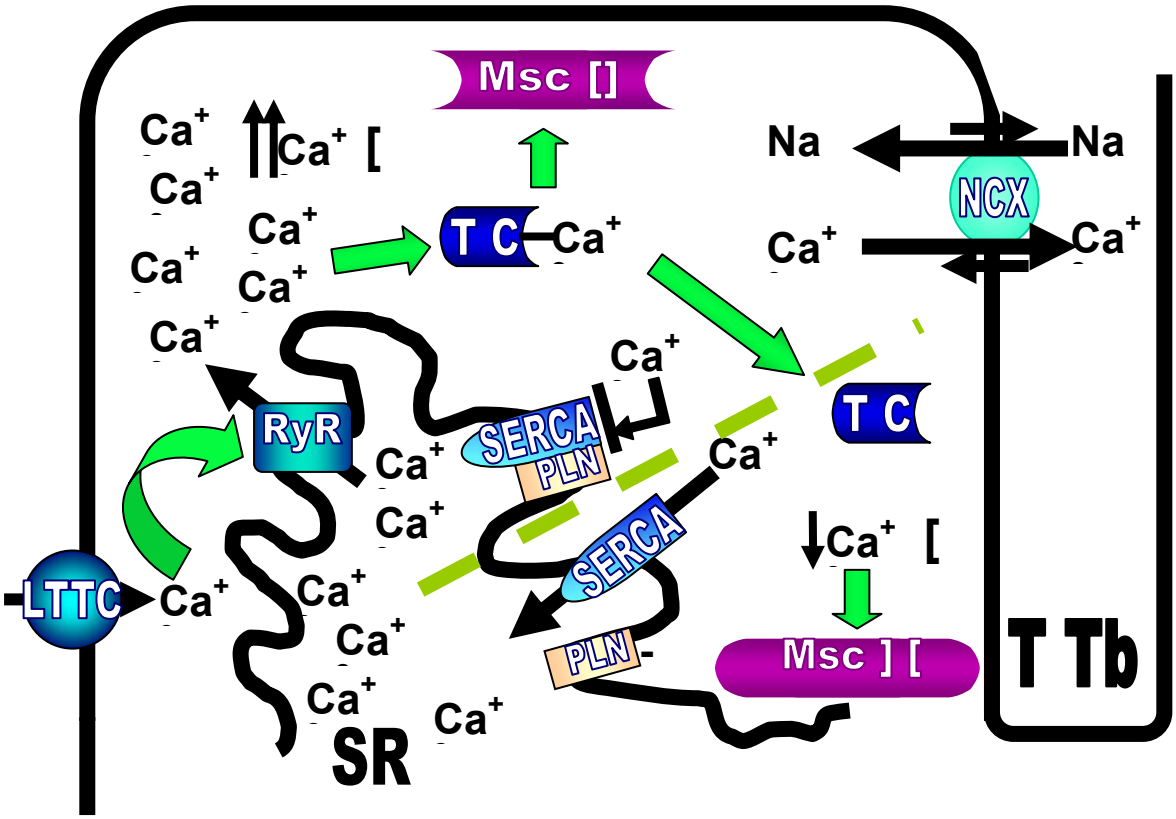

Figure 4. Regulation of cardiac contraction and relaxation by cytoplasmic calcium levels. In cardiac excitation- contraction coupling, a small amount of calcium enters through the L-type Ca+2 channels (LTCC) during membrane depolarization. This calcium influx triggers a large scale Ca+2 releases (high cytoplasmic calcium) through the Ca+2 release channel of the sarcoplasmic reticulum (SR) referred to as the ryanodine receptor (RyR). The released calcium binds to troponin C (T C) which induces muscle contraction. Relaxation is initiated by the dissociation of calcium from troponin C, followed by its reuptake into the SR via SERCA2a and the Na+/Ca+2 exchanger (NCX) operating in its forward mode. SERCA2a activity is regulated by phospholamban (PLN), which in its dephosphorylated form act as an inhibitor of SERCA2a. Phosphorylation of PLN increases SERCA2a activity. (Modified from Yano M 2005).

1.13. Atrial Natriuretic Peptide (ANP).

ANP is produced predominantly in atria and vascular endothelium. Human ANP is synthesized as a 151- amino acids (152-amino acids in the rat) pre-pro hormone. Removal of a 25 amino acid (24 amino acids in the rat) signal peptide from the pre-pro hormone results in the 126 amino acid pro ANP form. Pro ANP is vectorially transported through the Golgi complex and packaged into membrane bound granules. Upon secretion, pro ANP is further processed at the monobasic cleavage site Arg198-Ser99 into its circulating biologically active form (ANP 99-126) and its N terminal fragment (ANP 1-98). (Forero M., 2005).

ANP plays an integral role in the regulation of fluid and salt homeostasis under normal and pathological conditions that arise from vasorelaxation, increased diuresis, increased natriuresis and reduction of venous return by a shift of plasma into the interstitium. In the kidney the natriuretic and diuretic actions of ANP result in enhanced glomerular filtration and/or decreased tubular reabsorption of sodium and water, suppression of renin, aldosterone and vasopressin release that are mediated by angiotensin II effects. The ANP gene is strongly activated in response to hypertrophic stimuli in the heart and prevents hypertrophy by inhibition of protein synthesis in cardiac myocytes via a cGMP-dependent process. (Jankowski M., 2001). Expression of ANP mRNA in the atrium increases gradually with aging, whereas its expression decreases abruptly after birth. However ANP mRNA expression in the ventricles can be induced by increased cardiac workload with cardiac hypertrophy. Thus increased ventricular ANP expression is part of the fetal phenotype reprogramming that occurs with cardiac hypertrophy (Semmekrot B., 1991; Wei Y., 1987; Lattion AL.,1986; van Bilsen M., 1993). Estradiol replacement therapy in human patients and in animal models results in elevated plasma levels of ANP and reduction of hypertension. Increased ANP expression and secretion in response to ER-α activation may thus be a protective mechanism in the heart that lower blood pressure and

hence cardiac afterload (Jankowski M, 2001; Belo NO, 2004; Tsai EM, 2005; Karjalainen AH, 2004).

1.14. Renin-Angiotensin-Aldosterone System (RAAS).

The renin-angiotensin-aldosterone system (RAAS) plays an important role in regulating blood volume and systemic vascular resistance. The RAAS becomes activated upon the loss of blood volume or a drop in blood pressure (such as in a haemorrhage). As the name implies, there are three important components to this system: 1) renin, 2) angiotensin, and 3) aldosterone. Under the effects of renin the circulating angiotensinogen is converted into angiotensin I. Angiotensin I is the substrate of the angiotensin converting enzyme (ACE) that catalyzes the production of angiotensin II , which stimulates the release of aldosterone from the adrenal cortex. Renin is a proteolytic enzyme that is released into the circulation primarily by the kidneys. Its release is stimulated by:

1) sympathetic nerve activation (acting via b1-adrenoceptors).

2) renal artery hypotension (caused by systemic hypotension or renal artery stenosis).

3) decreased sodium delivery to the distal tubules of the kidney. (Klabunde RE, 2007).

Juxtaglomerular (JG) cells associated with the afferent arteriole entering the renal glomerulus are the primary site of renin storage and release. A reduction in afferent arteriole pressure causes the release of renin from the JG cells, whereas increased pressure inhibits renin release. Beta1-adrenoceptors located on the JG cells respond to sympathetic nerve stimulation by releasing renin. Specialized cells (macula densa) of distal tubules lie adjacent to the JG cells of the afferent arteriole. The macula densa senses the amount of sodium and chloride ion in the tubular fluid. When NaCl is elevated in the tubular fluid,

renin release is inhibited. In contrast, a reduction in tubular NaCl stimulates renin release by the JG cells. When afferent arteriole pressure is reduced, glomerular filtration decreases, which reduces Na+ and Cl- secretion in the distal tubule. This serves as an important mechanism contributing to the release of renin during hypotension. When renin is released into the blood, it converts its circulating substrate, angiotensinogen, to the decapeptide angiotensin I. Vascular endothelium, particularly in the lungs containing the angiotensin converting enzyme, that cleaves off two amino acids from angiotensin I to form the octapeptide, angiotensin II (AII) (Fig 6) (Klabunde RE, 2007). Although angiotensin I may have some biological activity, angiotensin II is pharmacological much more potent and exert a variety of biological effects by itself including:

- Vasoconstriction. Angiotensin II constricts resistance vessels thereby increasing systemic vascular resistance and arterial pressure.
- In the kidney AII constricts glomerular arterioles, having a greater effect on efferent arterioles than afferent. As with most other capillary beds, the constriction of afferent arterioles increases the arteriolar resistance, raising systemic arterial blood pressure and decreasing blood flow. Despite this, the kidneys maintain glomerular filtration pressure up via constriction of the efferent arterioles.
- In the adrenal cortex, AII causes the release of aldosterone. Aldosterone acts on the tubules (i.e. the distal convoluted tubules and the cortical collecting ducts) in the kidneys, causing them to reabsorb sodium and water from the glomerular filtrate. In exchange, potassium is secreted into the tubule.
- Release of Anti Diuretic Hormone (ADH) from the pituitary gland, which increases fluid retention by the kidneys.
- Facilitates norepinephrine release.

- Together, these mechanisms results in the maintenance of fluid and electrolytes homeostasis.

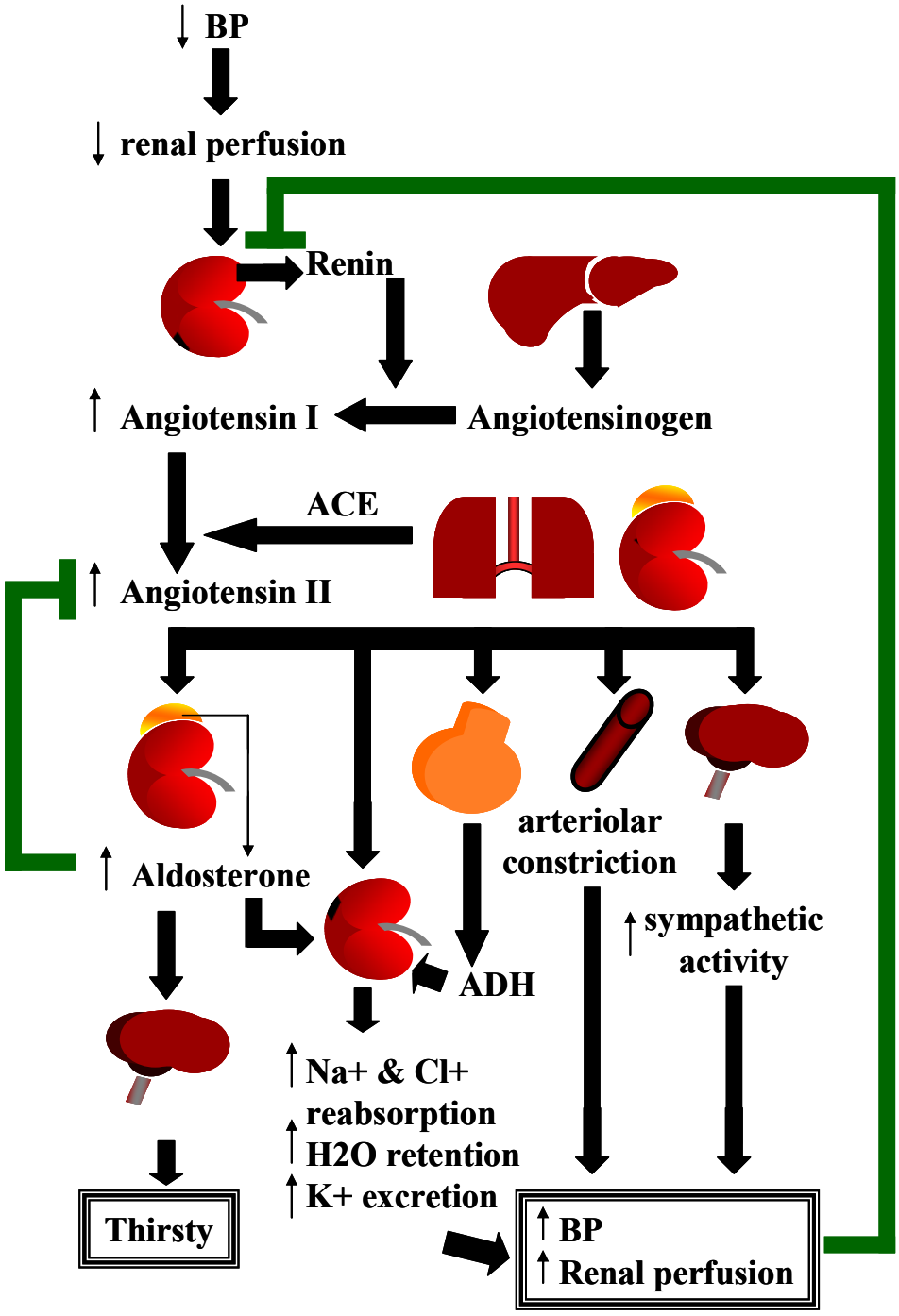

Figure 5. The renin angiotensin aldosterone system is activated by a loss of blood volume or a drop in blood pressure that induce secretion of renin by the kidney. Renin cleaves angiotensinogen to angiotensin I which itself is cleaved by ACE to angiotensin II. Multiple actions of angiotensin II in different tissues result in water and salt retention and thereby maintain fluid and electrolyte homeostasis.

The actions of AII are mediated by two receptors termed AT_1 and AT_2 showing a complex pattern of regulation and function In rat and mouse, two AT_1 subtypes have been cloned and characterized; they are termed AT_{1A} and AT_{1B} (Inagami T, 1995). The AT_1 and AT_2 subtypes show similar properties of AII binding but different genomic structure and localization as well as tissue-specific expression and regulation (De Gasparo M, 2000). Whereas most of the well-known actions of AII such as vasoconstriction and aldosterone release are mediated by the AT_1 receptor, less is known of the function of the AT2 receptor, in fetal development, AT2 receptor plays a role in kidney morphogenesis. In the adult, AT_2 mediated actions have been shown to counteract AT_1 effects such as cell proliferation (Stoll M, 1995; Morishita R, 1993). Increasing evidence supports a role of AT_2 in the regulation of growth, differentiation, and regeneration of neuronal tissue (Steckelings UM, 2005).

Aldosterone by itself has two main actions at the late distal tubule and the collecting ducts:

- By acting on mineralocorticoid receptors (MR) in the distal convoluted tubule of the kidney, aldosterone increases the permeability of the apical (luminal) membrane to potassium and sodium and activates their basolateral Na+/K+ pumps, stimulating ATP hydrolysis, reabsorbing sodium (Na+) ions and water into the blood, and secreting potassium (K+) ions into the urine.
- Aldosterone also stimulates H+ secretion by intercalated cells in the collecting duct, regulating plasma bicarbonate (HCO3−) levels and its acid/base balance.(Brenner, 2004).

Aldosterone is responsible for the reabsorption of about 2% of filtered sodium in the kidneys, which is nearly equal to the entire sodium content in the human blood pool under normal glomerular filtration rate. (Sherwood L, 2001). It also potentiates the sympathetic nervous system activity and reduces the conversion of angiotensin I to angiotensin II in a

negative feedback mechanism that prevents excessive RAAS activity. (Chatterjee K., 2004).

The renin-angiotensin-aldosterone system is regulated not only by the mechanisms that stimulate renin release, but it is also modulated by natriuretic peptides (ANP and BNP) that are released by the heart (Klabunde RE, 2007). Besides of the major physiological role of the RAAS in fluid homeostasis, it also exerts local tissue effects that have an important role in mediating diverse physiological functions such as ovary atresia modulation and fluid balance in the intestinal tract. The genes for all components of the RAAS have been cloned and gene expression studies could verify their mRNA expression and regulation in many tissues, demonstrating the possibility of local AII synthesis. In some tissues local RAAS appears to be regulated independently of the systemic RAAS as concluded from experiments in which nephrectomy (down regulating the formation of AII in the plasma) did not reflect local formation of AII in the adrenal gland or the brain. (Klabunde RE, 2007). It has also become increasingly clear that the local RAAS system is not an isolated entity but interacts with the systemic RAAS as well as other peptide systems (such as the endothelin system) on multiple levels. (Paul M, 2006). The concept of local or tissue-based RAAS, therefore is not an opposing or alternative but rather a complementary or integrated functional concept of angiotensin formation and function. (Paul M, 2006).

As many cardiovascular pathologies include the activation of the RAAS, therapeutic manipulation of its components is very important in treating hypertension and heart failure. ACE inhibitors, AII receptor blockers and aldosterone receptor blockers, are used to decrease arterial pressure, ventricular after load, blood volume and hence ventricular preload, as well as to inhibit and eventually reverse cardiac and vascular remodelling. (Pinto YM, 1993).

1.15. Gender influence on the RAAS.

Components of the RAAS are influenced by gender and sex hormones (Fischer M, 2002). Pregnant women present higher aldosterone serum levels as non-pregnant woman, contributing to the blood volume increase observed during pregnancy. The characterization of an estrogen-responsive element in the 5'-flanking region of the angiotensinogen gene has been an important early finding to prove interactions of sex hormones with the RAAS at the molecular level (Feldmer M, 1991, Gordon MS, 1992). In addition, angiotensinogen expression in the liver is reduced by castration and increased by testosterone treatment in rats (Ellison KE, 1989). While earlier studies suggested that renin activity in plasma is enhanced by estrogens, more recent studies indicated that renin is actually suppressed by estrogens (Fischer M, 2002; Oelkers WK, 1996; Schunker H, 1997). Collectively these studies are in line with the observation that renin plasma levels are lower in women compared with men (Fischer M, 2002; Schunker H, 1997). However, the expression of ACE mRNA was significantly lowered in response to estradiol in kidney, lung, and aorta in rats (Gallagher PE, 1999). In the heart, ventricular ACE is more abundant in male than female mice at both, mRNA and protein levels, after reaching sexual maturity (Freshour JR, 2002). In humans, circulating ACE levels appear also to be lower in response to estrogens (Proudler AJ, 1995; Schunker H, 1997; Seely EV, 2004). Similarly, AT_1 receptor expression is down-regulated by estrogens while estrogen deficiency resulted in an up-regulation of this receptor (Nickenig G, 1998). In female transgenic rats with activated tissue RAAS, estrogen treatment protected against hypertension by reducing the vasoconstrictor response to ANG II independently of changes from plasma renin activity (Brunswig-Spickenheier B, 1992). In summary, the net

effect of estrogens seems to be a suppression of the RAAS both systemically and at the tissue level (Fisher M, 2002; Paul M, 2006).

More evidence for a relation between aldosterone and gender are provided by Schunkert et al. (1997) who described a positive association of serum aldosterone levels with left ventricle mass index in women and by the Framingham Study (Vasan 2004). All these evidence suggest a role of gender in the cardiovascular effects of aldosterone and the possibility that ligand dependent activation of estrogen receptors may attenuate unfavorable effects of MR activation. However despite the evidence that both, the activity of the RAAS and cardiovascular morbidity and mortality, show gender differences, it is not fully understood to what extent gender and sex hormones regulate the RAAS (Fisher M, 2002; Rossouw JE, 2002). Further studies of gender effects on the RAAS appears of interest to understand cardiac and vascular remodelling processes as well as the role of hormone replacement therapy in cardiovascular disease.

1.16. Role of aldosterone in cardiovascular disease.

A number of deleterious effects have been reported for aldosterone in the cardiovascular system, such as vascular inflammation, fibrosis, hypertension and cardiac hypertrophy. In humans, it has been proposed that elevated aldosterum plays a causative role in up to 10% of patients previously diagnosed with essential hypertension (Mulatero P, 2004; Brilla CG, 1992; Rocha R, 2002). Mineralocorticoid receptor (MR) antagonists such as spironolactone and eplerenone prolong survival in heart failure patients and improve clinical symptoms which results from decreased left ventricle end-diastolic pressure and reduction of left ventricle mass, collagen deposition and myocardial fibrosis, increase nitric oxide bioavailability and improve endothelial vasodilatory function (MacFadyen RJ, 1997; Farquharson CA, 2000; Tiyyagura S, 2006; Mizuno Y, 2001).

The detailed roles of aldosterone and mineralocorticoid receptors in the development of myocardial fibrosis and hypertension have been studied in vivo by **A**ldosterone **S**alt **T**reatment (AST) in rats and by gene manipulation in mice. Brilla and Weber (Brilla CG, 1992) demonstrated that the administration of aldosterone in conjunction with uninephrectomy and a high salt diet produces hypertension, cardiac hypertrophy and cardiac fibrosis. In this model cardiovascular damage is explained by increased water and sodium retention that leads to hypertension and perivascular fibrosis. However this is not the only damage mechanism involved and currently it is well accepted that these pathological actions of aldosterone, although dependent on the salt status of the animal, are partially independent of systolic blood pressure, hypokalemia, and cardiac hypertrophy (Young M, 1995). More recent investigations of early time points in the establishment of cardiac fibrosis have now identified oxidative stress and early vascular inflammatory events as key mediators of MR activation in the blood vessel wall and essential steps in the progression to cardiac fibrosis. (Young M,2003; Rocha R, 2002, Sun Y, 2002).

Knock out mice for the MR develop symptoms of pseudohypoaldosteronism including hyponatremia, hyperkalemia and strong activation of the renin-angiotensin-aldosterone system. MR over expressing mice had normal blood pressure, but presented tachycardia and a high occurrence of dysrrhytmia, suggesting an arrhytmogenic effect of MR. (Le Menuet D, 2001; Ouvrard-Pascaud A, 2005). Another genetically modified mouse has been generated by spatio-temporal control of the expression of an antisense mRNA of the mMR by using the tet-OFF system together with the α-myosin heavy chain promoter. Double transgenic mice develop severe cardiomyopathy leading to premature death. This phenotype was accompanied by extensive cardiac fibrosis which was reversible upon doxycycline administration (Beggah AT, 2002). Collectively the phenotypes of

mineralocorticoid receptor transgenic mice clearly indicate that the mineralocorticoid receptor plays an important role in the cardiovascular function.

1.17. Drospirenone.

Drospirenone or Dihydrospirenone (6β, 7β, 15β, 16β –dimethylen-3-oxo-17- α-pregn-4-ene-21, 17-carbo-lactone) is a novel progestogen that simultaneously act as a potent mineralocorticoid receptor antagonist. As such drospirenone promotes the maintenance of pregnancy, inhibits ovulation and stimulates endometrial transformation. Like progesterone, drospirenone has been shown to have an antimineralocorticoid effect in rats and humans. It has been demonstrated that drospirenone has a long lasting natriuretic activity on administration of a daily dose of 10 mg s.c. for 3 weeks. The compound is devoid of androgenic, estrogenic, glucocorticoid and antiglucocorticoid activity (Muhn P, 1995) and suppresses ovulation in women at a daily dosage of 2 mg. (Oelkers W, 1991). Used as an oral anticonceptive drospirenone leads to a relevant decrease of elevated blood pressure (Oelkers W, 1995). Drospirenone has only been tested in combination of 17-β estradiol, combination that effectively prevented hot flushes and other menopausal symptoms. (Oelkers W, 2004).

1.18. Oxidative stress in cardiovascular disease.

Oxidative stress refers to a disturbance in the oxidation reduction state of the cell. The process of ATP generation from high energy substrates involves a host of oxidation reduction reactions that transfer reducing agents (electrons) to oxygen. However the uncontrolled production of these Reactive Oxygen Species (ROS) or a reduction of antioxidant capacities (super oxide dismutase, catalase, glutathione) results in oxidative stress and is thought to be deleterious by altering gene expression and protein structure and function (Molavi B, 2004).

The important forms of ROS in the cardiovascular system include superoxide (O2-), hydrogen peroxide (H2O2), hydroxyl radical (OH-) and nitric oxide (NO-), which is produced mainly by the NADPH oxidase, xanthine oxidase system and by nitric oxide synthases.

- NADPH oxidase. The membrane bound enzyme complex is activated in response to acute stress, such as stretch, laminar shear stress, and a disturbed oscillatory flow that occurs at vascular branch points (Griendling KKP, 2003). The enzyme complex is composed of electron transfer groups (gp91phox also known as nox 2, nox 1 and nox 4), p22phox and regulatory subunits (p47phox, p67phox and rac 1) and mainly produces O2- (Fig 7).One of the unique features of this system, which is present in vascular smooth muscle cells, endothelial cells, neutrophils and fibroblasts, is that it is responsive to a variety of neurohormonal stimuli, including angiotensin II, tumour necrosis factor-α, and growth factors (Molavi B, 2004).
- Xanthine oxidase system. This membrane bound system generates extracellular superoxide and peroxide moieties and metabolizes hypoxanthine, xanthine and NADH to form O2- and H2O2. It is implicated in a variety of pathophysiologic states, including ischemia-reperfusion injury, and responds to cellular ATP/ADP balance.
- Nitric Oxide synthases. Under physiological conditions, this system produces endothelium derived relaxing factor nitric oxide (NO) from L-arginine in a reaction catalyzed by tetrahydrobiopterin. However, in the absence of adequate quantities of L-arginine or tetrahydrobiopterin, nitric oxygen synthase becomes uncoupled and generates significant amounts of O2- (Molavi B, 2004). NO can mediate the S-nitrosylation of proteins at specific cystein residues, this process in the heart has significant functional implications, especially with regard to calcium flux and excitation-contraction coupling. S-nitrosilation is facilitated by O2-, when O2- is present at physiological levels. Increased

O2- interacts with NO to form deleterious reactive molecules including peroxinitrite (Giordano F, 2005).

- The electron transfer chain in mitochondria and the myeloperoxidase system of the macrophages provide additional sources of ROS in the cardiovascular system.

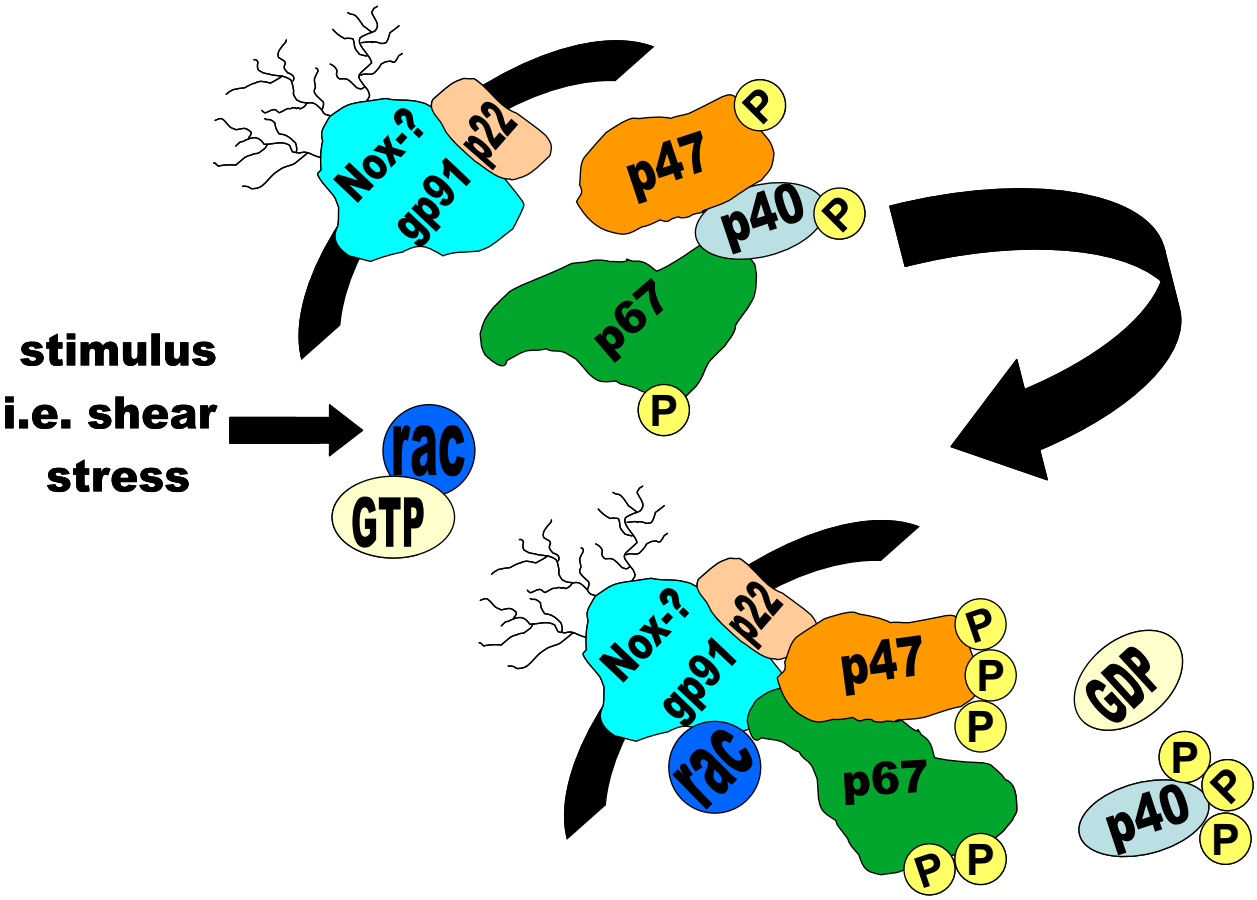

Figure 6. Components of the NADPH oxidase (neutrophil NADPH oxidase). Gp91phox and p22phox form the electron transfer component of the oxidase, and p47phox and p67phox are cytosolic components that interact with these 2 proteins to modulate its activity. The low molecular weight G protein rac also serves as regulatory unit (Adapted from: De Leo F, 1996).

In the myocardium, as in other tissues, antioxidant enzymes protect cellular integrity by maintaining anion super oxide and hydrogen peroxide levels. Three known types of super

oxide dismutase (SOD) are present in mammalian tissues: MnSOD, which is encoded on the nuclear genome but localizes to mitochondria, makes up to 70% of the SOD activity in the heart and 90% of the activity in cardiac myocytes (Assem M, 1997). The remaining SOD consists primarily of Cu/ZnSOD which is located in the cytosol and the EcSOD which is located in the extracellular and intracellular spaces (Sawyer DB, 2002).

Oxidative stress has been implicated in chronic heart failure and ischemia reperfusion injury. Markers of oxidative stress are also elevated in heart failure and have been correlated with myocardial dysfunction and overall severity of heart failure (Grieve D, 2003). Similarly, there is evidence of increased oxidative stress in the myocardium of animal models of hemodynamic overload, including myocardial infarction, pressure overload and pacing induced heart failure (Hill MF, 1996; Dhalla AK, 1996; Ide T, 1999; Kinugawa S, 2000). Singal et al. (Singal PK,1993) showed evidence of increased oxidative stress in the myocardium of animals at the transition from compensated hypertrophy to overt failure in association with decreased levels of antioxidant enzymes.

ROS generation has also been related to endothelial dysfunction. Several atherosclerosis risks factors, including hypertension, diabetes, and smoking, are associated with increased ROS in endothelial cells. Treatment with ROS scavengers such as super oxide dismutase (SOD) has been shown to restore the normal endothelium dependent vasoreactivity (Mugge A,1991).

ROS modulate the activity of diverse intracellular signalling pathways and molecules. For example, key proteins involved in myocardial excitation-contraction coupling, such as ion channels, sarcoplasmatic reticulum calcium release channels and myofilament proteins, can undergo redox sensitive alterations in activity (Byrne J, 2003). ROS modulate fibroblast proliferation and collagen synthesis and are involved not only in matrix metalo proteinase activation, but also in its expression (Spinale FG, 2002). The most widely recognized biological effects of ROS are those that occur when cellular antioxidant

defences are overwhelmed and ROS reacts directly with cellular lipids, proteins and DNA, causing cell damage and death. Lipid peroxidation for example is a well-characterized effect of ROS that results in damage to the cell membrane as well as to the membranes of cellular organelles. Modification of proteins by ROS can cause inactivation of critical enzyme and can induce denaturation that renders proteins non-functional (Giordano F, 2005).

1.19. Aim of the study.

Hormone Replacement Theraphy (HRT) consisting in the combination of equine estrogens plus Medroxyprogesterone acetate in clinical trials resulted in an increased risk of thromboembolic events and no cardiovascular protective effects. However unopposed estrogens in vitro and in animal studies have been proved to be beneficial for the cardiovascular system. One possible source of this controversy is the use of MPA, which may have limited estrogens beneficial effects in the clinical trials. Different studies have analyzed the role of estrogens in the cardiovascular system; however the effects of the combined Hormone Replacement Therapy (estrogen plus progestins) in vivo are still understudied. Therefore the main aim of this work was to evaluate the cardiovascular effects of the regime medroxyprogesterone acetate plus 17β-estradiol in comparison to the ones of unopposed 17β-estradiol during cardiac disease. So far very rare data on the mechanisms responsible for the cardiovascular effects of a combinational HRT were available, therefore the second aim of this work was to analyse putative mechanisms involved in the differential cardiovascular effects between the regimes MPA plus 17β-estradiol and unopposed 17β-estradiol. Furthermore progestins are known to be able to bind to different steroid receptors, besides of the progesterone receptor, resulting in unique pharmacological profiles. Therefore an additional aim of this work was to differentiate the progestin class effects of MPA from the ones exclusive to this compound.

With the pourpose to acchive these aims 3 different animal studies were initiated to characterize the role of estrogens and progestins in cardiovascular disease:

- Rat myocardial infarction model in wich ligation of the left descending coronary artery emulates a coronary artery occlusion that leads to local ischemia and development of heart failure. In this model cardiac function, morphology, hormonal status and gene expression changes were evaluated.

- Aldosterone-Salt-Treatment (AST) to otherwise normotensive rats, model in which activation of the Renin-Angiotensin-Aldosterone-System (RAAS) is assured by constant infusion or aldosterone. In this model cardiac function, morphology, cardiac and vascular histo-morphology, hormonal status and gene expression changes were evaluated
- Considering that the kidney is one of the major targets of the RAAS and plays an important role for the development and aggravation of hypertension a second study in the AST rats was conducted in order to characterize fluids exchange, kidney morphology and renal protein expression.

Chapter 2. Medroxyprogesterone acetate aggravates left ventricular dysfunction and oxidative stress in rats with chronic myocardial infarction.

2.1. Study design.

The role of the hormone supplementation during myocardial ischemia was studied in the rat myocardial infarction model by comparing sham operated rats vs. ovariectomized rats receiving: placebo, 17β-estradiol or 17β-estradiol plus MPA. Female Wistar rats were obtained from Harlan (Germany) at the age of 8 weeks and randomized into 6 treatment groups. Animals in groups 1 and 3 were sham ovarectomized (intact) and the remaining animals in group 2, 4, 5 and 6 were ovarectomized (ovx). A ligature was placed around the proximal left anterior descendent coronary artery in all animals including groups 1 and 2 but myocardial infarction was induced only in groups 3 to 6 by tightening the ligature (Pelzer, 2005). Pharmacological treatment was as follows: group 1 (intact): none; group 2 (ovx): placebo; group 3 (intact): none; group 4 (ovx): placebo; group 5 (ovx): 17β-estradiol (2 µg/kg BW/d); group 6 (ovx): 17β-estradiol (2 µg/kg BW/d) plus MPA (3 mg/kg/d). Echocardiography was performed 3 days and 8 weeks following myocardial infarction. Animals in groups 3 to 6 without detectable or with minor infarcts were excluded from further analysis upon echocardiography at day 3. After the 8 weeks of treatment, hemodynamic measurements were performed and followed by measurement of body weight, heart weight, uterus weight and tibia length. Relative heart weight was calculated from absolute heart weight and tibia length. To control hormone supplementation estradiol and progesterone serum levels were quantified by radio immunoassays (RIA). Considering that progesterone is the precursor of most of the steroid hormones and as neuro-humoral factors have been reported to have high relevance for the development of heart failure we analyzed 17-OH-progesterone, androstenedione, cortisol, corticosterone, aldosterone and angiotensin II serum levels also by RIA. Heart frozen samples were subject of histological analyses for

determination of infarct size, cardiomyocyte cross sectional area and reactive oxygen species generation. Protein extract were prepared from the remote non-infarcted myocardium and subject of western blot analysis.

2.2. Results.

2.2.1. Histo-morphological analysis and infarct size determination.

After 8 weeks of treatment with myocardial infarction presented major morphological left ventricular remodelling. In gross morphology the infarcted ventricles thinned and necrotic cardiomyocytes were substituted by collagen fibbers forming a scar, as can be seen in mid-ventricle transversal sections stained with PSR (Picro Sirius Red) (Figure 7). All animals that received myocardial infarction presented similar scar size. To study the effects of the treatment on heart failure only animals with an infarct size between 40 to 50 % were included since animals with small infarcts don't develop heart failure (Table 1). Selection of the animals with an infarct size between 40 to 50% did not lead to statistical significant changes in infarct size among the animals excluded from the study. Therefore neither ovariectomy nor 17-β estradiol nor the combination 17-β estradiol plus MPA affected infarct size and responded only to random variations.

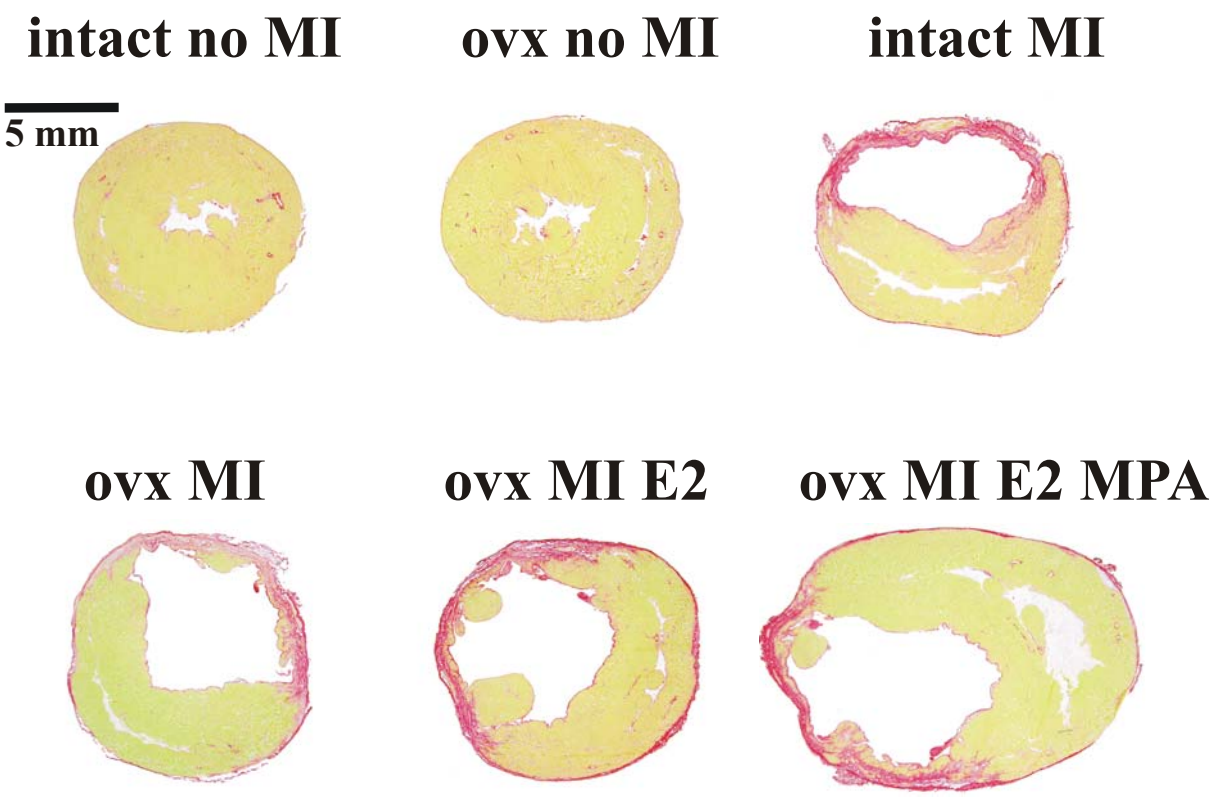

Figure 7. Representative cardiac cross sections stained with Picro Sirius Red illustrate extensive scar formation (stained in red) and hypertrophy of the remaining myocardium as well as the comparable size of myocardial infarction. Abbreviations: MI= Myocardial Infarction; ovx = ovarectomy; E2= 17 β–estradiol: MPA= Medroxyprogesterone acetate.

Table 1. Morphometric left ventricular analysis for infarct size determination.

	intact Sham MI	ovx sham MI	intact MI	ovx MI + placebo	ovx MI + E2	ovx MI + E2 + MPA
n	***10***	***8***	***7***	***8***	***8***	***6***
septum mm	2,4 ± 0,1	2,5 ± 0,1	2,0 ± 0,1	2,3 ± 0,1	2,1 ± 0,2	2,4 ± 0,2
free wall mm	2,6 ± 0,1	2,9 ± 0,1	0,8 ± 0,1	0,8 ± 0,1	0,9 ± 0,1	1,0 ± 0,1
cavity area mm2	7,2 ± 0,7	7,7 ± 1,0	25,2 ± 3,1 *§	28,6 ± 3,1*§	21,6 ± 2,9*§	26,8 ± 2,6*§
LV area mm2	53,6 ± 1,2	57,3 ± 2,1	61,1 ± 4,3 *§	69,6 ± 3,5*§	56,5 ± 4,4	69,7 ± 3,6*§
infarcted epicardium (A)	0	0	12,8 ± 0,6	14,4 ± 0,7	11,9 ± 0,9	14,7 ± 0,9
total epicardium (B)	26,8 ± 0,3	27,7 ± 2,1	29,1 ± 1,0	30,9 ± 0,8	28,4 ± 0,9	31,8 ± 0,5
infarcted endocardium (C)	0	0	10,4 ± 0,6	10,5 ± 0,9	9,9 ± 0,9	12,0 ± 0,8
total endocardium (D)	12,2 ± 0,5	12,6 ± 0,7	21,5 ± 0,9	22,3 ± 1,0	19,8 ± 1,3	24,0 ± 1,1
LV diameter mm	8,3 ± 0,2	8,6 ± 0,2	8,9 ± 0,5	10,1 ± 0,3*§	8,8 ± 0,5	9,9 ± 0,2*§
infarct size %	0	0	46,3 ± 1,3	47,0 ± 2,2	46,0 ± 4,0	48,1 ± 2,1

Abbreviations: MI= Myocardial Infarction; ovx = ovarectomy; E2= 17 β–estradiol: MPA= Medroxyprogesterone acetate; LV= left ventricle. Infarct size was calculated using the following formula: %infarcted myocardium= (A/B+C/D)/2, where A= Infarcted epicardium; B= Total epicardium; C= Infarcted endocardium; D= total endocardium. * $p<0,01$ vs. intact + sham MI; § $p<0,01$ vs. ovx + sham MI; # $p<0,01$ vs. ovx + E2 + MPA. Values represent mean ± standard error of the mean.

2.2.2. Global measurements.

Body weight was higher in ovarectomized compared to intact rats and not altered by myocardial infarction (table 1). Hormone substitution with 17 β-estradiol but not with 17β-estradiol plus MPA reduced the body weight in ovariectomized rats. Uterus atrophy among ovarectomized animals were efficiently prevented by hormone replacement with 17β-estradiol and not affected by additional MPA treatment. Lung weight was increased in all rats following myocardial infarction but reached statistical significance only in animals receiving 17β-estradiol plus MPA (table 2).

2.2.3. Cardiac hypertrophy.

Myocardial infarction resulted in a significant increase of absolute as well as relative heart weight normalized to tibia length in comparison to the hearts of sham operated rats (table 2). The increase of absolute heart weight reached a similar extent in animals with intact ovaries, ovariectomized or estradiol substituted rats. Combined treatment with 17β-estradiol plus MPA further aggravated cardiac hypertrophy following myocardial infarction (+19.4 % vs. ovx + MI + E2, $p<0.05$). Analysis of cardiac hypertrophy at the single myocyte level revealed increased cardiac myocyte cross-sectional areas in infarcted rats that was similar in the ovariectomized, non ovariectomized and estrogen substituted rats (Fig. 8). Combination of 17β-estradiol with MPA further increased the cardiomyocyte cross sectional area. (+33,3 % vs. ovx + MI + E2).

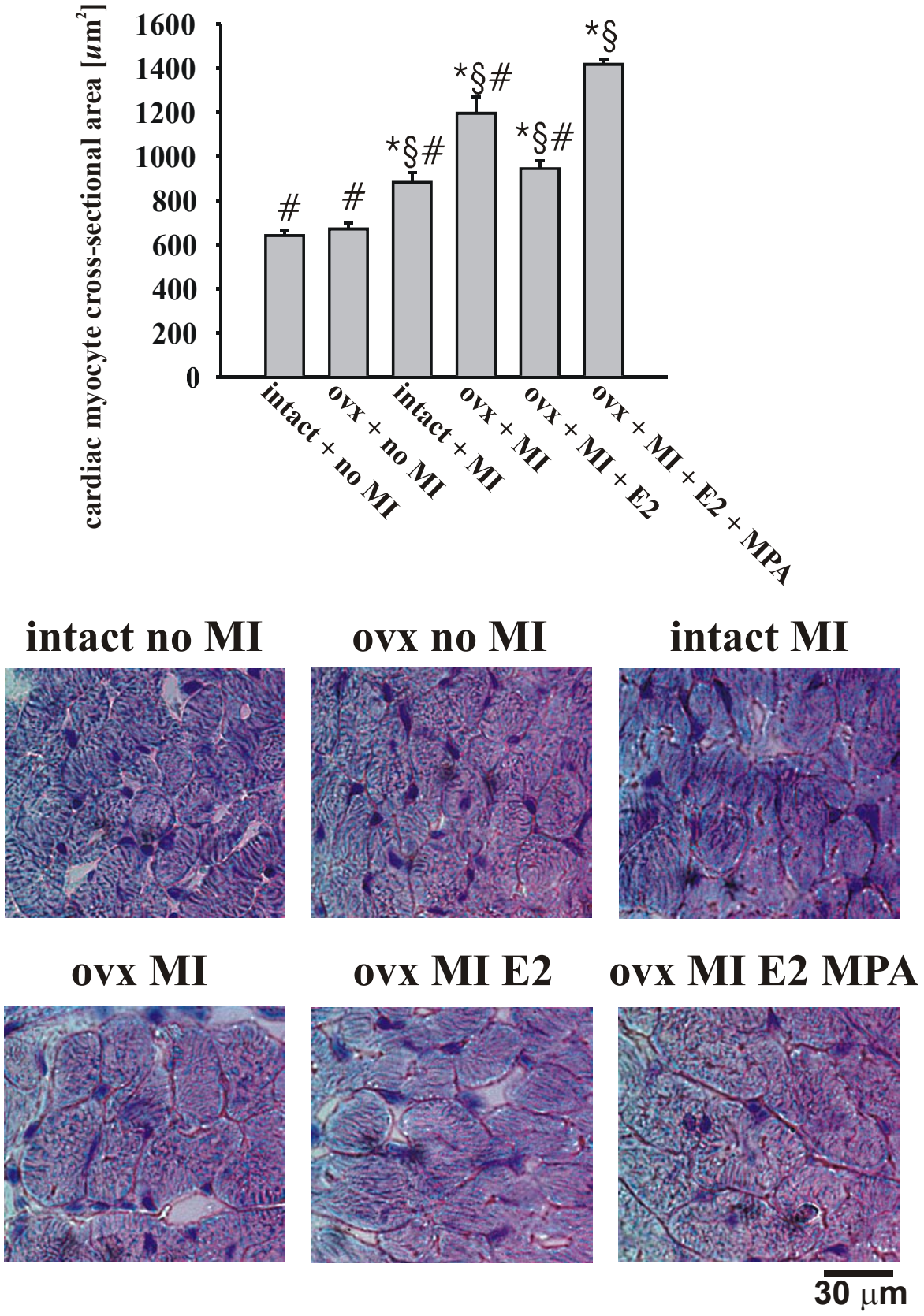

Figure 8. Cardiomyocyte Cross Sectional Area. Cardiomyocyte hypertrophy was present in all animals post myocardial infarction. Hypertrophy was increased by supplementation with 17 –estradiol plus MPA. (A) Absolute values; (B) Microphotographs of cardiac cross sections stained with hematoxilin.

2.2.4. Hemodynamic analysis.

Mean blood pressure and heart rate were similar among all animals indistinct from treatment. Left ventricular end diastolic pressure (LVEDP) was elevated post myocardial infarction and highest in rats receiving 17β- estradiol plus MPA (+17,6% vs. ovx MI E2 Table 2).

2.2.5. Echocardiography.

Echocardiographic data demonstrated that systolic and diastolic diameters were significantly increased in infarcted vs. non infarcted rats. Left ventricular systolic areas were significantly larger in infarcted compared to sham operated rats (Table 2, Fig. 9). Ovariectomy or hormone substitution with 17β-estradiol didn't altered increased left ventricle systolic area. The combination of estrogen supplementation with MPA resulted in a further and significant enlargement of LV systolic areas ($p<0.01$ vs. ovx + MI + E2). Increased systolic and diastolic dimensions resulted in impaired left ventricular performance as indicated by lower left ventricular fractional shortening in the rats receiving 17 β–estradiol plus MPA ($p<0.01$ vs. ovx + MI + E2; Table 2 and Fig. 9).

Table 2. Global, hemodynamics and echocardiography measurements.

	intact sham MI	ovx sham MI	intact MI	ovx MI + placebo	ovx MI + E2	ovx MI + E2 + MPA
n	***10***	***10***	***8***	***7***	***8***	***6***
body weight [g]	231 ± 6§	288 ± 6*	242 ± 4§	285 ± 9*	247 ± 6§	264 ± 15
rel. heart weight	203	236	309	305	295	366
[mg/cm tibia]	± 6,5#	± 8#	± 22*§	± 7*§	± 19*§#	± 30*§
heart weight [mg]	669	787	1027	1001	974	1210
	± 21#	± 28#	± 72*§#	± 30*§#	± 56*§#	± 110*§
lung weight [mg]	1131 ± 23	1195 ± 40	1867 ± 316	1547 ± 138	1864 ± 263	2092 ± 417
uterus weight [mg]	635 ± 96§	140 ± 27*	680 ± 101§	92 ± 11*#	441 ± 72§*	348 ± 21*
hemodynamics						
heart rate [bpm]	404 ± 11	360 ± 10	364 ± 19	362 ± 12	348 ± 14	402 ± 19
LVEDP [mmHg]	5 ± 1	7 ± 1	24 ± 4*§	19 ± 3*§#	28 ± 4*§	34 ± 7*§
mean BP [mmHg]	121 ± 3	127 ± 2	112 ± 5	118 ± 6	112 ± 4	114 ± 5
LVEDA [mm2]	33 ± 2 #	44 ± 2#§	72 ± 6*§#	75 ± 9*§#	68 ± 4*§#	91 ± 6*§
LVESA [mm2]	12 ± 2 #	19 ± 1#§	53 ± 4*§#	54 ± 8*§#	53 ± 4*§#	65 ± 2*§
FS %	48 ± 5	37 ± 2	13 ± 2*§#	15 ± 4*§#	16 ± 2*§#	7 ± 1*§

* p<0,01 vs. intact+ sham MI; § p<0,01 vs. ovx + sham MI; # p<0,01 vs.ovx+E2+MPA; LVEDP= Left Ventricle End Dyastolic Pressure; FS= Fractional Shortening; LVESA= Left Ventricle End Systolic Area; LVEDA= Left Ventricle End Diastolic Area. Values represent mean ± standard error of the mean.

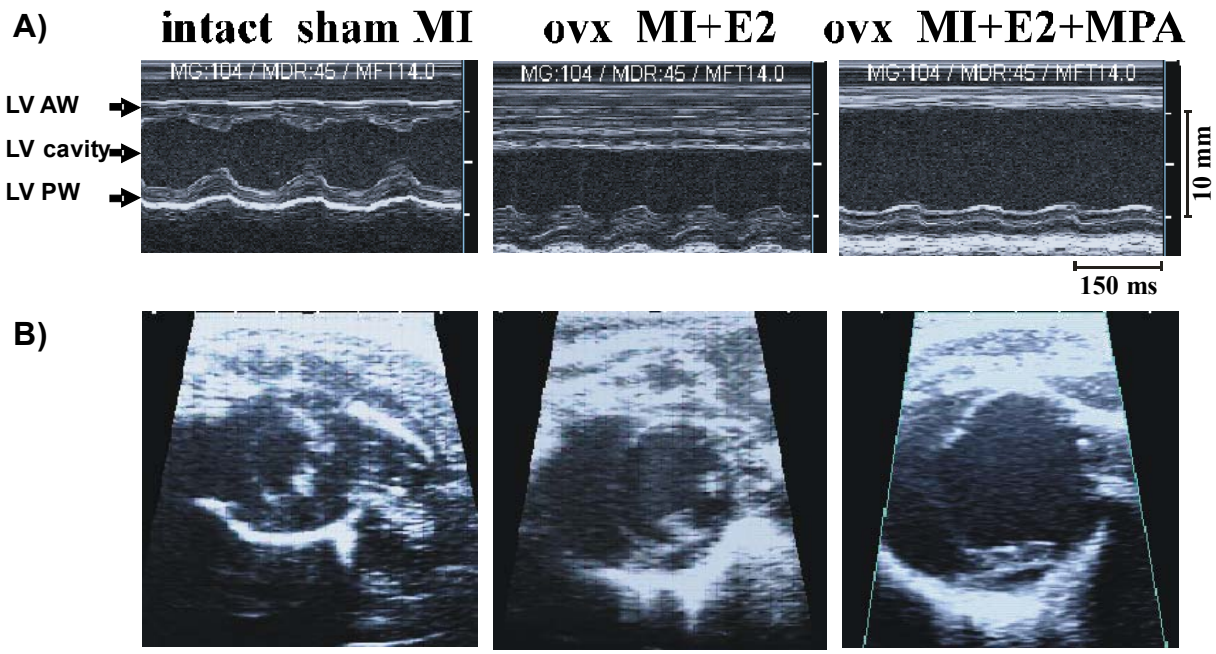

Figure 9. Echocardiographic analysis. Representative echocardiograms obtained from non-infarcted (sham) and infarcted rats receiving hormone supplementation with 17β- estradiol or 17β- estradiol plus MPA. Myocardial infarction resulted in ventricular dilatation and loss of anterior wall motion. 17β- estradiol plus MPA treatment aggravated left ventricular dysfunction post myocardial infarction (A: M-mode) and ventricular dilatation (B: 2D). LV= left ventricule; AW= anterior wall; PW= posterior wall.

2.2.6. Left ventricular ROS generation

APF staining and detection by fluorescence microscopy of left ventricular cross-sections from non-infarcted ovariectomized and intact rats showed low signal intensities indicating low levels of basal ROS generation (Fig. 10). Fluorescence intensity was below the detection range in negative control sections pre-treated with SOD and catalase. Signal intensity increased modestly in LV sections from intact, ovarectomized and estrogen substituted rats subjected to myocardial infarction. However, maximum fluorescence intensities were detected in LV sections obtained from infarcted rats receiving combined treatment with 17β-estradiol and MPA.

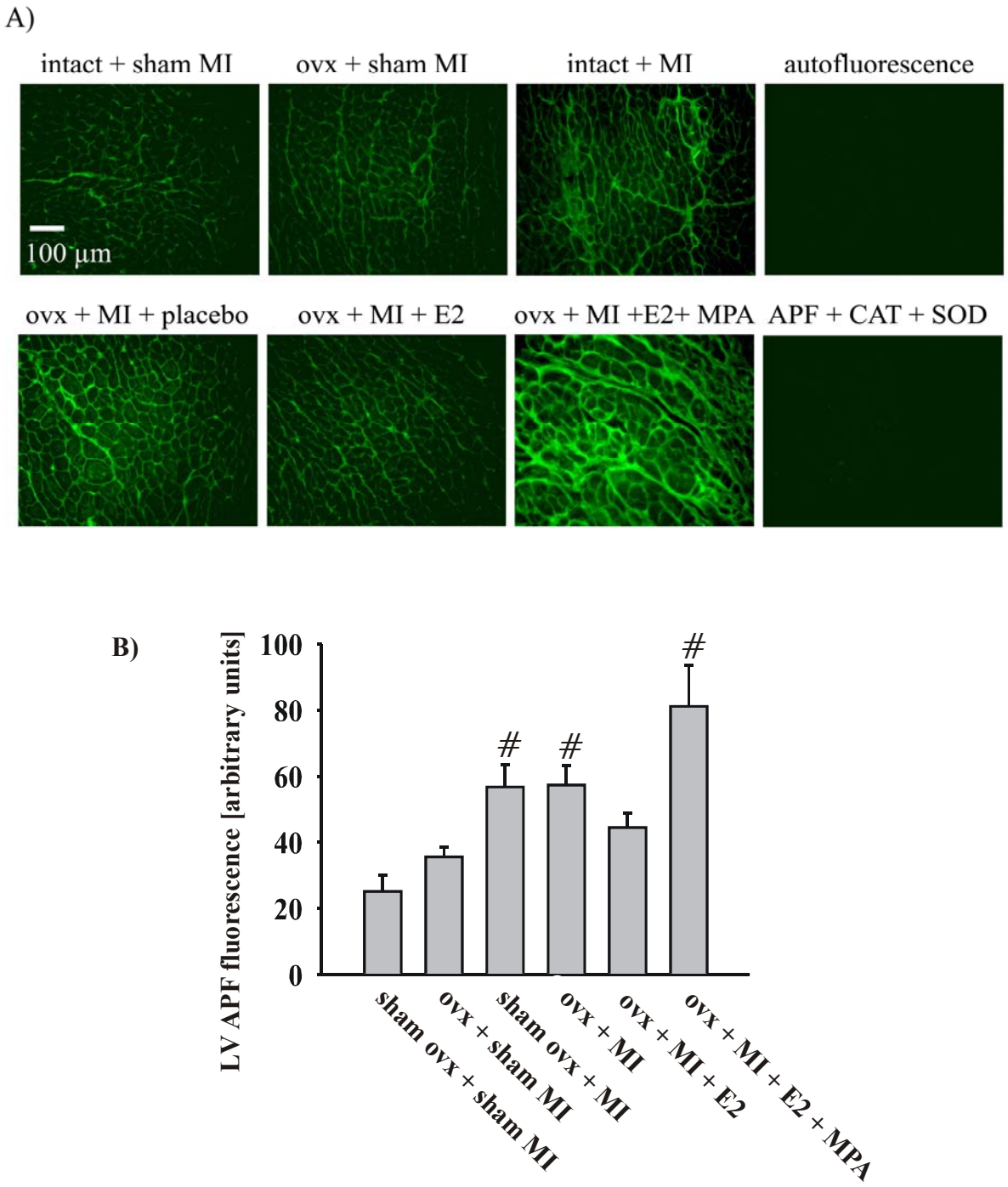

Figure 10. Left ventricle ROS generation. Myocardial ROS production was detected by APF staining and was only slightly increased in the ovariectomized myocardial infarction group. However supplementation with 17β-estradiol plus MPA induced a marked increase in the fluorescent signal. Specificity of the signal was proved by the absence of fluorescence in sections pre-treated with catalase and SOD. No autofluorescence was detected in the absence of APF. (A: representative microphotographies, original magnification X200; B: bar graph that illustrated the APF staining intensity analysis).

2.2.7. Cardiac gene expression.

Myocardial gene expression was analyzed in extracts from the left ventricle remote of the infarct. P67phox and rac-1 basal expression in sham operated rats was low and was not affected by ovariectomy or myocardial infarction. Hormone supplementation with 17β–estradiol plus MPA during myocardial infarction resulted in a significant increase of the expression of rac-1 and p67phox (Fig.11).

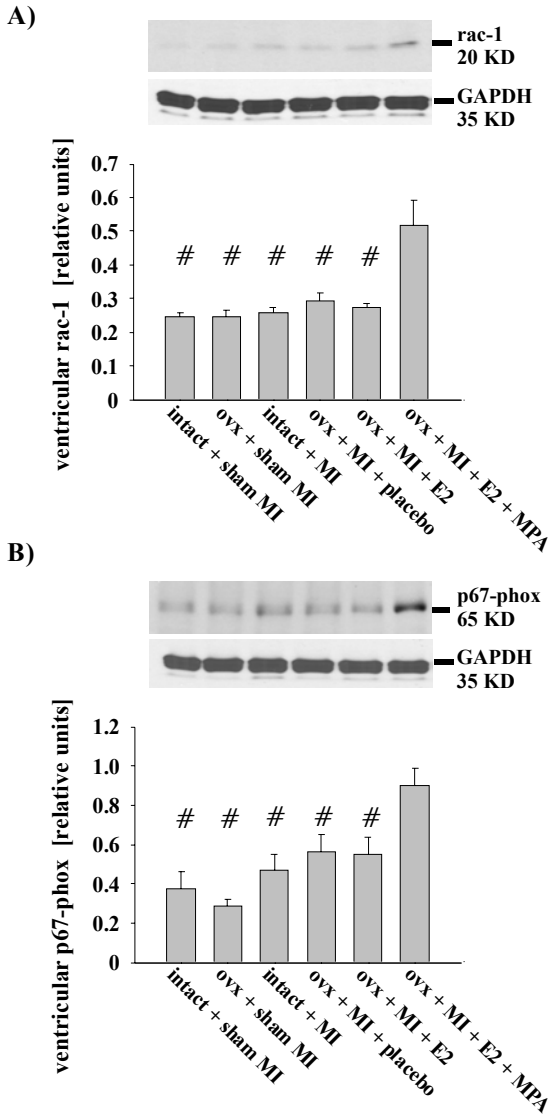

Figure 11. Left ventricular expression of the NAD(P)H oxidase sub-units, rac-1 (A) and p67phox (B), exhibited low baseline levels which were not significantly changed by myocardial infarction or hormone status. Treatment of myocardial infarcted rats with 17β –estradiol plus MPA induced significant increases of p67phox and rac-1.

The expression levels of proteins that block ROS generation such as MnSOD, eNOS, P-eNOS^{ser1177}, iNOS and nNOS were not different among all treatment groups (Fig 12). Local left ventricular pro-ANP expression didn't change due to myocardial infarction, however hormone supplementation with 17β– estradiol plus MPA resulted in increased pro-ANP levels (Fig 13). SERCA2a expression was homogeneous in all groups, levels of its inhibitory protein phospholamban were also similar between the groups (Fig 14). Phospholamban phosphorylation, decreased to a significant extent in infarcted rats receiving placebo or 17 β–estradiol plus MPA (Fig 13). Protein levels of ER-α and ER-β were not different among all treatment groups (Fig 14).

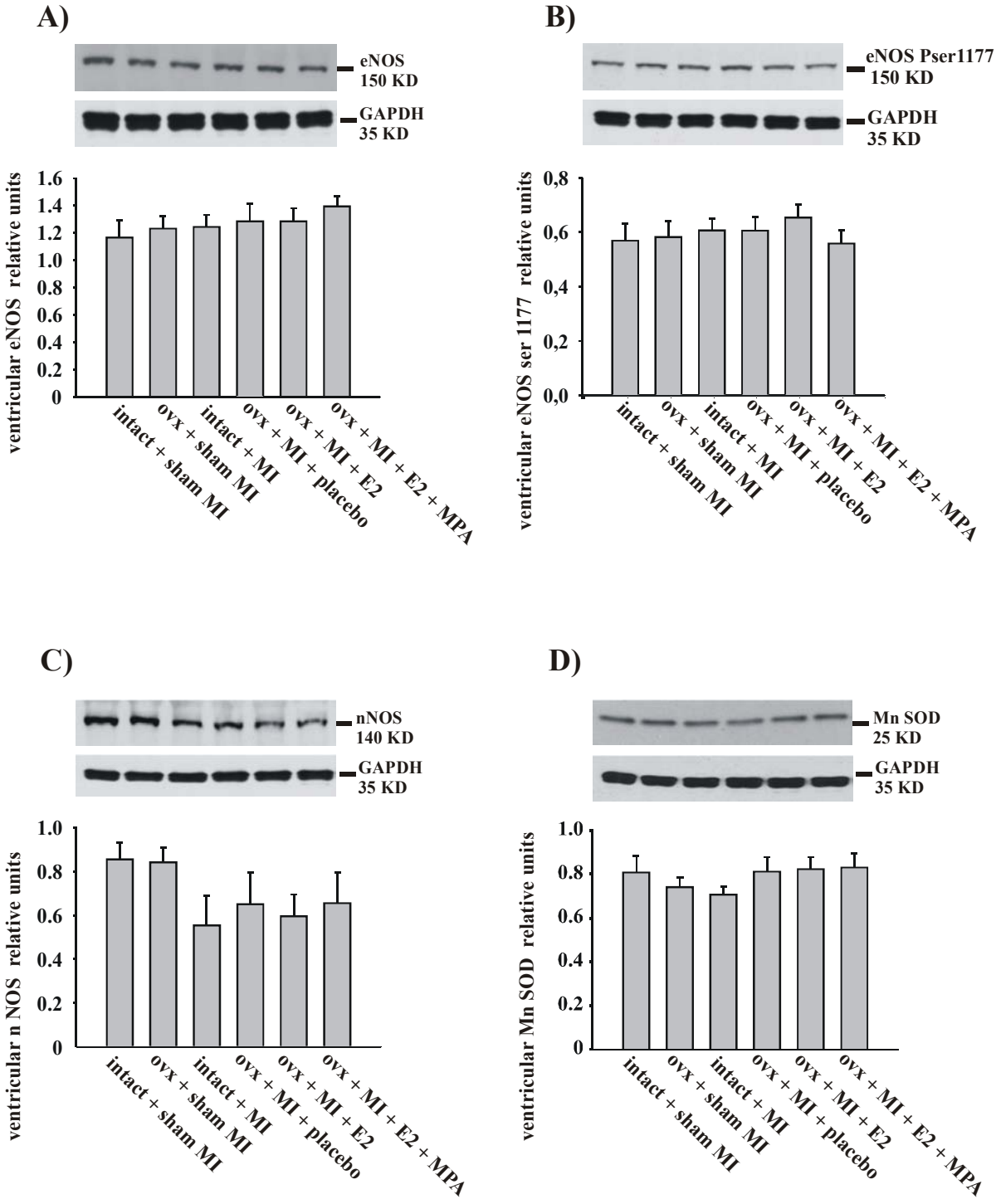

Figure 12. Left ventricular expression of proteins involved in anti-oxidative mechanisms (A) eNOS, (B) P-eNOS^{ser1177}, (C) nNOS and (D) MnSOD ,were not significantly affected by the treatments.

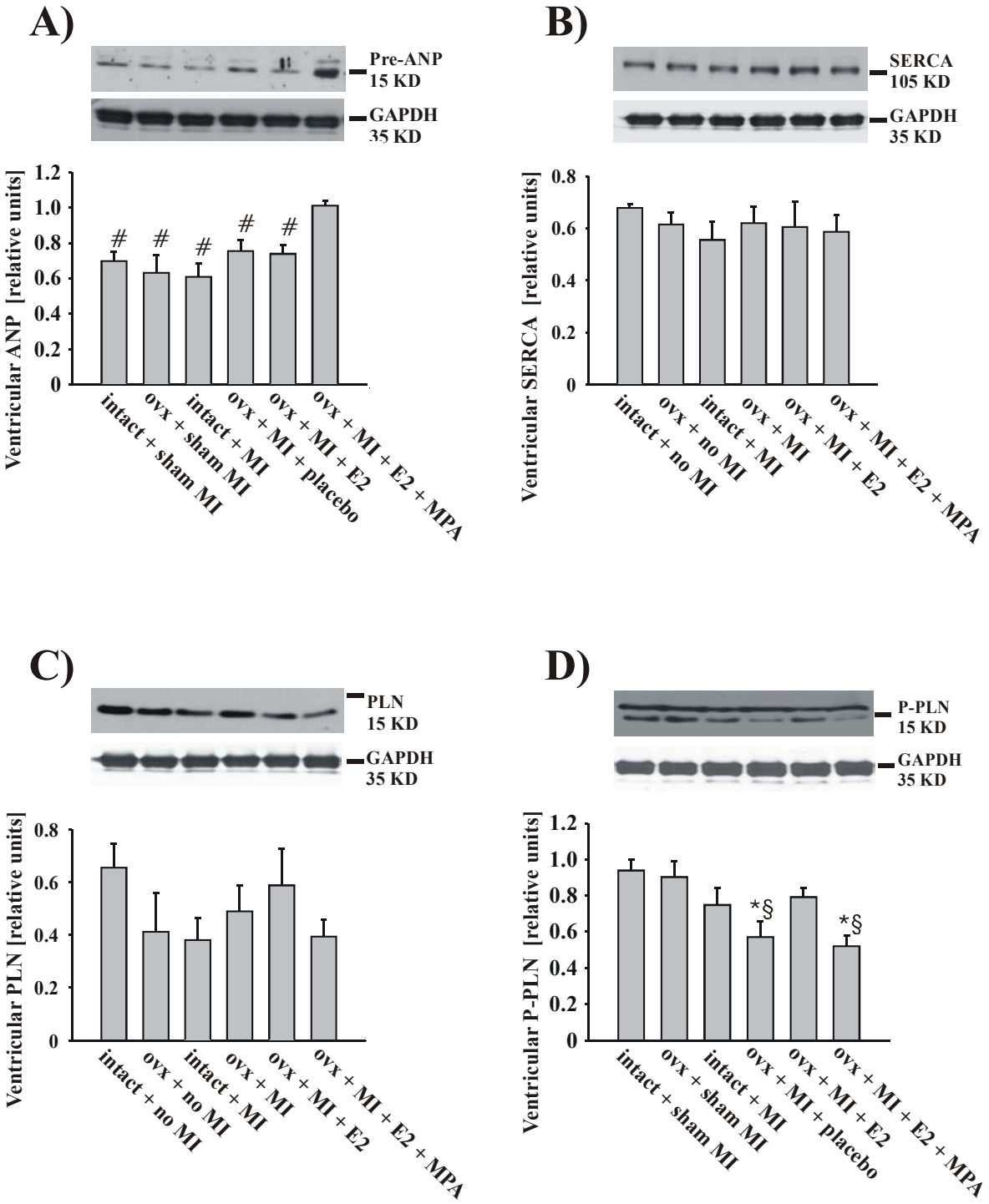

Figure 13. Left ventricular expression of calcium handling proteins and ANP. Ventricular pre-ANP (A)was increased only in infarcted rats treated with 17β- estradiol plus MPA. SERCA (B) and phospholamban (C) expression didn't change significantly due to the treatments, the phosphorylated form of phospholamban (D) was significantly reduced in ovariectomized infarcted rats and in 17 β–estradiol plus MPA treated rats.

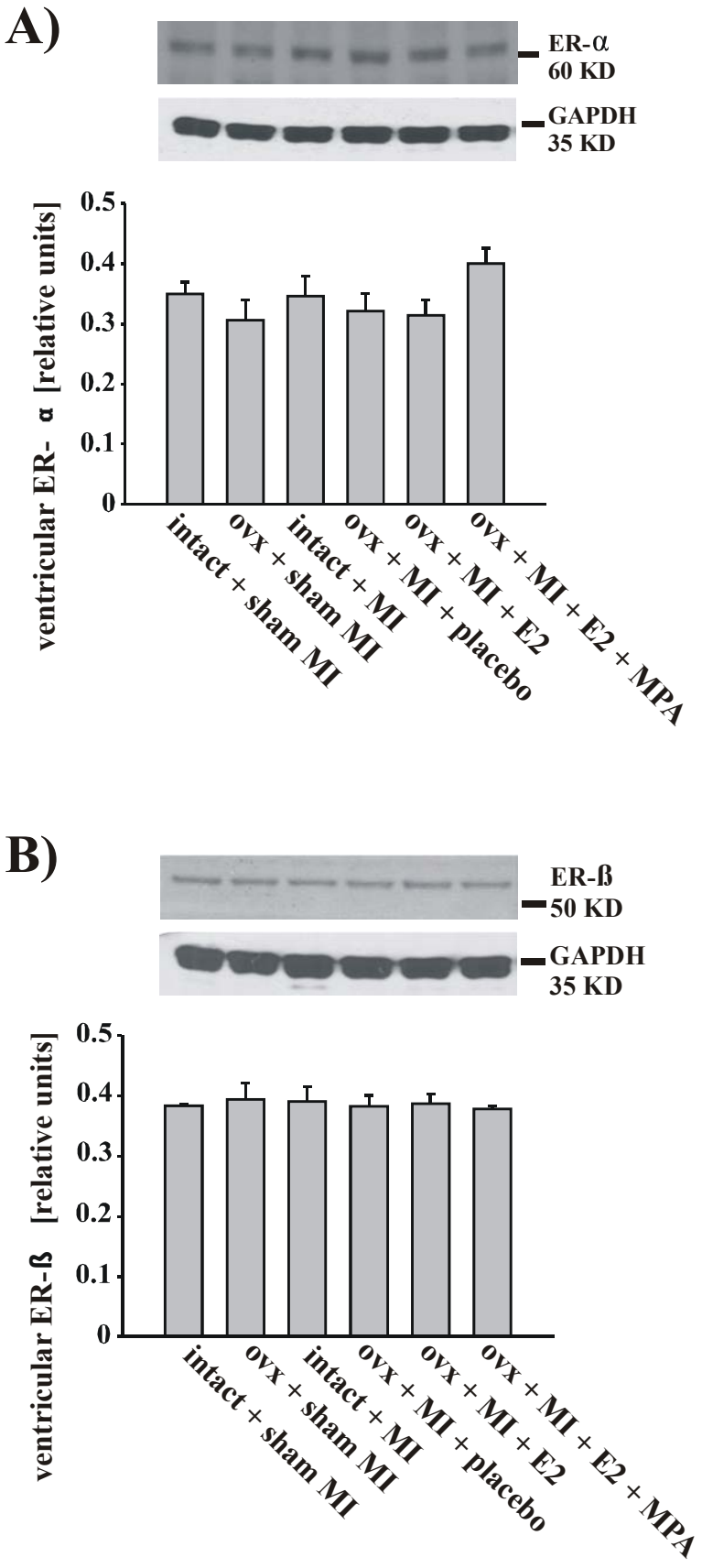

Figure 14. Protein expression of ER-α (A) and ER-β (B) were comparable among all groups.

2.2.8. Serum hormone levels

As a result of the ovariectomy the synthesis of the three major steroid precursor-hormones (progesteron, 17-hydroxy progesterone and androstenedione) were decreased significantly (Fig 15). Corticosterone, aldosterone and cortisol serum levels were only decreased by the co-treatment estradiol plus MPA (Fig 16 A,B,C). Serum levels of ACTH (adrenocorticotrophin hormone), hormone that controls the hypothalamic-pytuitary axis, were also low in the estradiol plus MPA group (Fig 17). Possible physiological evidence of hypoaldosteronism or lack of MR activity we analyzed by measuring angiotensin II serum levels and serum electrolytes, which were similar among all groups (Table 3). In a similar way glucose serum levels, as a marker of glucocorticoid activity, were not different among groups. Finally ovariectomy resulted in low estradiol serum levels that were recovered by hormone replacement with 17β-estradiol and were not affected by additional MPA treatment or myocardial infarction (Fig. 16 D).

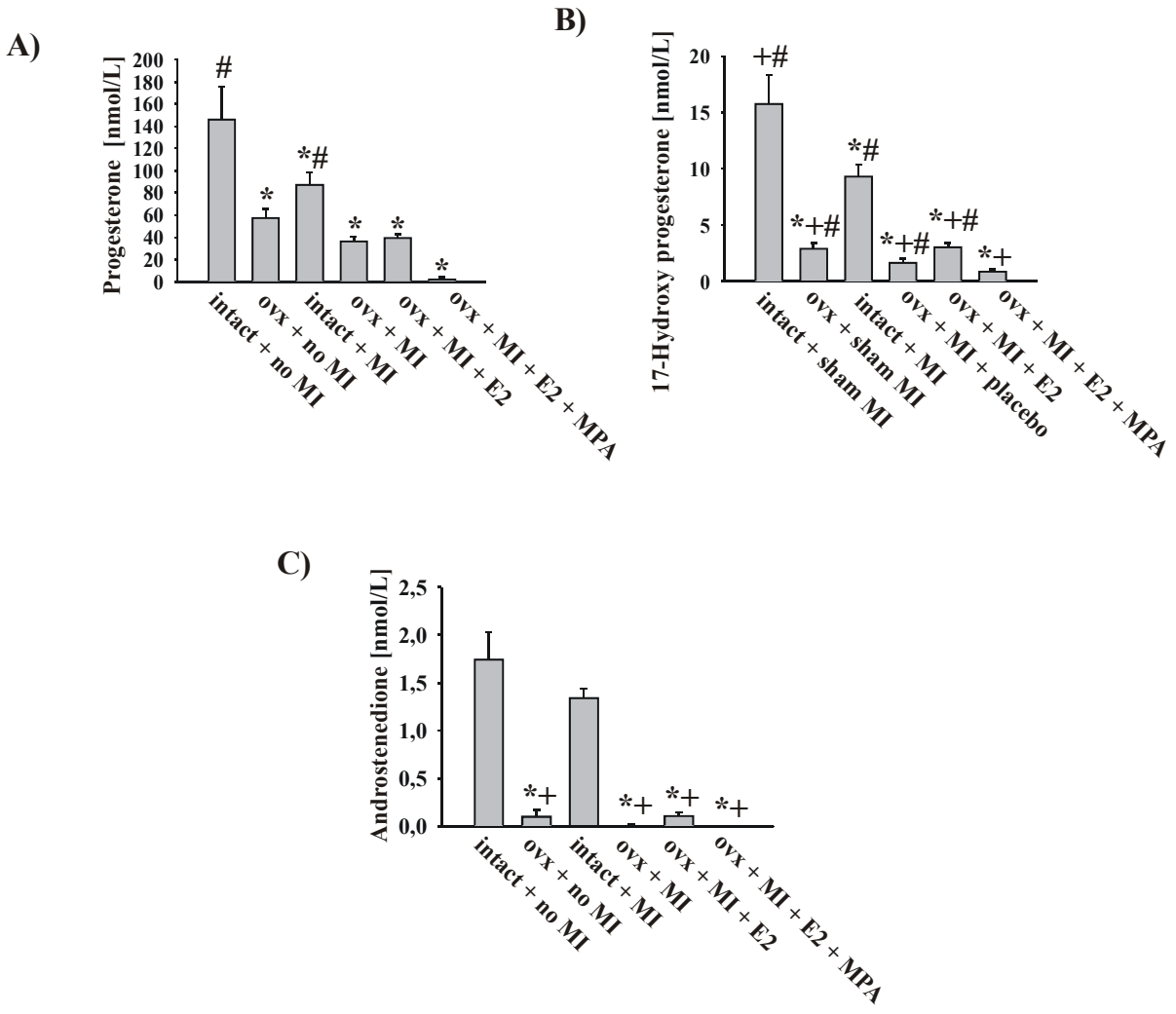

Figure 15. Analysis of progesterone, 17-OH progesterone and androstenedione serum levels by RIA. Progesterone serum levels (A) are reduced significantly by ovariectomy, treatment with estradiol and MPA result in a further reduction. Serum levels of 17-OH progesterone (B) and androstenedione (C) show the same pattern.***** $p<0,01$ vs. intact+ sham MI; **#** $p<0,01$ vs.ovx+E2+MPA; **§** $p<0,01$ vs. ovx + E2; **+** $p<0,01$ vs. intact + MI.

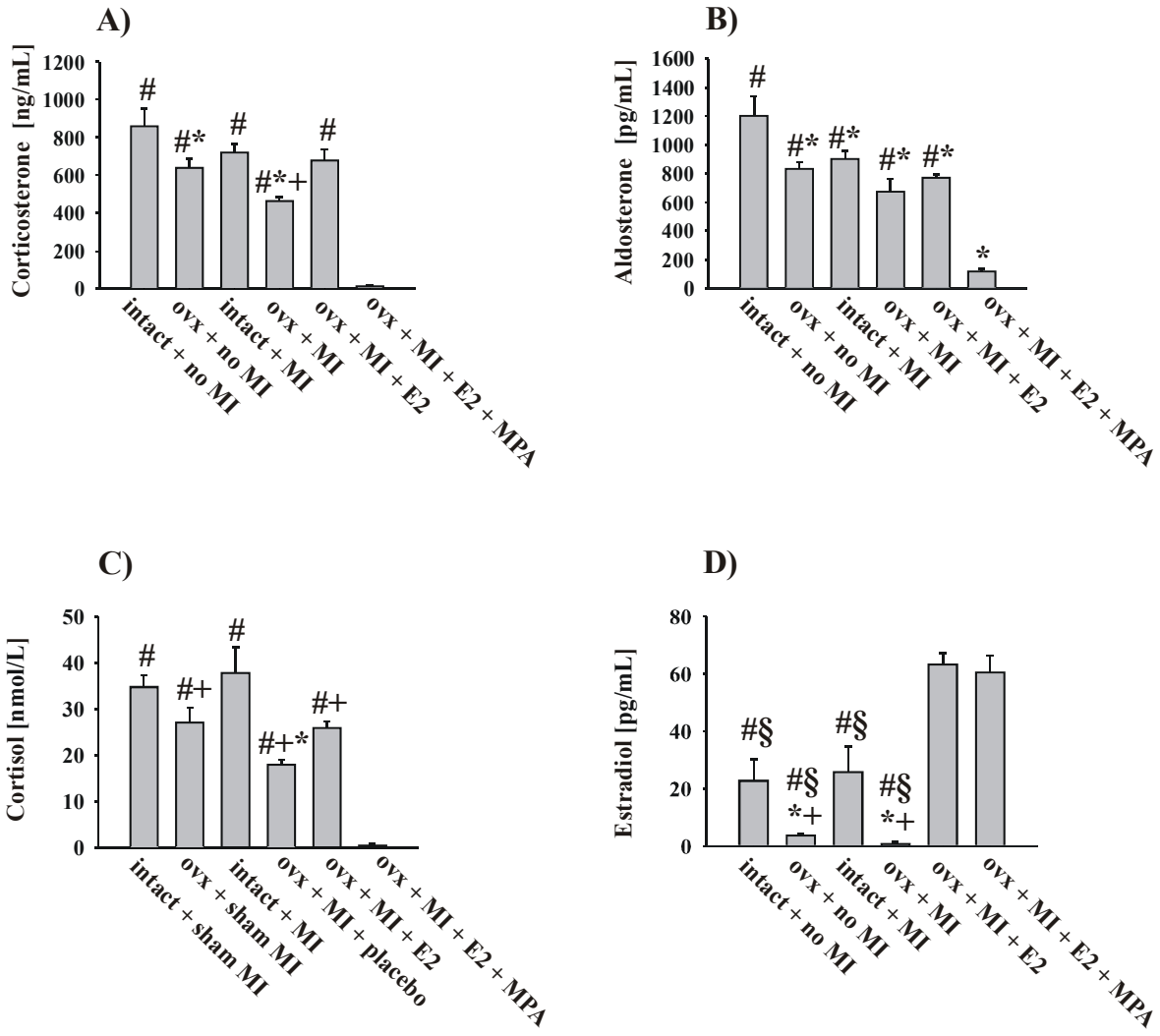

Figure 16. Analysis of corticosterone, aldosterone, cortisol and estradiol serum levels by RIA. Aldosterone (B) serum levels and its precursor corticosterone (A) show a marked suppression by treatment with estradiol plus MPA. Cortisol (C) serum levels follow the same pattern. Estradiol (D) serum levels are lower in ovariectomized rats and increase upon estradiol supplementation**.** * $p<0,01$ vs. intact+ sham MI; # $p<0,01$ vs.ovx+E2+MPA; § $p<0,01$ vs. ovx + E2; + $p<0,01$ vs. intact + MI.

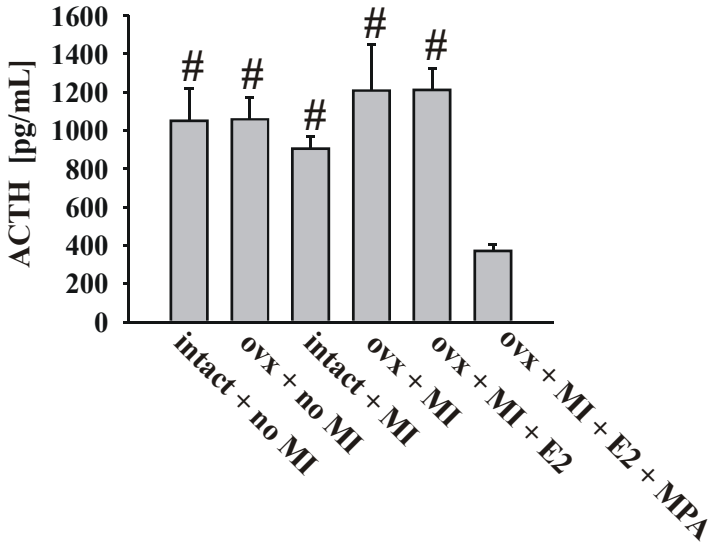

Figure. 17. ACTH serum levels are suppresed by estradiol plus MPAtreatment. # p<0,01 vs.ovx+E2+MPA.

Table 3.Serum parameters for the evaluation of mineralocorticoid and glucocorticoid activity.

	intact sham MI	**ovx sham MI**	**intact MI**	**ovx MI + placebo**	**ovx MI + E2**	**ovx MI + E2 + MPA**
n	***10***	***8***	***7***	***8***	***8***	***6***
angiotensin II [pg/100 uL]	130 ± 9	60 ± 8 *+	110 ± 23	60 ± 3*+	74 ± 7*	98 ± 8
sodium [mmol/L]	138 ± 0,52	138 ± 0,91	137 ± 0,59	138 ± 0,43	137 ± 0,44	140 ± 0,77
potasium [mmol/L]	4,5 ± 0,12	5,3 ± 0,10	4,8 ± 0,27	5,4 ± 0,15	5,3 ± 0,13	5,5 ± 0,21
glucose [mg/dL]	227 ± 21	232 ± 22	247 ± 18	221 ± 16	209 ± 14	231 ± 28

Abbreviations: MI= Myocardial Infarction; ovx = ovarectomy; E2= 17 β–estradiol: MPA= Medroxyprogesterone acetate. Values represent mean ± standard error of the mean. * p<0,01 vs. intact+ sham MI; + p<0,01 vs. intact + MI.

2.3. Discussion.

The results presented indicate that treatment with 17β- estradiol plus MPA aggravates the symptoms of heart failure in rats with myocardial infarction. Native ovarian hormones or 17β-estradiol supplementation did not affect infarct size or development of chronic heart failure post myocardial infarction. Therefore the deleterious effects of MPA are most likely not due to the activation of the progesterone receptors or inhibition of estradiol beneficial effects, but maybe intrinsic to this compound.

The role of 17β- estradiol during myocardial infarction has been studied by several groups, however due to different experimental designs its effects remained unclear. Cavasin et al (Cavasin, 2003) concluded that estrogens prevent the deterioration of cardiac function and remodelling after MI in mice. Whereas van Eickels et al. (van Eickels, 2003) concluded that, despite of a reduction of infarct size and cardiomyocyte apoptosis, estrogen replacement increases ventricular remodelling and mortality in mice after MI. In ovariectomized rats, Smith et al reported an increase in MI size with E2 replacement, whereas Nekooeian and Pang (Nekooeian, 1998) reported that E2 replacement had no effect on MI size and caused minor changes in LV function. After chronical myocardial infarction (8 weeks) on rats Hügel et al (1999) (Hugel, 1999) concluded that endogenous estrogen deficiency does not have major effects on cardiac hyperthrophy, dilatation and dysfunction. With a similar design and treatment time Beer S. et al 2006 (Beer, 2006) using supra physiological dosis reported that estradiol treatment prevented development of post-MI remodelling. Also using supra-physiological dosis Dean S. 2006 (Dean, 2006) reported that estradiol does not enhance or hinder the development of LV dysfunction. Taken together, these observations indicate a neutral effect of estradiol replacement. Differences among various studies might arise from other variables as estradiol dosage, treatment time, via of hormone substitution, exclusion or inclusion of animals with small infarct sizes as well as different estrogen receptor expression between rats and mice. However unopposed estrogen supplementation do not resemble HRT

in women with an intact uterus, because women require a combination of estrogens and progestins to prevent endometrial proliferation and malignancy. Experimental animal studies conducted without a progestin co-treatment do not mimic combined HRT and could not thus answer the question whether MPA might have contributed to the unfavourable outcomes of clinical studies on the safety of HRT such as the HERS and WHI trials. The results published by the WHI study support the view that HRT with a combination of CEE and MPA increases risks of coronary heart disease. Although informative for the understanding of the relationships between HRT and cardiovascular disease, these results can not be extrapolated to all kinds of HRT as the CEE only arm of the WHI study did not suggest an excess of adverse outcomes. In addition different animal models and in vitro experiments provide evidence that MPA, and hence the HERS and WHI studies, have unique features, different from the native progesterone. The specific purpose of this work was thus to evaluate the effects of combined HRT (estradiol plus MPA) vs. unopposed estradiol supplementation during heart failure. A standard dosage of 17-β estradiol that resulted in physiological estradiol serum levels was employed. Under these dosage simultaneous activation of both estrogen receptors by 17-β estradiol or natural ovarian hormones, post myocardial infarction, didn't exert a significant effect on cardiac damage and functionality. However combination of 17 β- estradiol plus MPA aggravated symptoms of heart failure. The relevance of these data is significant as the combination of estradiol plus MPA is the most used standard protocol in HRT. In fact besides of the micronized progesterone, MPA is the most prescribed treatment for HRT in postmenopausal women (Stefanick, 2005).

There is growing evidence suggesting that MPA elicits harmful effects in the cardio-vascular system by either ablating the beneficial roles of estradiol or conferring unfavourable effects by itself. Previous studies in vitro and in vivo have shown that MPA may act very different from native progesterone. In HUVEC cells, the combination of estradiol plus progesterone is innocuous to estrogen induced NO production, while co-treatment with MPA resulted in an

attenuation of NO generation due to eNOS phosphorylation, which decreased eNOS activity (Oishi, 2004; Simoncini, 2004). In primates co-treatment of estradiol with different progestines revealed very different effects, since administration of progesterone did not alter whereas MPA inhibited the vasodilatory effects of estrogen responses by 50% (Williams, 1990; Williams, 1994). In humans, acute 17-β estradiol treatment induced rapid increases in blood flow in peripheral as well as in coronary arteries due to vasodilatation (Gilligan, 1994; Collins, 1995). Similar to the observations in Cynomolgus monkeys co-treatment of estradiol plus progesterone or MPA on coronary blood flow, in postmenopausal women, showed that progesterone has synergistic vasodilatory effects when added to estrogens, while MPA do not share this action. (Rosano, 2000). Along the same line, Jeanes et al. (Jeanes HL, 2006) in an ex vivo model of global ischemia, reported that after 45 minutes of ischemia, pre-treatment with estradiol and MPA inhibited the protective estradiol effect on reduction of the necrotic zone. However effects of MPA on long term myocardial damage after myocardial infarction and the mechanisms by which MPA may aggravate the development of chronic heart failure were not studied or determined. The present results, indicate that deleterious effects induced by MPA are intrinsic and probably exclusive to this compound as native ovarian progesterone did not exert similar effects.

After myocardial infarction the remaining myocardium goes through compensatory mechanisms that eventually can develop in to a failing myocardium with increased hyperthrophy, left ventricle enlargement and impaired systolic function. In the groups receiving myocardial infarction ventricular enlargement was similar independently of the hormonal status (ovariectomized, intact or 17β-estradiol substituted). However co-treatment with 17β-estradiol and MPA induced a further increase of heart weight, cardiomyocyte cross sectional area and cavity dilatation. The deleterious effects of the combination 17β-estradiol and MPA on myocardial function were not due to different infarct size, which was not

different among all animals. Thus, progressive LV dilatation in MPA treated rats arises from increased remodelling of the remote region.

Impairments in blood circulation that accompany heart failure can be traced, in part, to alterations in the activity of the sarcoplasmic reticulum Ca+2 (SERCA) pump that are induced by its interactions with the reversible inhibitor phospholamban. Myocardial infarction in female rats did not affect the expression levels of SERCA nor PLN, but phosphorylation of PLN in the animals receiving 17- estradiol plus MPA was decreased. Suggesting an impairment of calcium re-uptake by the sarcoplasmatic reticulum as part of the mechanism of delayed contractility (fractional shortening) of the MPA plus estradiol treated rats. However this is probably not the only cause of the impaired function induced by MPA as the ovariectomized myocardial infarcted rats receiving only placebo also presented a decrease in PLN phosphorylation. Therefore alternative mechanisms may have play a role in the LV dysfunction of MPA treated rats, one of them could be increase ROS generation. Different lines of evidence have suggested that the myocardial ROS generation could play an important role in remodelling of the failing myocardium.(Bendall, 2002; Nadruz, 2004; Asimakis, 2002; Yoshida, 2000). In addition, in patients with chronic heart failure, increased oxidative stress is associated with reduced left ventricular function and correlates with the severity of left ventricle dysfunction. (Bendall, 2002; Nadruz, 2004; Maack, 2003). Stretch causes ROS production in primary cultures of cardiomyocytes and level dependent hyperthrophy, fetal gene expression and apoptosis (Pimentel 2001). Therefore increased ROS production in the hearts of animals co-treated with 17-β estradiol plus MPA could have contributed to increased heart failure and cardiomyocytes hyperthrophy.

The source of ROS generation in the hypertrophic and failing myocardium has not been clearly identified, but activation of the myocardial NAD(P)H oxydase appears be a major source (Bendall, 2002; Nadruz, 2004; Grieve, 2001; Li, 2002). NADPH oxidase consists of two membrane-bound subunits (gp91and p22phox) and at least three cytoplasmic regulatory

subunits (p47phox; p67phox and Rac). Rac 1 plays an important role in NADPH oxidase activity and hence in ROS generation, because mice expressing a constitutively activated rac-1 protein develop either a dilatative cardiomyopathy and/ or cardiac hypertrophy (Sussman, 2000). Activation of rac-1 in cell free systems and in vascular smooth muscle cells culture is a pre-requisite for NAD(P)H oxidase activation. In the failing human myocardium, up-regulation of rac-1 expression as well as increased rac-1-GTPase activity seems to play a damaging role (Price, 2002; Gorzalczany, 2000; Maack, 2003). Thus it appears possible that increase ROS production in myocardium of myocardial infarcted rats receiving 17-β estradiol plus MPA was a result of an up-regulation of rac-1. Furthermore Pracyc et al 1998 also reported that overexpression of rac-1 in cardiomyocytes leads to an increase of atrial natriuretic peptide secretion similar to what was observed in the local left ventricle ANF production that was only increased in rats co-treated with 17-β estradiol plus MPA. Major differences in rac-1 or p67phox expression induced by myocardial infarction were not detected. However this doesn't exclude the role of the NADPHoxidase or ROS generation in the other groups as the expression and phosphorylation of other subunits of the NADPH oxidase were not studied. Also to consider, rac-1 function in the heart is not only regulated by its over-expression but also by membrane translocation and activation through binding to GTP, parameters that could have been influenced by myocardial infarction. Other sources of ROS including mitochondria, xanthine oxidase, cytochrome P450 based enzymes and dysfunctional NO synthases that were not studied here, might have also contributed to increased ROS generation.

Ovaries are the primary source of sex steroid hormones. Therefore, ovariectomy resulted in reduced serum levels of sexual hormones including progesterone. Interestingly progesterone serum levels were further reduced by estradiol plus MPA. Similarly corticosterone, aldosterone and cortisol were not affected by ovariectomy but dramatically reduced by estradiol plus MPA co-treatment. Despite this significant reduction of serum gluco- and

mineralo- corticoid levels, MPA treated rats did not exhibit systemic signs of gluco- or mineralo- corticoid deficiency such as hypotension, hypoglycemia or serum electrolyte disturbances. In addition, plasma ACTH, which raises adrenal aldosterone and cortisol biosynthesis and release, was suppressed in rats receiving MPA plus estradiol. Previously MPA has been reported to be able to bind and transactivate the mineralocorticoid and glucocorticoid receptor (Sitruk-Ware R, 2004; Schindler AE, 2003; Thomas CP, 2006). Thus the known MR, GR agonist function of MPA could explain both of these findings, the negative feed-back suppression of the hypothalamic-pituitary-adrenal axis and the absence of clinical symptoms of gluco- and mineralo- corticoid deficiency. In support of this concept, serum aldosterone levels have been reported to increase in patients receiving MR-antagonists whereas MPA treatment resulted in a reduction of systemic aldosterone levels in postmenopausal women. (Miya Y, 2002; White WB, 2003; Fraccarollo D, 2005). Because disproportionate MR and GR activation promotes cardiac remodelling and worsens chronic heart failure then increased activation of the MR-GR by MPA may explain the aggravated cardiac phenotype in these rats. In conclusion the combination of estradiol plus MPA, but not estradiol alone aggravates heart failure symptoms in a model of myocardial infarction; corticoid receptor activation and oxidative stress seems to play an important role in the mechanism of damage.

Chapter 3. Medroxyprogesterone-acetate ablates the cardio-protective function of 17β-estradiol and promotes renal damage in aldosterone-salt treated rats.

3.1. Design.

3.1.1. Design for the analysis of cardiovascular effects.

The effects of different hormone replacement therapies regimes on the development of cardiac hypertrophy and hypertension was tested in the Aldosterone Salt Treated rats. Female Wistar rats obtained from IFFA CREDO (Lyon, France) at the age of 12 weeks were randomized into 11 separate groups. Animals in group 1 were sham operated; animals in group 2 were ovarectomized (ovx) and uni-nephrectomized (npx) by right-sided nephrectomy. Groups 3 to 10 were subjected to ovarectomy and uni-nephrectomy plus additional **a**ldosterone-**s**alt **t**reatment (AST) consisting of continuous aldosterone infusion (0.75µg/h via Alzet minipumps model 2004) and 1% NaCl added to tap water. AST rats were treated as follows; group 3: placebo; group 4: 17β-estradiol (2µg/kg/d); group 5: estradiol plus spironolactone (20mg/kg/d); group 6: estradiol plus drospirenone *low dose* (3mg/kg/d); group 7: estradiol plus drospirenone *medium dose* (9mg/kg/d); group 8: estradiol plus drospirenone *high dose* (30mg/kg/d); group 9: estradiol plus medroxyprogesterone-acetate (3mg/kg/d) group 10: placebo plus medroxyprogesterone-acetate and group 11: placebo non ovariectomized rats. After continuous treatment for 8 weeks hemodynamics analysis were performed and body weight, heart weight, uterus weight, kidney weight and tibia length were measured. Relative heart weight was calculated from absolute heart weight and tibia length. Serum estradiol, and angiotensin II levels were measured by radio immunoassays (Estradiol: DPC-Biermann and Angiotensin II: Peninsula). Mid-ventricle sections were employed for histological analysis to measure cardiomyocyte cross sectional area and perivascular collagen. The base of the heart was freeze at -80 and afterwards used for conventional western blot analyzes. In parallel thoracic aortas were isolated and prepared for histology and used for measurement of aorta media area as well as aorta osteopontin expression.

3.1.2. Design for the analysis of renal effects.

In parallel renal damage by different hormone replacement therapies was studied in the Aldosterone Salt Treated rats. In a similar setting as for the analysis of the cardiovascular effects female Wistar rats obtained from IFFA CREDO (Lyon, France) at the age of 12 weeks were randomized into 9 separate groups, corresponding to the same treatment as the groups 1 to 9 described previously. After continuous treatment for 8 weeks the animals corresponding to the groups 1, 2, 3, 4, 8 and 9 were placed in metabolic cages to record water intake and urine production. Afterwards hemodynamics were performed and body weight, heart weight, uterus weight, kidney weight and tibia length were measured. Relative right kidney weight was calculated from absolute right kidney weight vs. tibia length. Serum estradiol, angiotensin II, progesterone, 17-OH progesterone, cortisol, corticosterone and ACTH levels were measured by radio immunoassays (Estradiol, progesterone, 17-OH progesterone, cortisol and corticosterone: DPC-Biermann; angiotensin II: Peninsula; ACTH: DRG). Right kidneys were collected and divided in inter-polar sections that were kept for histology and western blot analysis.

3.2. Results

3.2.1. Cardiovascular effects.

3.2.1.1. Global and hemodynamic measurements

Relative heart weight was significantly higher in aldosterone-salt treated rats compared to sham operated or ovarectomized and nephrectomized animals (+27.3±3.5% vs. sham; $p<0.01$; table 4). Cardiac mass decreased significantly in AST rats receiving 17β-estradiol substitution (-14±2.4% vs. AST placebo, $p<0.01$) but remained elevated in rats treated with estradiol plus medroxyprogesterone-acetate (+24±5% vs. AST + E2; $p<0.01$). The combination of drospirenone and 17β-estradiol reduced relative heart weight to near-baseline levels (Table 4).

Very similar results were obtained for absolute heart weight. Significantly elevated systolic and mean blood pressure levels in aldo-salt treated rats compared to sham operated or ovx/npx rats were reduced substantially by co-treatment with 17β-estradiol (-15±3% vs. placebo, $p<0.01$) (Table 4). Combining drospirenone or spironolactone with E2 substitution caused a further reduction of blood pressure whereas medroxyprogesterone-acetate treatment resulted in increased blood pressure in estradiol substituted rats. Uterus atrophy was observed in ovarectomized rats but not in sham operated or estradiol substituted animals. Uterus weight was lower in estrogen substituted rats receiving drospirenone, unaltered by spironolactone and increased in medroxyprogesterone-acetate treated AST rats. Estrogen serum levels were low in ovarectomized and increased to physiological levels in E2 substituted rats. Endometrial epithelium height was significantly lower in rats receiving estradiol plus medroxyprogesterone-acetate or drospirenone compared to unopposed estrogen substitution. Body weight was higher in estrogen depleted animals and decreased upon estradiol substitution. Heart rate was similar and within physiological limits among all animals (Table 4).

Table 4. Global and hemodynamic measurements for cardiovascular effects of AST treatment.

n	**sham** *10*	**ovx npx** *5*	**ovx AST placebo** *10*	**ovx AST + E2** *10*	**ovx AST+ E2 + spiro** *10*	**ovx AST + E2 + dro 3mg** *10*	**ovx AST + E2 + dro 9mg** *10*	**ovx AST + E2 + dro 30mg** *10*	**ovx AST + E2 + MPA** *10*	**ovx AST + MPA** *5*	**sham ovx +AST** 9
rel. heart weight [mg/cm]	171 ± 2*	176 ± 2*	259 ± 10	219 ± 6*	190 ± 5*	217 ± 14*	196 ± 6*	195 ± 5*	272 ± 11	270 ± 14	221 ± 6*
abs. heart weight [mg]	650 ± 7*	669 ± 6*	997 ± 40	858 ± 24*	741 ± 21*	837 ± 55*	756 ± 24*	763 ± 18*	1055 ± 46	1028±54	863±34
systolic BP [mmHg]	145 ± 3*	143 ± 6*	190 ± 9	161 ± 5*	143 ± 5*	149 ± 4*	150 ± 6*	154 ± 7*	191 ± 12	189±10	160±3*
mean BP [mmHg]	128 ± 2*	132 ± 6*	164 ± 7	131 ± 6*	116 ± 6*	123 ± 4*	120 ± 6*	121 ± 6*	155 ± 8	152±12	139±3*
serum AII [pg/100µl]	79 ± 5*	63 ± 6*	7 ± 1	9 ± 2	46 ± 3*	29 ± 6*	50 ± 5*	43 ± 8*	6 ± 2	6 ± 2	4 ± 1
uterus weight [mg]	699 ± 88*†	101 ± 6	107 ± 6	447 ± 47*	497 ± 64*	275 ± 9*†	283 ± 20†	242 ± 12†	533 ± 23*	233±16†	660±113*†
serum E2 [pg/ml]	25 ± 8*	2 ± 1	4 ± 3	22 ± 4*	19 ± 3*	14 ± 3*	19 ± 3*	26 ± 7*	18 ± 7*	2 ± 1*	16 ± 5*
epithelium height [µm]	*nd*	*nd*	*nd*	26 ± 1	*nd*	*nd*	*nd*	18 ± 1†	17 ± 1†	*nd*	*nd*
body weight [g]	268 ± 5*	297 ± 8	326 ± 8	287 ± 5*	287 ± 6*	295 ± 8	286 ± 5*	296 ± 12	277 ± 14*	298±5	257±8*
heart rate [bpm]	420 ± 12	411 ± 20	395 ± 15	394 ± 7	414 ± 9	410 ± 7	416 ± 9	418 ± 11	381 ± 14	361±17	367±12

All measurements were performed after 8 weeks of treatment. Significances: * $p<0.05$ vs. ovx AST placebo; † $p<0.05$ vs. ovx AST + E2. Abbreviations: *nd* = not done; AST (aldosterone-salt treatment); MPA (medroxyprogesterone-acetate); E2 (17□-estradiol), ovx (ovarectomy); npx (nephrectomy); dro (drospirenone); spiro (spironolactone); AII (angiotensin II); bpm (beats / minute); BP (blood pressure).

3.2.1.2. Serum angiotensin II and electrolyte levels.

Constant infusion of aldosterone resulted in a significant decrease of serum angiotensin-II levels compared to sham operated or ovx/npx animals (-90±2.5% vs. sham, $p<0.001$). Serum angiotensin II levels were not affected by estradiol or medroxyprogesterone-acetate treatment. Combination of estradiol with the mineralocorticoid antagonist spironolactone or with drospirenone blocked the suppression of serum angiotensin II levels (Table 4).

3.2.1.3. Cardiac myocyte cross-sectional area.

Cardiac hypertrophy and a patchy pattern of perivascular fibrosis were evident from the gross-morphological appearance of cardiac cross-sections (figure 18A and 18B). Average cardiac myocyte cross-sectional area was elevated in AST compared to sham operated rats (+128±12% vs. sham, $p<0.001$; figure 19) and decreased substantially with estradiol substitution (-57±1.3% vs. AST-placebo, $p<0.001$). No additional effect was observed in groups receiving estradiol plus drospirenone or spironolactone. Medroxyprogesterone-acetate blocked the decrease of cardiac myocyte cross-sectional area in estrogen substituted rats (+45±5% vs. AST-E2) but, by itself, did not aggravate myocyte hypertrophy.

3.2.1.4. Perivascular collagen accumulation.

Aldosterone-salt treatment resulted in significantly higher levels of perivascular collagen deposition compared to sham treatment or uni-nephrectomy / ovarectomy (+300±33% vs. sham, $p<0.001$; figure 20). Estradiol treatment completely blocked perivascular fibrosis in AST rats (-277±31% vs. AST-placebo, $p<0.001$) and no further reduction of collagen deposition was observed in animals co-treated with E2 plus drospirenone or spironolactone. Medroxyprogesterone-acetate aggravated perivascular fibrotic lesions and blocked the inhibitory effect of 17β-estradiol on perivascular fibrosis.

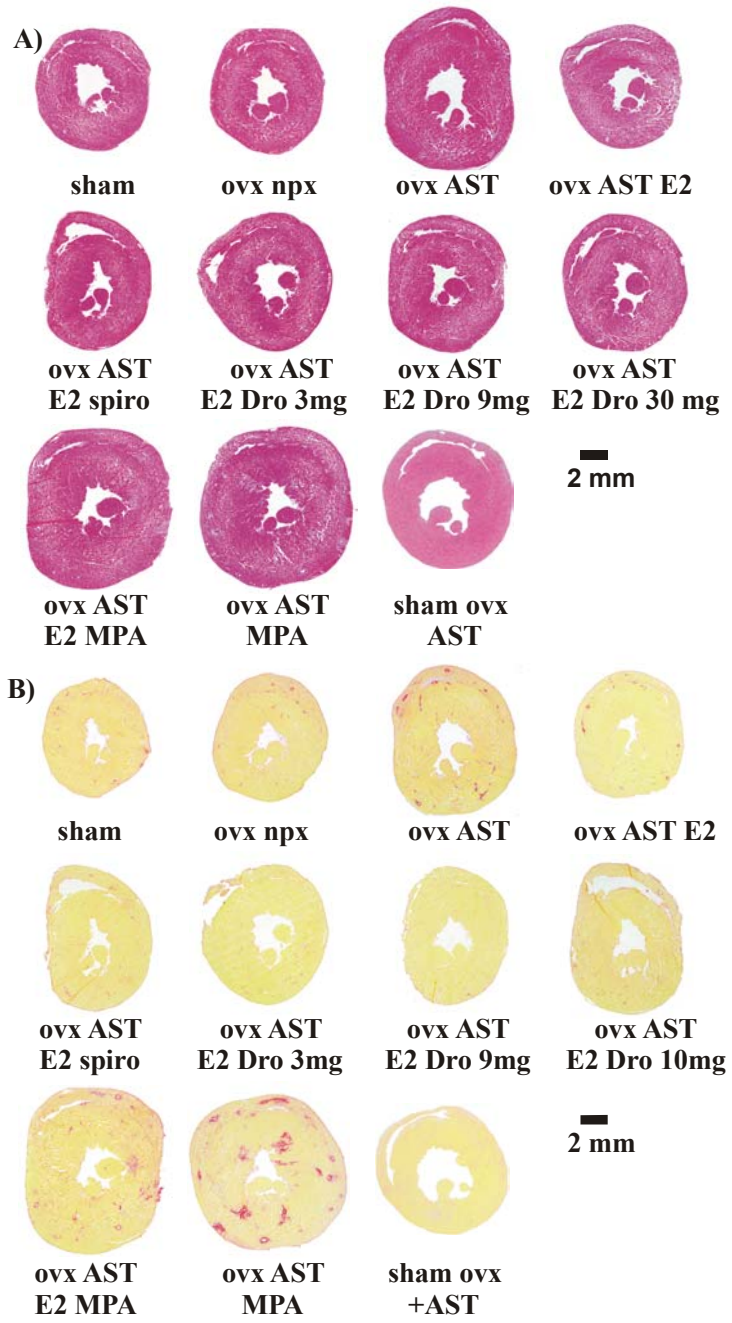

Figure 18: Panel A Cardiac histo-morphology. HE stained cross-sections illustrate cardiac hypertrophy in ovarectomized AST rats compared to sham operated or ovarectomized-npx rats. Cardiac mass decreased in AST rats treated with 17β-estradiol (E2), E2 plus spironolactone, E2 plus drospirenone (3, 9, 30mg/kg/d) but not in AST rats treated with medroxyprogesterone-acetate (MPA) or E2 plus MPA. Panel B Cardiac fibrosis. Cardiac cross-sections stained with picro sirius red illustrate increased perivascular fibrosis in ovarectomized AST rats that was attenuated by E2, intact ovarian hormones, E2 plus spironolactone, E2 plus drospirenone whereas MPA increased cardiac collagen deposition.

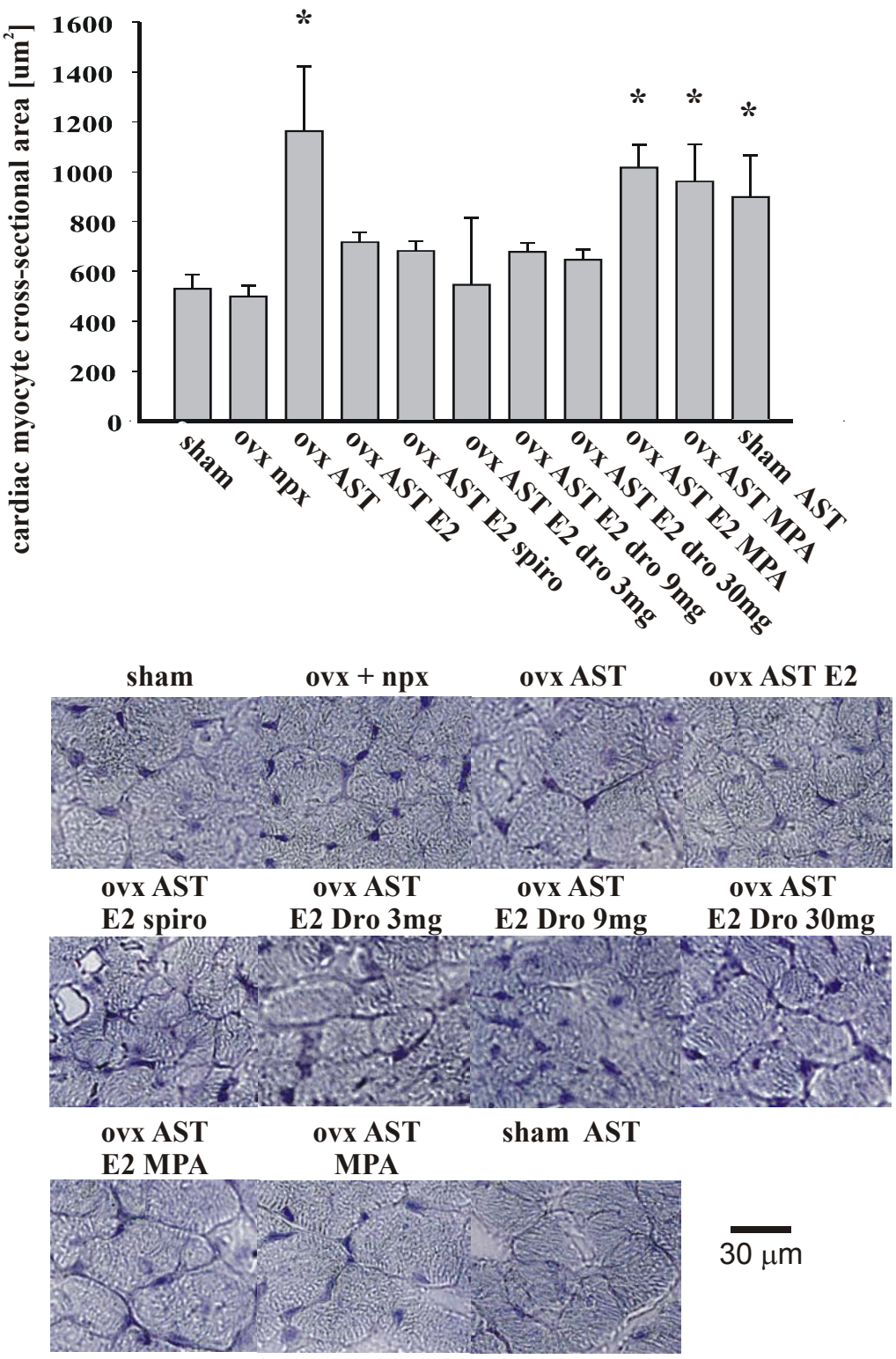

Figure 19: Cardiac myocyte cross-sectional area. Increased cardiac myocyte cross-sectional areas in ovarectomized aldo-salt treated rats compared to control were normalized by 17β-estradiol, intact ovarian hormones, E2 plus spironolactone and E2 plus drospirenone but remained elevated in medroxyprogesterone-acetate or medroxyprogesterone-acetate plus estradiol treated rats. A) Bar graph; B) Photomicrographs illustrate representative HE stained myocardial cross-sections. n=5-10 animals/group; * $p<0.05$ vs. sham.

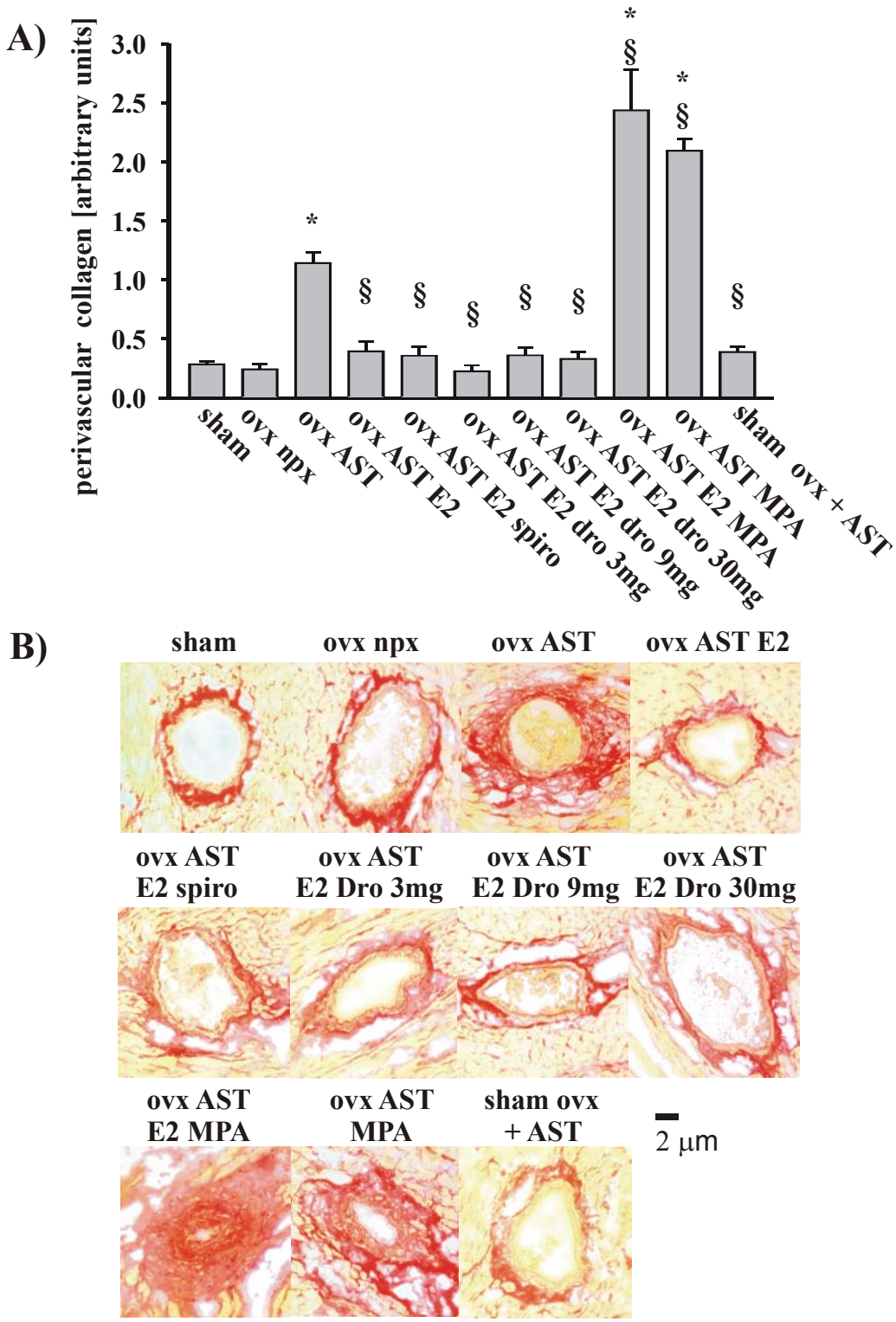

Figure 20: Perivascular collagen content. Increased perivascular collagen accumulation in ovarectomized AST rats compared to control was attenuated by treatment with either 17β-estradiol, intact ovarian hormones, E2 plus spironolactone and E2 plus drospirenone whereas medroxyprogesterone-acetate resulted in increased perivascular fibrosis. A) Bar graph; B) Photomicrographs illustrate representative vascular cross-sections (picro sirius red stain). n=5-10 animals/group; * $p<0.05$ vs. sham; § $p<0.05$ vs. ovx AST.

3.2.5. Aortic intima to media ratios

Aortic intima to media ratios increased in placebo treated AST rats compared to sham operated or ovarectomized and uni-nephrectomized animals (figure 21). Combining estrogen substitution with AST treatment resulted in reduced intima to media ratios that nearly reached physiological levels. No additional effect on vascular remodeling was observed in animals receiving 17β-estradiol plus drospirenone or spironolactone. Highest intima to media ratios were observed in AST rats receiving estradiol plus medroxyprogesterone-acetate.

3.2.6. Vascular osteopontin expression

Osteopontin expression was higher in aortic specimens of ovarectomized aldo-salt treated rats compared to sham operated or ovarectomized and uni-nephrectomized animals (figure 22). 17β-estradiol blocked the local accumulation of osteopontin in the aortic media and intima layer. Similar results were obtained when E2 substitution was combined with drospirenone or spironolactone. In contrast, medroxyprogesterone-acetate prevented the reduction of vascular osteopontin expression in estrogen substituted AST rats. But by itself MPA did not increase aortic osteopontin content.

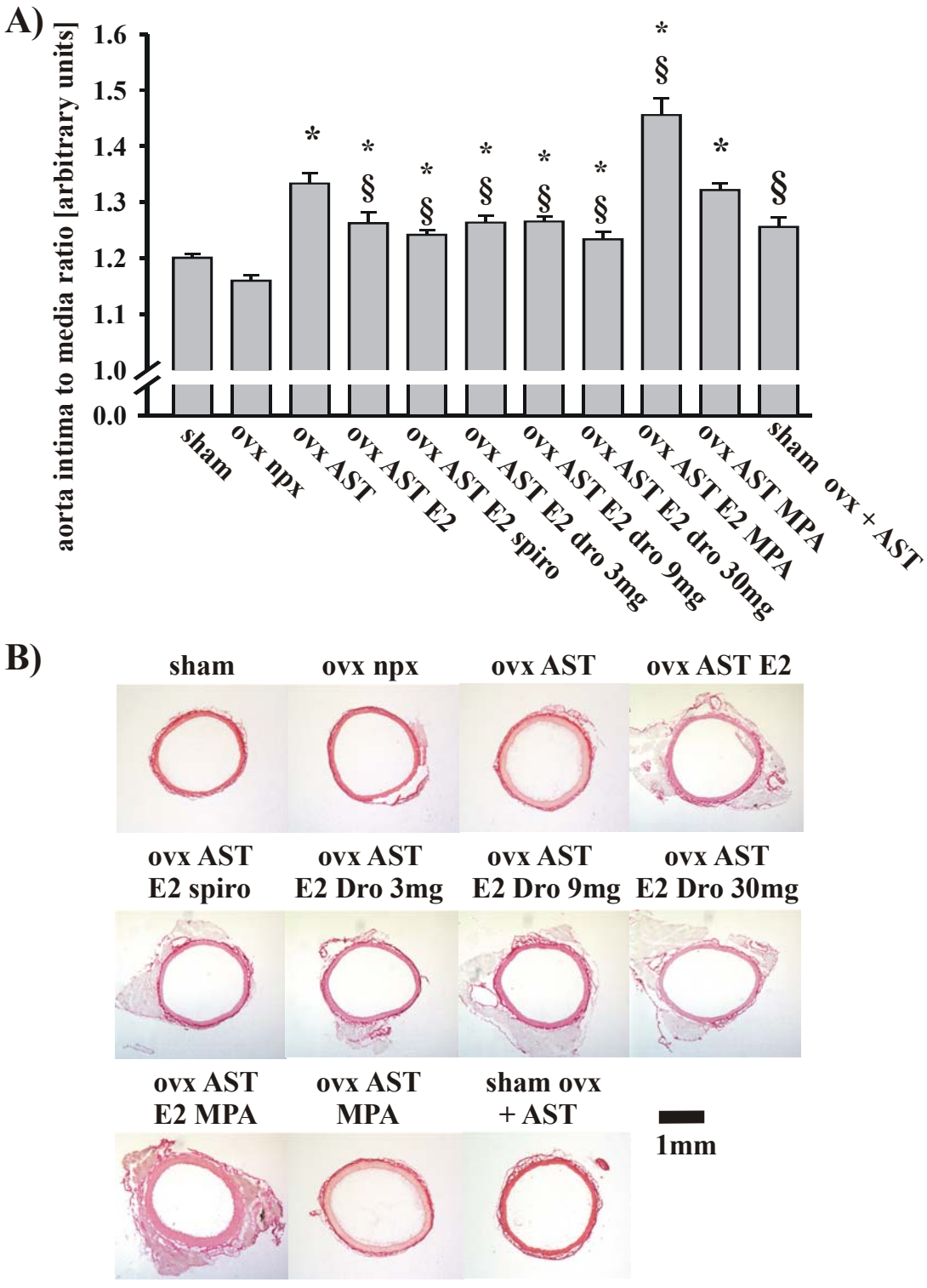

Figure 21: Aortic intima to media ratios. Increased aortic intima to media ratios in ovarectomized AST rats compared to control were decreased by treatment with either 17β-estradiol, intact ovarian hormones, E2 plus spironolactone and E2 plus drospirenone whereas co-treatment with medroxyprogesterone-acetate resulted in media hypertrophy and increased intima to media ratios. A) Bar graph; B) Photomicrographs illustrate representative HE stained aortic cross-sections. n=5-10 animals/group; * $p<0.05$ vs. sham; § $p<0.05$ vs. ovx AST.

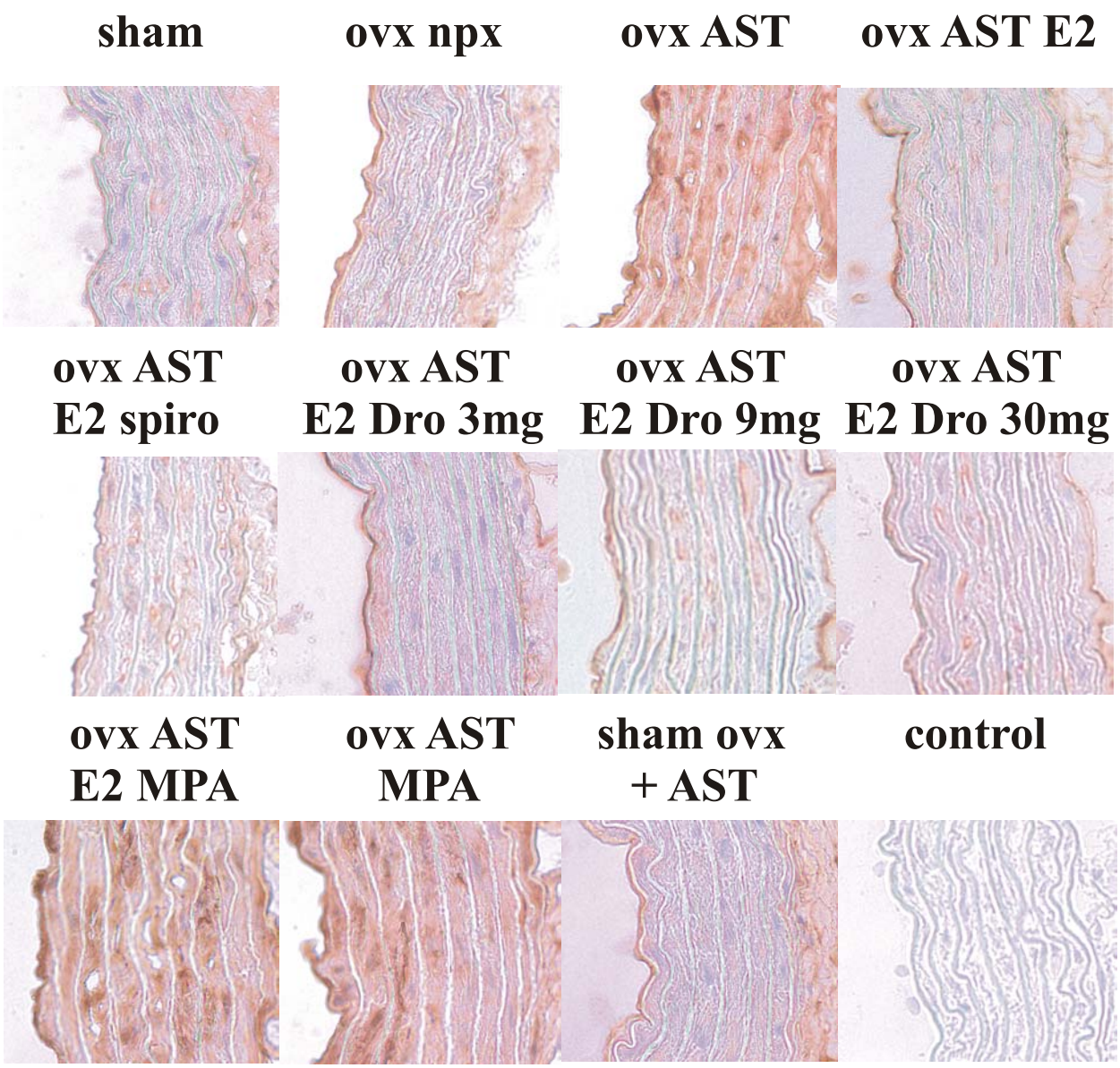

Figure 22: Vascular osteopontin expression. The photomicrographs illustrate representative immunohistochemistry stainings for aortic osteopontin expression from all treatment groups including negative control (opn antibody omitted). Increased vascular osteopontin expression in ovarectomized AST rats was attenuated by treatment with 17β-estradiol, E2 plus spironolactone and E2 plus drospirenone but remained elevated with administration of medroxyprogesterone-acetate.

3.2.1.7. Cardiac protein expression.

Left ventricular expression of cox-2 was significantly increased upon MPA treatment and co-treatment with estradiol prevented this up-regulation (Fig 23). Analysis of the regulatory subunits of the NADPH oxidase rac-1 and p67 phox, revealed that while rac-1 expression was homogeneous among all groups p67phox was induced by MPA (Fig 24). The expression of MnSOD and eNOS, iNOS, ER-α, ER-β, ANP, AT1 and eNOS phosphorilation in SER1177 were similar in all groups (Fig 25).

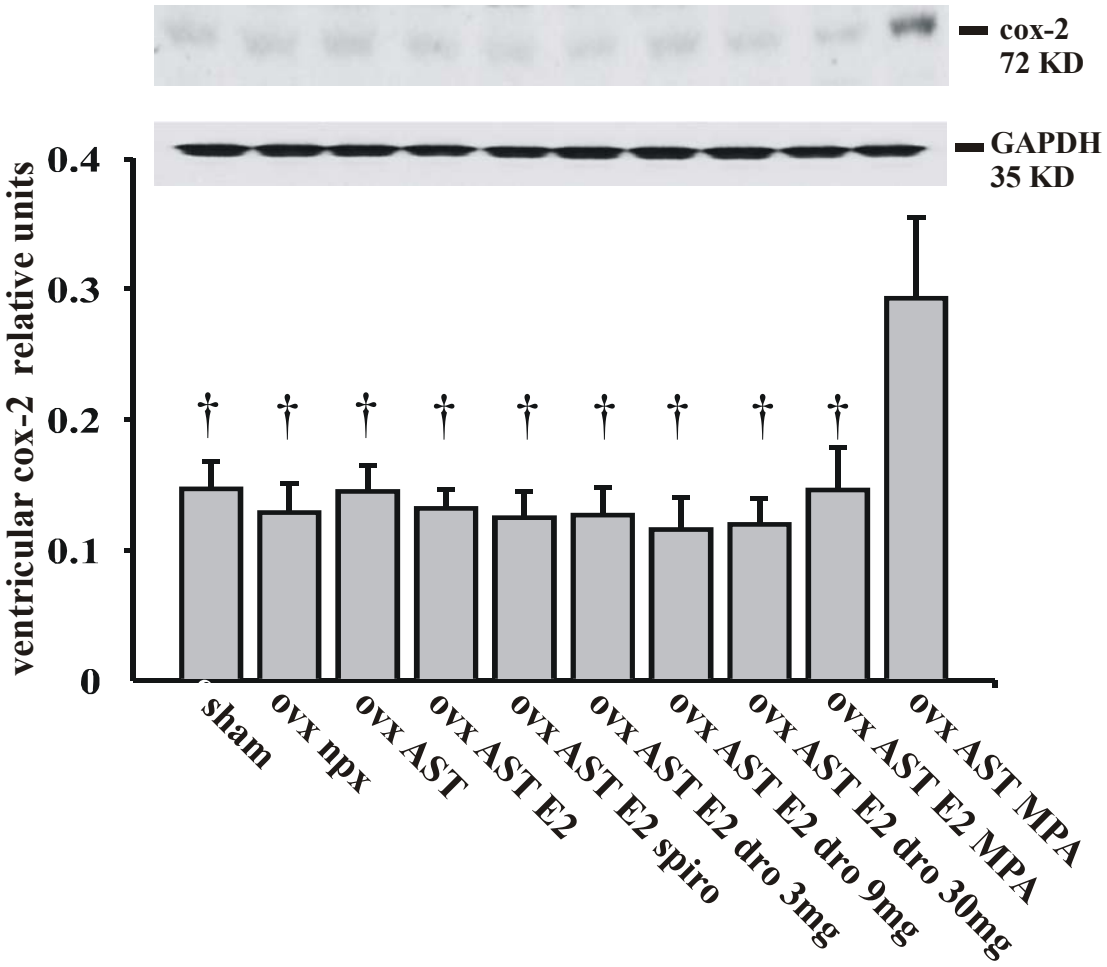

Figure 23. Expression of the inflammation marker cox-2 in cardiac extracts. Cox-2 was increased only in rats treated with MPA, while co-treatment with estradiol decrease its expression to normal baseline levels. Estradiol or AST by itself didn't present any effect in this marker. † p<0,001 vs. MPA AST.

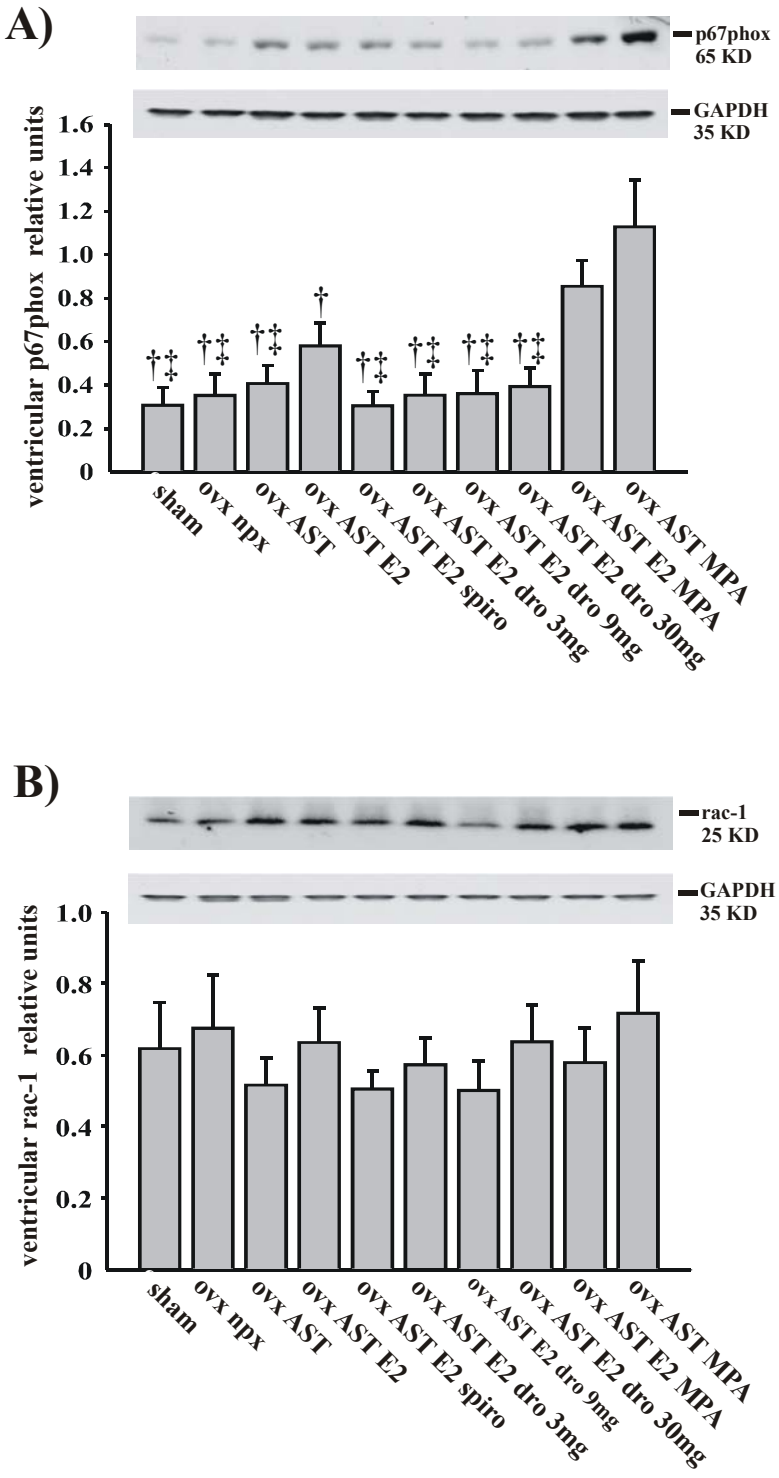

Figure 24. NAD(P)H oxidase sub-units expression, p67phox (A) and rac-1 (B). Expression of rac-1 was similar in all treatment groups. p67phox exhibited low baseline levels which were not significantly changed by AST, however co-treatment with MPA increased its expression. † p<0,001 vs. MPA AST; ‡ p<0,001 vs. MPA+E2 AST.

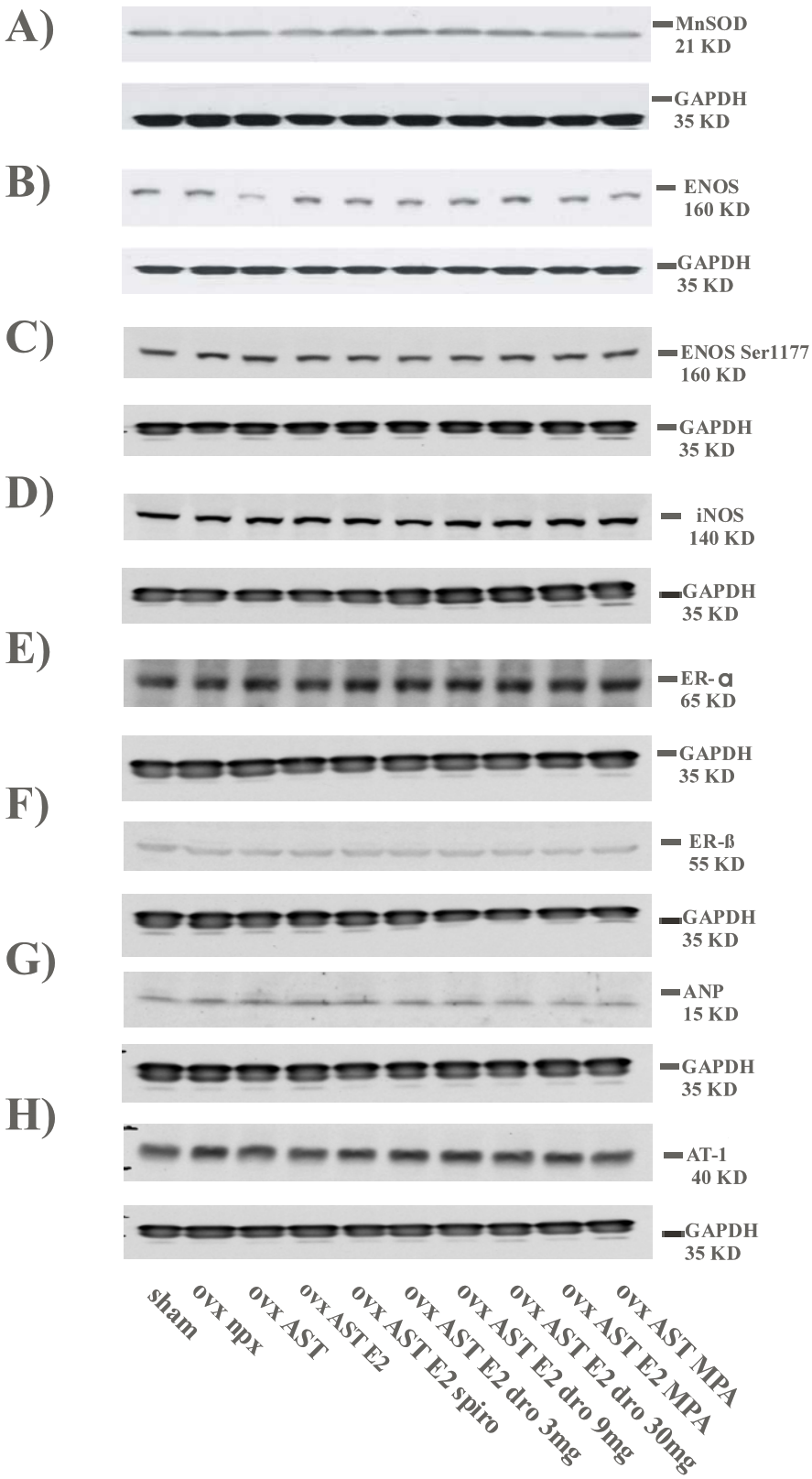

Figure 25. Cardiac expression of different proteins. A) MnSOD; B) eNOS; C) eNOS1177; D) iNOS; E) ER-α ; F) ER-β ; G) ANP; H) AT-1, were not significantly affected by treatments.

3.2.2. Kidney effects.

3.2.2.1. Liquid balance and electrolytes.

Individual animals were monitored for water consumption and urine excretion for 24 hrs in metabolic cages. Water intake as well as urine excretion were significantly increased by AST. 17β- estradiol treatment resulted in a further increase of water consumption. Co-treatment of E2 with drospirenone decreased elevated water in AST rats to baseline levels. In contrast treatment with E2 plus MPA resulted in a further increase of water consumption. These effects of E2 plus MPA co-treatment in the fluid balance were accompanied by a significant reduction of serum potassium levels, which were lowered by AST treatment (Fig. 26).

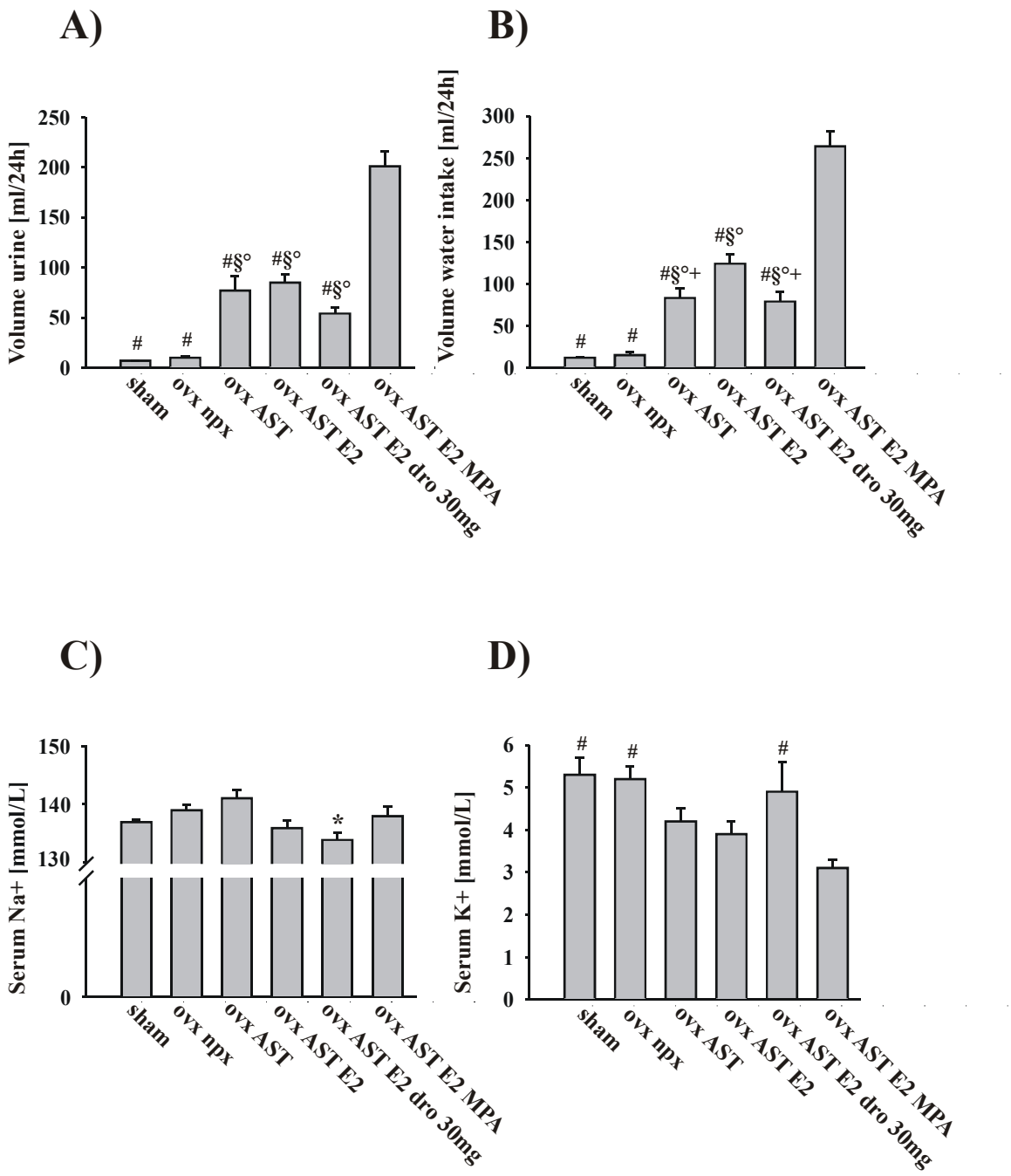

Figure 26: Fluid homeostasis and serum electrolytes. Water uptake and urine excretion were significantly increased in ovarectomized aldo-salt treated rats compared to control. 17β-estradiol further increased water consumption; however co-treatment with E2 plus drospirenone blocked this effect. Co-treatment of E2 plus MPA leaded to a doubling of the fluid exchange. E2 plus MPA effects in fluid balance were accompanied by effects in serum electrolytes expressed by a significant reduction of serum Potasium. A) Urine excretion; B) Water uptake; C) Serum Sodium; D) Serum Potasium. n=4 animals/group; * p<0.05 AST; + p<0,05 vs. AST plus E2; # p<0,05 vs. E2 plus MPA; ° p<0,05 vs. sham; § p<0,05 vs. ovx npx.

3.2.2.2. Global measurements.

Body weight, heart weight, uterus weight, blood pressure and aorta media area, followed the same tendencies as described earlier for the cardiovascular effects of hormone replacement therapy in the aldo-salt treated rats (Table 5). In addition kidney weight was significantly increased by aldo-salt treatment, to values that went beyond the compensatory hypertrophy due to the loss of one kidney, as can be seen by the lower kidney weight in the nephrectomized placebo animals (group 2). 17-β estradiol treatment did not have any effect on kidney hypertrophy but the combination of 17-β estradiol with the anti-mineralocorticoid spironolactone as well as with the medium and high dosis of drospirenone resulted in a significant reduction of kidney weight. Whereas MPA treatment resulted in a further increase of kidney weight (Table 5).

3.2.2.3. Kidney histomorphology.

5 μm kidney section of the interpolar axis were stained with hematoxilin-eosin and trichrom Masson followed by histopathological analysis. Significant renal lesions beyond what would be expected as spontaneous renal pathology background were only reported in the AST rats that received E2 plus MPA. Typical findings in this group were mesangiolysis, glomerular necrosis, glomerular sclerosis and periglomerular fibrosis (Fig 27). With respect to the vasculature myointimal proliferation as well as perivascular fibrosis was presented. Tubular degeneration and dilated tubules containing protein casts were also present only in MPA treated rats (Fig 27).

Table 5. Global measurements for kidney effects.

	sham	ovx npx	Ovx AST placebo	ovx AST+E2 ovx	ovx AST+ E2 + spiro	ovx AST + E2 + dro 3mg	ovx AST + E2 + dro 9mg	ovx AST + E2 + dro 30mg	ovx AST + E2 + MPA
n	**4**	**4**	**4**	**4**	**4**	**4**	**4**	**4**	**4**
body weight [g]	258 ± 5*	317 ± 5	311 ± 11	267 ± 4*	291 ± 8*	294 ± 17	281 ± 8*	300 ± 12	270 ± 24*
uterus weight [mg]	566 ± 73*†	108 ± 9	113 ± 4	405 ± 19*	448 ± 105*	261 ± 29†	252 ± 45†	275 ± 25†	524 ± 34*
abs. kidney weight [mg/cm]	711 ± 9#†*	932 ± 58#†*	1534 ± 79#	1556 ± 81#	1279 ± 58#†	1419 ± 60#	1174 ± 51#†*	1237 ± 51#†*	2407 ± 140 †
rel. kidney weight [mg/cm]	187 ± 2#†*	245 ± 15#†*	403 ± 21#	409 ± 21#	337 ± 15#†	374 ± 18#	307 ± 31#†*	315 ± 4#†*	621 ± 39†
rel. heart weight [mg/cm]	168 ± 3*#	187 ± 6*#	243 ± 9#	200 ± 5*#	207 ± 5#	221 ± 19#	194 ± 8*#	192 ± 4*#	279 ± 20*
abs. heart weight [mg]	637 ± 11*#	709 ± 21*#	922 ± 35	762 ± 20*#	788 ± 18*#	838 ± 68*#	744 ± 32*#	752 ± 16*#	1082 ± 81*
heart rate [bpm]	417 ± 9	385 ± 26	380 ± 2	403 ± 5	421 ± 4	406 ± 6	420 ± 4	410 ± 18	391 ± 6
systolic BP [mmHg]	139 ± 4*#	150 ± 3*#	182 ± 6	158 ± 3#	154 ± 7#	154 ± 8#	136 ± 4#	155 ± 12#	200 ± 15
mean BP [mmHg]	126 ± 4*#	133 ± 4	157 ± 3	144 ± 4	128 ± 7*#	118 ± 7*#	108 ± 4*#	125 ± 12*#	157 ± 11
aorta media area [mm2]	1,20±0,1*#	1,18±0,02*#	1,30± 0,03	1,21±0,01*#	1,25 ± 0,01*#	1,26 ± 0,03*#	1,26 ± 0,02*#	1,22 ± 0,02*#	1,52 ± 0,01

All measurements were performed after 8 weeks of treatment. Significances: * $p<0.05$ vs. ovx AST placebo; † $p<0.05$ vs. ovx AST + E2. Abbreviations: AST (aldosterone-salt treatment); MPA (medroxyprogesterone-acetate); E2 (17□-estradiol), ovx (ovariectomy); npx (nephrectomy); dro (drospirenone); spiro (spironolactone); bpm (beats / minute); BP (blood pressure).

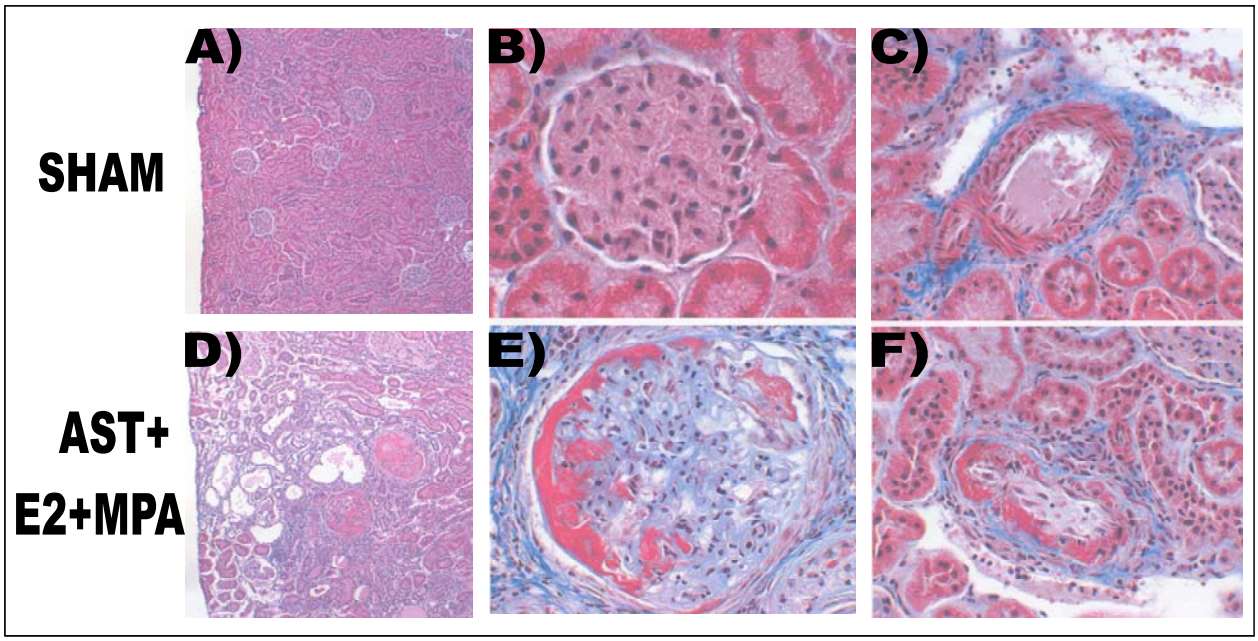

Figure 27: Kidney Histomorphology induced by AST co-treatment with E2 plus MPA. A) and D) gross morphology of the kidney, A) sham kidney shows a compact distribution of glomeruli and tubuli, D) kidney of an AST plus E2 and MPA treated rat show fibrinoid necrosis that completely destroyed the 2 central glomeruli, there is marked tubular fibrosis with destruction of tubules. B) and E) closer magnification of a single glomeruli, B) healthy glomeruli, D) fibrinoid necrosis and periglomerular fibrosis. C) and F) closer look of renal small arteries, C) normal artery, F)artery with fibrinoid necrosis and perivascular inflammatory cells infiltration. Staining Trichrom Masson.

3.2.2.4. Serum hormone levels.

Estradiol serum levels were low in ovariectomized rats and increased by estradiol substitution. A decrease of angiotensin II serum levels due to a negative feedback regulation of the RAAS as a long term effects of AST treatment was also observed. The combination of estradiol with spironolactone or drospirenone recovered the decrease of angiotensin II serum levels. Serum progesterone and 17OH-progesterone were significantly reduced by ovariectomy and remained low in all treatment groups except by the estradiol plus MPA co-treatment that further reduced progesterone serum levels. Corticosterone and cortisol serum levels were not affected by AST treatment and were significantly reduced by estradiol plus MPA co-treatment. ACTH serum levels were also not altered by AST treatment but significantly reduced by estradiol plus MPA treatment (Table 6).

Table 6. Serum Hormones.

	sham	ovx npx	ovx AST placebo	ovx AST+E2 ovx	ovx AST+ E2 + spiro	ovx AST + E2 + dro 3mg	ovx AST + E2 + dro 9mg	ovx AST + E2 + dro 30mg	ovx AST + E2 + MPA
n	**4**	**4**	**4**	**4**	**4**	**4**	**4**	**4**	**4**
estradiol [pg/ml]	27 ± 8*	3 ± 1	2 ± 1	44 ± 4*	29 ± 5*	21 ± 5*	22 ± 4*	28 ± 4*	24 ± 9*
angiotensin II [pg/100ul]	82 ± 10 #	70 ± 9 #	8 ± 2	11 ± 1	52 ± 4 #	28 ± 6 #	50 ± 6 #	47 ± 6 #	4 ± 3
progesterone [nmol/L]	133 ± 10 #	62 ± 10 #	45 ± 8 #	78 ± 14 #	55 ± 8 #	57 ± 11 #	45 ± 3 #	76 ± 20 #	12 ± 4
17OH-progesterone [nmol/L]	10,8 ±1,0#	4,9 ± 0,5 #	3,0 ± 0,5 #	2,7± 0,2	3,8 ±0,6 #	3,0 ± 0,2	2,3 ± 0,3	2,1 ± 0,4	0,8±0,2
corticosterone [ng/ml]	831 ± 83#	688 ± 66#	538 ± 58°#	676 ± 32#	672 ± 80#	551 ± 40#	602 ± 58#	616 ± 77#	108 ± 39
cortisol [nmol/L]	34,5±2,6#	37,9± 8,3#	22,1± 3,8#	21,9±2,7#	21,7±3,0	21,8±4,1#	32,6±3,9#	27,7±3,9#	1,5± 0,7
ACTH [pg/ml]	652 ± 46#	846 ± 99#	818 ± 79#	781 ±43#	701 ± 127#	580 ± 70#	523 ± 15#	545 ± 71#	338 ± 35

Significances: * $p<0.05$ vs. ovx AST placebo; # $p<0,05$ vs. ovx AST+E2+MPA. Abbreviations: AST (aldosterone-salt treatment); MPA (medroxyprogesterone-acetate); E2 (17β-estradiol), ovx (ovariectomy); npx (nephrectomy); dro (drospirenone); spiro (spironolactone).

3.2.2.5. Kidney protein expression.

Western blot analysis of the steroid hormones receptors revealed that ER-α , ER-β , PR-A, PR-B, MR and GR were all expressed in rat kidney and were not altered by treatment (Fig 28 and 29). The expression levels of the most important receptors of angiotensin II: AT1 and AT2 were analyzed because angiotensin II serum levels were affected by AST treatment and replacement therapies and also because angiotensin II is an important mediator of cardiovascular and renal damage. While AT1 receptor kidney expression was similar between all groups, AT2 receptor expression increase upon antimineralocorticoid treatment (spironolactone and drospirenone) (Fig. 29). The epithelial sodium channel (ENaC), which mediates sodium retention in the kidney was more abundant in rats receiving AST plus E2 and MPA. P67phox as a marker of oxidative stress was also significantly increased in this group. AST treatment by itself presented a tendency to increase p67phox amount of protein that wasn't changed by estradiol treatment, however co-treatment of estradiol plus spironolactone or drospirenone decreased its expression. Analysis of rac-1 also a marker of oxidative stress and of MnSOD (manganese superoxide dismutase) an antioxidative stress protein didn't reveal differences among the different treatment. (Fig. 30).

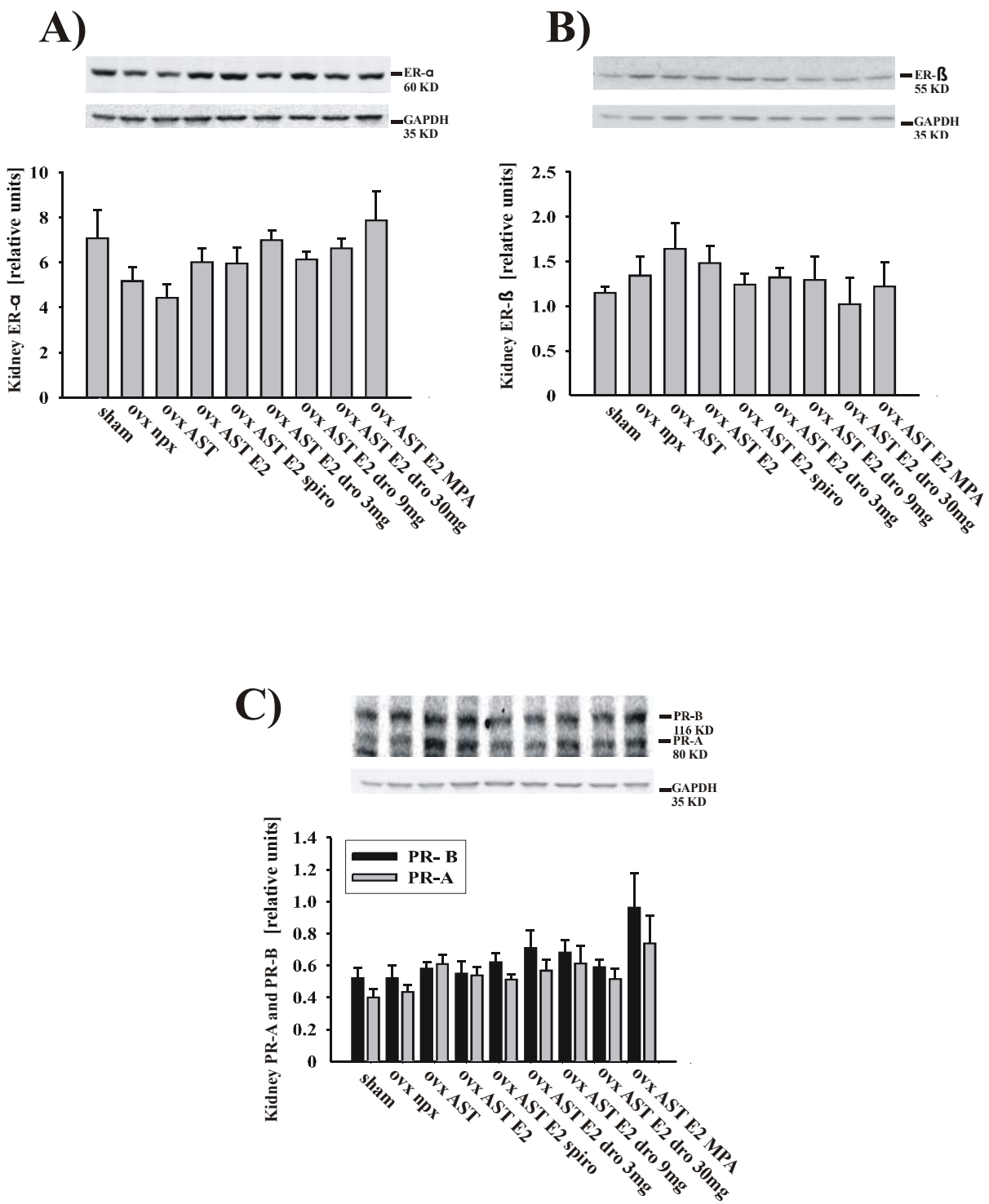

Figure 28. Expression of ER-α(A), ER-β (B) as well as PR-A and PR-B (C) in the kidney were similar in all the groups analyzed.

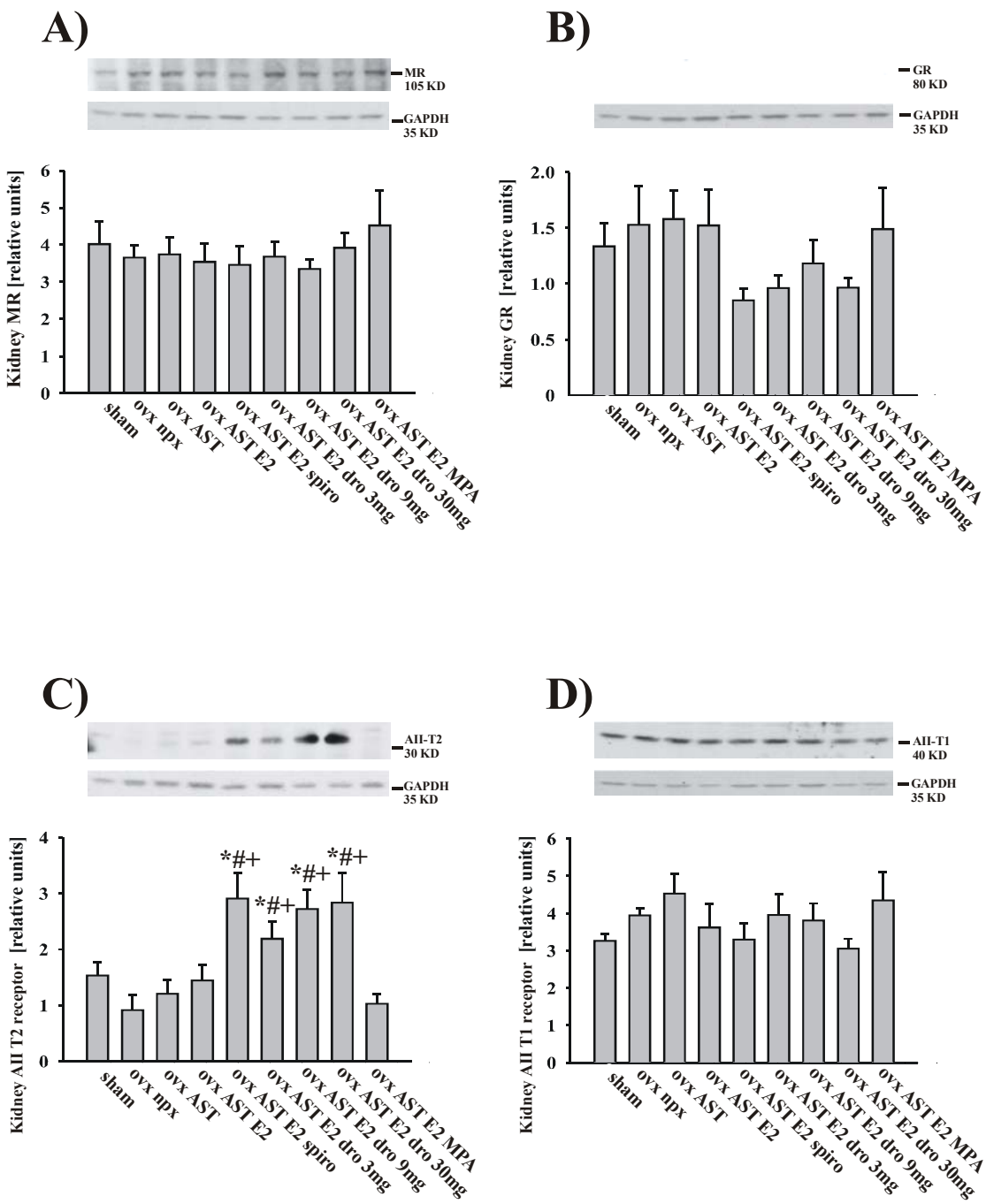

Figure 29. Expression of MR, GR, AT1 and AT2 receptors. MR (A), GR (B) and AT1 (D) expression in the kidney were similar in all groups. AT2 was significantly induced by the antimineralocortioid agents (spironolactone and drospirenone) and was not affected by AST or estradiol treatment. # p<0,001 vs. E2 MPA AST; *p<0,001 vs. AST; + p<0,001 vs. AST plus E2.

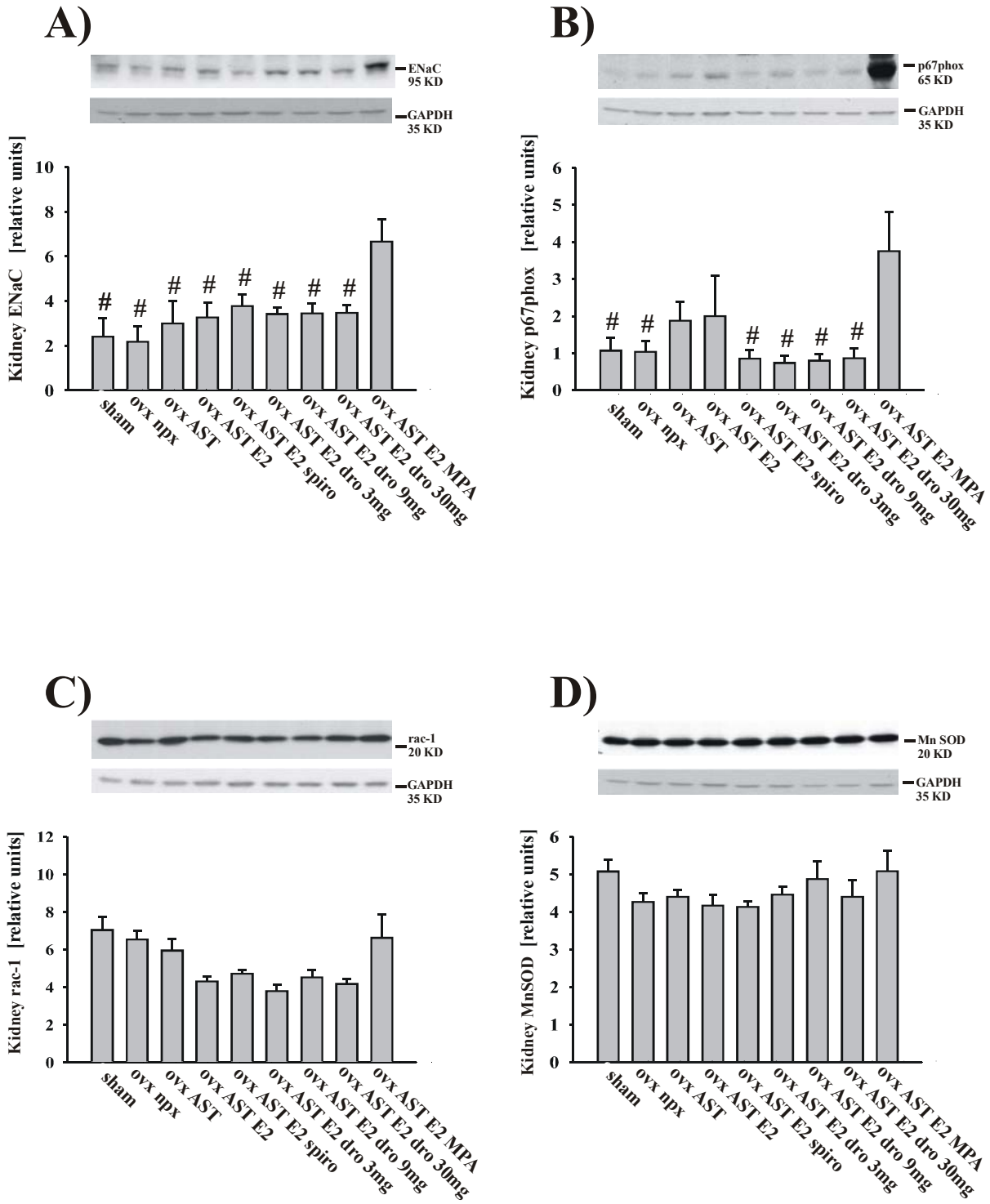

Figure 30. Expression levels of the alpha subunit of the sodium retention channel eNaC (A) and of the oxidative stress marker p67phox (B) were significantly increased by co-treatment of AST plus E2 and MPA. MnSOD (D) and rac-1 (C) expression were similar in all groups. # $p<0,001$ vs. MPA AST

3.2.2.5. Protein nitrosylation.

Oxidative stress in presence of nitrites/nitrates leads to the production of peroxinitrite and hence of protein nitrosylation. Protein nitrosylation damage proteins and promotes its degradation. In our set of experiments only the kidneys of rats receiving AST plus estradiol and MPA presented a significant increase of protein nitrosylation as detected by immunohistochemistry of nitrotyrosin (Fig. 32). Protein nitrosylation in the AST plus E2 and MPA was distributed in all the kidney transversal sections, both glomeruli and tubuli stained positive as did protein casts.

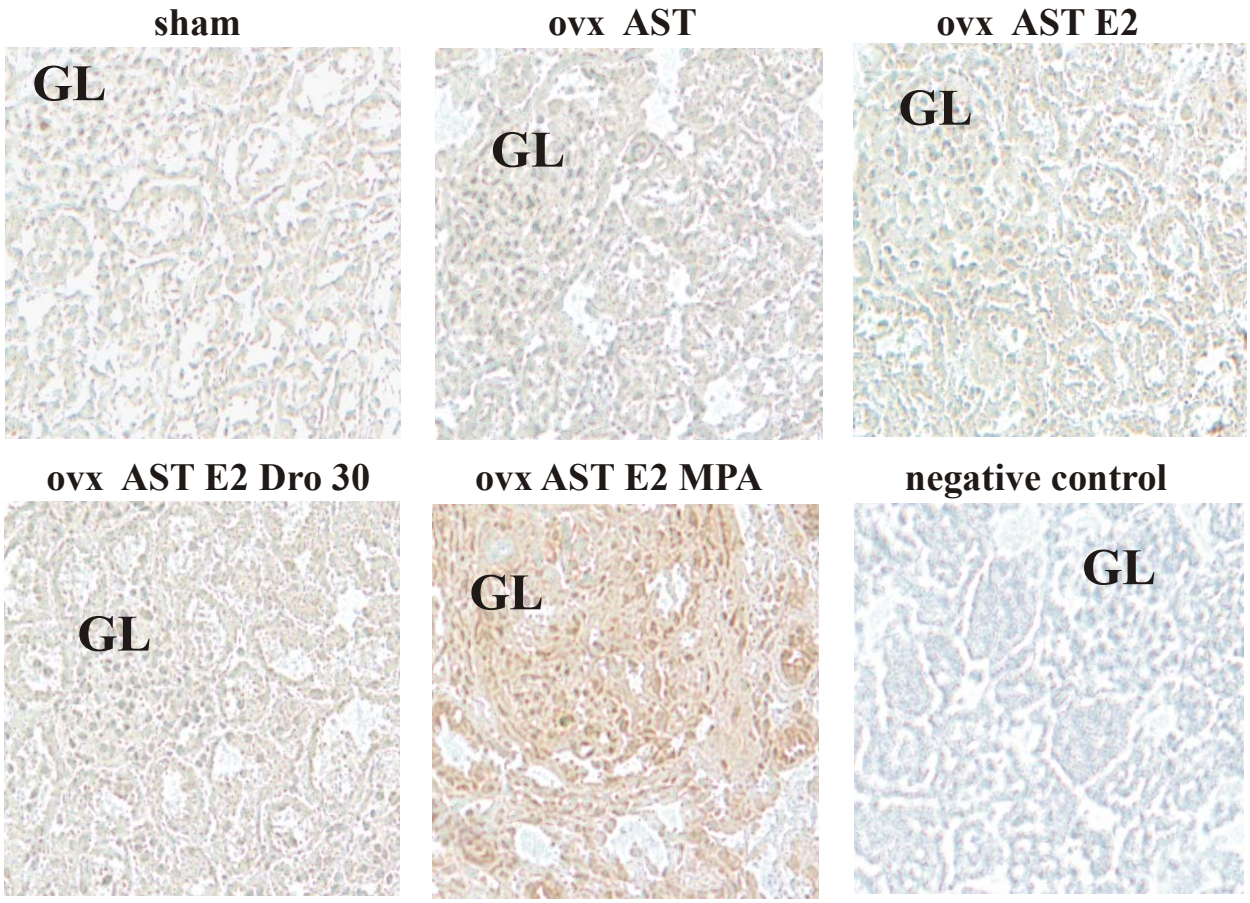

Figure 31: Kidney protein nitrosylation. Increased protein nitrosylation was observed only in the kidneys of the animals receiving aldosterone-salt treatment plus estradiol and MPA. Negative control consisted of the primary antibody preincubated with 3 nitro-tyrosin ON at 4 °C.GL=glomeruli. Immunohistochemistry with DAB as substrate. Original magnification X400.

3.3. Discussion.

Ten percent of all cardiovascular disease conditions are accompanied by increased aldosterone serum levels, in addition mineralocorticoid receptor antagonists in clinical trials have been able to reduce in 30% the rates of mortality, highlighting the role of aldosterone in the cardiovascular system. Aldosterone and its antagonists have been extensively studied in vitro, in vivo and in clinical trials however the gender effect during aldosterone induced damage is still unknown. The effects of aldosterone-salt treatment on cardiovascular pathology have so far only been studied in male but not in female rats. Of note, aldosterone and estrogen receptors share similar structure and a protective role of estradiol in two of the major targets of aldosterone (cardiovascular and renal systems) have been reported previously. Therefore it is interesting that signs of disproportionate MR activation such as cardiac hypertrophy, hypertension, perivascular collagen accumulation, endothelial dysfunction as well as increased vascular osteopontin expression were observed predominantly in ovarectomized AST rats whereas their severity was significantly reduced in aldo-salt treated rats substituted with 17 β-estradiol. One important finding reported here is the observation that physiological doses of 17 β-estradiol completely blocked or significantly improved several key features of cardiovascular injury under condition of disproportionate mineralocorticoid receptor activity.

Although the complex phenotype of AST rats suggests multifactorial mechanisms through which estrogens might attenuate cardiovascular injury and dysfunction, 17 β-estradiol could interfere directly with mineralocorticoid receptor activation. However this hypothesis is neither supported by the current literature nor by measurements of angiotensin-II serum levels, which were suppressed in aldosterone-salt treated rats irrespective of E2 substitution whereas MR-antagonist treatment with spironolactone and drospirenone resulted in increased serum AII levels. Therefore, it appears more likely that 17 β-estradiol interacts with downstream signal transduction pathways that become activated upon aldosterone-salt treatment in different organ systems.

The extent of vascular injury in aldo-salt treated rats was closely linked to vascular osteopontin expression levels. Osteopontin plays an important role in cardiac and vascular fibrosis because targeted deletion of osteopontin ameliorates cardiovascular collagen accumulation following myocardial infarction, aortic banding and chronic angiotensin-II infusion. (Rocha R, 2002; Collins AR, 2004; Trueblood NA, 2001; Xie Z, 2004). Decreased osteopontin expression in estrogen substituted AST rats thus appears as a likely mechanism to explain lower perivascular collagen accumulation and fibrosis. Although it is interesting to note that estrogens can increase osteopontin expression under in vitro conditions (Craig AM, 1991; Vanacker JM, 1999). The differential expression pattern of osteopontin under in vitro and in vivo conditions is most likely explained by very different experimental conditions. Further and more specific studies will be required to characterize the mechanisms by which mineralocorticoids and estrogens regulate vascular osteopontin expression in vivo.

In parallel to vascular osteopontin expression, aorta media hypertrophy was attenuated by estradiol treatment. However the protective estrogen effects in AST rats were not limited to the vasculature but extended to cardiac muscle as well since cardiac mass was lower and cardiac myocyte cross-sectional areas were smaller in estrogen substituted compared to placebo treated AST rats. These findings could primarily be explained by lower blood-pressure levels in aldosterone-salt treated rats receiving 17β-estradiol substitution. Beyond that, estrogens attenuate cardiac hypertrophy directly by regulation of signal transduction pathways that promote or inhibit the development of cardiac hypertrophy such as ANP expression or MAP-kinase activation (Pelzer T, 2005; van Eickels M, 2001). Based on the current results and experimental strategy, we can not rule out that similar mechanisms might be operative in AST rats as well, although the reduction of blood pressure already provides a direct mechanism for decreased cardiac mass in E2 substituted rats.

The second and functionally important finding of this study is the observation that medroxyprogesterone-acetate almost completely blocked the protective function of 17 β-

estradiol in AST rats. Unfavorable effects of medroxyprogesterone-acetate in aldosterone-salt treated rats parallel previous reports, in which progestins abrogated the reduction of vascular injury following carotid balloon injury upon estrogen substitution (Levine RL, 1996). Within this context it is important to note that medroxyprogesterone-acetate binds not only to the progesterone receptor but also acts as an androgen, mineralocorticoid and glucocorticoid receptor ligand (Sitruk-Ware R, 2002; Li X, 2003; Winnaker RC, 1981). The additional increase in E2 stimulated uterine weight by medroxyprogesterone-acetate is a clear sign of androgenic pharmacological activity and it may be argued that the androgenic activity of medroxyprogesterone-acetate may blunt the beneficial effects of estrogens (Nantermet PV, 2005). Similar observations have been made for the human situation in which progestins with androgenic partial activities can reverse the beneficial lipid profile of estrogens (Sitruk-Ware R, 2004). Collective evidence thus strongly suggests that synthetic progestins act differently due to different profiles in the activation of e.g. androgen and glucocorticoid receptors and it seems likely that some progestins increase vascular injury, whereas others exert rather beneficial effects (Muhn P, 1995; Selman PJ, 1996; Thomas CP, 2006).

The observation that medroxyprogesterone-acetate attenuated the cardiovascular protective effect of 17 β-estradiol in AST rats could be explained either by enhanced mineralocorticoid activity or, alternatively, by inhibition of estrogen action. To discriminate between these mechanisms, we analyzed the cardiovascular phenotype of ovarectomized aldosterone-salt treated rats upon long-term administration of medroxyprogesterone-acetate. Aggravation of perivascular fibrosis in medroxyprogesterone-acetate treated AST rats strongly suggest for an enhancement of vascular remodeling. Since long term androgen treatment in rats causes hypertension via an indirect mineralocorticoid activity, it is not possible to exclude that the androgenic partial activity of medroxyprogesterone-acetate may have contributed to perivascular fibrosis (Gallant S, 1991). Exacerbated vascular and perivascular damage in the MPA treated animals may also be linked to oxidative stress as p67phox expression was enhanced only in

MPA treated animals. MPA blocked the anti- inflammatory effect of estradiol because it reverted the down-regulation of OPN induced by 17 β-estradiol. MPA also increased the expression of the pro-inflammatory enzyme cox-2, similar to previous results showing that estradiol suppressed cox-2 expression and the inflammatory response in the brain vasculature, while MPA worsened the damage (Sunday L, 2006).

Drospirenone, which is a progestin with potent MR-antagonist properties and without additional androgenic and glucocorticoid activity, might exhibit different and protective effects on cardiovascular injury in estradiol substituted AST rats as compared to medroxyprogesterone-acetate (Muhn P, 1995; Oelkers W, 2004). If this hypothesis was true, cardiac and vascular damage, vascular reactivity and osteopontin expression should either be unaffected or even improved in drospirenone treated AST rats. The current results strongly support this hypothesis since drospirenone at all tested dosages conferred more favorable and protective effects on cardiac hypertrophy, perivascular fibrosis and osteopontin expression under conditions of disproportionate mineralocorticoid receptor activation than medroxyprogesterone-acetate. The observation, that drospirenone caused a further reduction of blood pressure and cardiac mass in estrogen substituted rats is in line with recent findings in postmenopausal women. Although it must be noted that, in contrast to the human situation, 17 β-estradiol already caused a significant reduction of blood pressure and cardiac mass in aldosterone-salt treated rats (Preston RA, 2002; Archer DF, 2005; White WB, 2005).

All components of the Renin Angiotensin Aldosterone System (RAAS) are present in the kidney and in normal conditions RAAS activity prevents excessive sodium loss and regulates blood pressure. During pregnancy sexual hormones play an important role in renal function and the induction of sodium retention by aldosterone is counterbalanced by the natriuretic effects of progesterone. Therefore it is possible that the cardiovascular effects of the HRT regimes in the aldosterone salt treated rats are accompanied by specifical renal effects. In here, it is described that estradiol beneficial effects are not the ones of a global antimineralocorticoid but may have

been limited to specifical functions, as the prevention of cardiovascular damage was not accompanied by antimineralocorticoid like kidney protective mechanisms (no difference in fluid turnover and kidney hypertrophy). Consequently, it is possible that most of the cardiovascular beneficial effects of estradiol are the result of local and extra-renal mechanisms and may include specifical gene modulation, anti-inflamatory effects and limitation of the excessive extracellular matrix turnover in blood vessels and myocardium.

In models of hypertension using male rats, activation of the mineralocorticoid receptor by aldosterone contributes to kidney damage and administration of aldosterone blockers or adrenalectomy attenuates renal injury (Rocha R, 1999; Rocha R, 2000). In addition AST treatment in male rats is characterized by vascular and glomerular sclerosis, besides of fibrinoid necrosis and tubular damage (Blasi E, 2003). In the observations reported here for female rats AST treatment didn't caused an extensive damage as reported for male rats although it did induce kidney hypertrophy. This difference may be explained by chromosomal effects and not by ovarian hormones. Intact female rats did not developed hypertension and ovariectomy was necessary for the development of the symptoms of AST treatment. Gender-associated differences in aggravation of renal hemodynamics in response to angiotensin II have only been analyzed previously in single nephrons from male and female rats. Nephrons from male rats presented lower preglomerular resistance that is a parameter associated with increased glomerular injury that is particularly relevant in company of high systemic blood pressure (Baylis 1994). Then it is possible that other structural differences between male and female rats may have played a role.

In here it has been described that several classical effects of aldosterone were induced by AST treatment including increased fluid exchange, serum electrolytes imbalance and kidney hyperthropy. While estradiol by itself didn't have any effect its combination with drospirenone or the classical anti-mineralocorticoid spironolactone reversed these parameters. Pointing to the possible beneficial renal effects of drospirenone in a clinical setting as anti-mineralocorticoid treatment of patients with primary hyperaldosteronism reversed glomerular hyperfiltration and

proteinurea. (Ribstein J, 2005; Sechi LA, 2006). Combining estradiol with MPA results in extensive renal damage that was associated with an increase of oxidative stress as MPA increased the expression of p67phox (regulatory subunit of the NADPH oxidase) and nitrotyrosin staining. Increased oxidative stress appears to be a general mechanism in response to MPA as p67phox induction was observed not only in the kidney but also in the heart of AST rats. Induction of oxidative stress by MPA may also be related to MR agonistic effects as progression of renal injury due to chronic elevation of aldosterone was related to ROS generation (Nishiyama A, 2004). Oxidative stress plays an important role in cardiovascular and renal pathology.

Angiotensin II serum levels were regulated in opposites directions by aldosterone and the antimineralocorticoids, considering that angiotensin II also plays an important role in the kidney the expression of its 2 important receptors was analyzed: the angiotensin type 1 receptor (AT1) and the type 2 receptor (AT2). None of the treatments included in this chapter changed the expression of AT1, but both antimineralocorticoids spironolactone and drospirenone, induced the expression of the AT2. The AT2 is highly expressed in the kidney during the development and its expression decreases after birth to low levels. Genetically engineered mice and pharmacological studies of the AT2 provided valuable insights to its role. A kidney protective role for the AT2 has been proposed, as AT2 knock out mice suffer a more pronounced parenchymal fibrosis following obstructive injury than wild type mice (Ma J, 1998; Stoneking BJTE,1998). Thus it is possible, that increased expression of AT2 induced by antimineralocorticoid treatment, may be a possible mechanism of renal protection. AT2 opposing the effects of AT1 have not only been proposed for the kidney but also for the cardiovascular system. This hypothesis is based on the observation that AT2 knock out mice exhibit increased blood pressure and sustained antinatriuresis (Blume A, 2001) and that blood pressure increases further upon angiotensin II infusion in knock out mice (Hein L, 1995). Besides angiotensin-dependent hypertension in rats is normalized by AT1 receptor blockade,

whereas AT2 receptor antagonism increases blood pressure (Siragy HM, 1999; Tsutsumi Y, 1999; Barber MN, 1999). Intracardial in vivo lentiviral vector-mediated gene transfer of AT2 resulted in a 85% attenuation of increased LV weight due to angiotensin infusion (Falcon B, 2004), affirming the hypothesis that AT2 activation plays a protective role. Thus it is possible that AT2 modulation by the antimineralocorticoids is an important factor for their protective role in the cardiovascular system.

The kidney plays a pivotal role in water and salt homeostasis. The epithelial sodium channel (ENaC) plays an important role in this regulation and mediates sodium retention in the kidney. ENaC is considered a mineralocorticoid target gene as increased aldosterone serum levels result in a rapid increase of the α–ENaC subunit expression, consistently absence of aldosterone under sodium loaded conditions result in minimal ENaC activity. However the regulation of ENaC activity do not only respond to aldosterone serum levels but also to salt loading conditions and other stimuli. Short term treatment with dexamethasone or very high aldosterone levels without salt induce ENaC expression and trafficking but, complete blockade of MR and GR with spironolactone and RU486 does not prevent ENaC trafficking nor the upregulation of ENaC protein abundance (Sayegh R, 1999; Nielsen J, 2007). In here, aldosterone salt treatment resulted in a moderate non-significant increase of α-ENaC abundance which may however translate into high sodium retention. Although unexpected these results can be explained by the particularities and differences of the AST treatment in comparison to the previous studies. Induction of α-ENaC by aldosterone has been shown before in normal salt diet, short term and high aldosterone dosage treatment (Thomas CP 2005; Nielsen J, 2007; Aoi W, 2006; Masimalani S, 1999), different from the results reported in here, in which the dosage of aldosterone was at least 200 times lower and the treatment time was at least 6 times longer. Thus it is likely that compensation mechanism may have limited the expression of α-ENaC to prevent excessive sodium overload. Also interesting is the fact that AST animals exhibited very low levels of angiotensin II, a peptide that has been previously shown to induce α-ENaC. It was also reported

that α-ENaC induction by angiotensin II wasn't affected by spironolactone co-treatment suggesting that the induction was a direct effect of AT1 activation (Beutler K, 2003). Moreover, AT1a receptor knock out mice under low salt diet show elevated plasma aldosterone levels but also a markedly decreased α-ENaC protein expression (Brooks HL, 2002). The significant increase of α-ENaC levels in MPA treated animals may however mediated the increase of fluid turnover and sodium retention in aldosterone salt treated rats. On the other side induction of ENaC may have responded to the combined activation of the MR and GR as both receptors promote the expression of α-ENaC (Thomas CP, 2005). Indeed renal-vascular aggravating effects of MPA are difficult to attribute to only one steroid receptor. Instead it seem to be the result of a simultaneous activation of different receptors, since AST rats treated with MPA showed clear signs of systemic GR (low ACTH and corticoids) and MR activation (low Angiotensin II and polydipsia). The observation that MPA and dexamethasone activate the α-ENaC promoter via the GR (Thomas CP, 2005) and that human patients receiving MPA have been anecdotally reported with Cushing´s syndrome, suggests that the aggravation of renal damage of MPA may have been mediated by the GR. (Donckier JE, 1990; Harte C, 1995; Learoyd D, 1990; Merrin PK, 1990; Siminoski K, 1989). However in the adrenalectomized rat remnant kidney model glucocorticoids supplementation improves glomerular filtration rate and renal morphology but aldosterone increased renal injury (Quang ZY, 1992; Greene EL, 1996), therefore is possible that even if MPA induces α–ENaC expression the activity of ENaC and renal damage induced by MPA are mediated by the MR.

In conclusion MPA but not DRO exerted opposing effects in the cardiovascular and renal system of aldosterone-salt-treated rats, while MPA reverted the beneficial effects of estradiol in the cardiovascular system and aggravated renal injury, drospirenone didn't affect estradiol effects and attenuated renal damage. Differential effects of both progestins on the renin angiotensin aldosterone system may be responsible of these effects.

Chapter 4. General conclusions and perspectives.

4.1. Conclusions.

- The cardiac effects of 17β–estradiol depend on the origin and characteristics of the cardiovascular disease. In aldosterone-salt-treated female rats 17β-estradiol exerted a beneficial role attenuating hypertension, cardiac hyperthrophy and vascular fibrosis. However during myocardial infarction 17β-estradiol played a neutral role. Differences in the efficacy of estradiol to improve cardiovascular function can be related to the characteristic of each animal model. While aldosterone salt treatment reflects the effects of a progressive increase in blood pressure accompanied by activation of the mineralocorticoid receptor, the myocardial infarction model mimics the cardiac remodelling and changes in cardiac function occurring after an ischemic event. Therefore estradiol seems to have an important role in hypertension but is neutral on post-ischemic ventricular remodelling.
- Beneficial effects of estradiol are organ specific an improvement in cardiac morphology and hypertension in the AST rats does not fully extended to renal disease.
- Unopposed estrogen supplementation does not reflect accurately the effects of treatment with a combination of MPA plus estrogens. 17β– estradiol played a neutral role in both models of cardiovascular disease, however its combination with MPA resulted in an aggravation of most of the symptoms of heart failure during myocardial infarction and in a reversion of the beneficial cardiovascular effects of 17β– estradiol in aldosterone salt treated rats, besides of causing extensive renal damage. Therefore the controversy between the negative outcomes of the clinical trials on HRT and the in vivo models could be partially explained by the use of MPA in the human patients.
- Preservation of intact ovarian hormones or a combination of 17β-estradiol plus drospirenone did not exert the harmful symptoms described for the combination of

MPA plus 17β-estradiol, suggesting that tissular damage was not produce just by activation of the progesterone receptor, but may be a property of the combination of MPA with 17β-estradiol. Indeed the lost of the 17 β-estradiol beneficial effects by co-treatment with medroxyprogesterone acetate in the AST rats was not presented by combination of 17β-estradiol with drospirenone and therefore may be intrinsic to MPA and should not be extrapolated to other progestins.

- A common mechanism of damage of MPA treatment seem to consist in the increased reactive oxygen species generation, possible through an increased expression of the NADPH oxidase regulatory subunit p67phox, that was equally up-regulated by MPA treatment in the ventricle of the myocardial infarcted rat and ventricle and kidney from the AST rats.
- Treatment with MPA in both animal models resulted in inhibition of the hypothalamus-pituitary axis without symptoms of lack of glucocorticoids or hypoaldosteronism, suggesting that excessive activation of the glucocorticoid and mineralocorticoid receptors is involved in the physiological effects of MPA and may be the initial signal leading to the harmful cardio-renal effects.
- In summary, MPA attenuated the beneficial effects of 17 β -estradiol treatment and aggravated symptoms of cardiac and renal disease in 2 different animal models. These observations contribute to explain the unfavourable outcome of clinical endpoint studies that employed combined estrogen and MPA treatment to prevent the development of cardiovascular disease. Alternative progestins that are devoid of MR agonist activity as drospirenone do not confer the unfavourable cardiovascular effects of MPA and could potentially improve the safety of HRT in women with heart disease.

4.2. Perspectives.

- The term HRT has been classically referred to the combination of estrogens and MPA and the lack of positive effects of this combination in clinical trials has been extended to all regimes of HRT, without considering the particularities of each synthetic steroid hormone. MPA and drospirenone are both synthetic progestins, however they show different effects in the cardiovascular and renal system of the AST rats. While MPA aggravated the damage, drospirenone was protective. Subsequently a novel strategy of a drospirenone based HRT in clinical trials could be advantageous compared to MPA.
- Drospirenone in vivo profile is superior to the one of MPA and further studies of this compound may not only help to characterize its mechanisms of protection but may also help to understand the role of antimineralocorticoids in the cardiovascular and renal systems. Indeed drospirenone not only blocked the harmful effects of aldosterone but it also induced expression of the AT2 receptor that has been previously related with cardiovascular and renal protection. Mechanisms of induction of AT2 receptor expression are not completely understood, thus further analysis of anti-mineralocorticoids treatment and combinations with antagonists of the AT2 receptor (PD 123177; 123319) may help to understand regulation of the expression and functional role of this receptor.
- MPA induced cardiovascular and renal damage through increased oxidative stress, which has not only been related to impairment organ function but also to tissular stability and ageing. Thus it is possible that the MPA based HRT is not only affecting cardiovascular function but also general well being. Considering that the female population taking HRT is also a common user of antioxidant therapies (vitamin C and E) some of the negative effects in MPA users may have been counterbalanced by the antioxidants. Studying the role of antioxidants counterbalancing MPA effects and

possible potentiation of the effect of other progestins in the cardiovascular system may be relevant.

- Female aged population present also several risk factors for cardiovascular disease and patients requesting HRT may also be under other medication regimes, therefore studying the effect of a combination of HRT with standard medications as ACE inhibitors may approximate better to the real situation. Further more studying the activation of a single steroid receptor is informative but do not reflect the naturally occurring complex pattern of interactions that occurs within the members of the nuclear steroid receptor family in vivo. Therefore HRT may benefit from the knowledge obtained from a deeper characterization of steroid receptors cross talk and non-specifical binding of ligands.

Chapter 5. Materials.

5.1. Animal strains.

Female *Rattus norvegicus* Wistar Kyoto outbred rats 8 weeks old HsdCpb:WU (Harlan-Winkelmann; Borchen, Germany)

Female *Rattus norvegicus* Wistar Kyoto outbred rats 12 weeks old HsdCpb:WU (Charles River- Lyon, France).

5.2. Compounds for animal treatment.

17 β-estradiol (Sigma-Aldrich)

MPA (Medroxyprogesterone acetate, Sigma-Aldrich)

Drospirenone (Schering AG)

Spironolactone (Sigma-Aldrich)

Aldosterone (Sigma-Aldrich)

Peanut oil. (Sigma-Aldrich)

5.3. Equipment.

Autoclave	Sanoclav	KL-12-3
Centrifuge	Sigma	2K15, Sigma 2-15
Centrifuge	Hettich	Mikro 12-24
Centrifuge	Beckmann	GPKR Centrifuge
Centrifuge	Eppendorf	
Centrifuge	Heraeus	Biofuge pico
Criostat	Leica	Jung CM 3000
confocal microscope	Nikon	EZ-C1
ELISA reader	Tecan	Tecan Spectra
fluroescence microscope	Zeiss	Axiovert 135
freezer (-20°C)	Liebherr	Economy
freezer (-20°C)	Bosch	Economic
freezer (-80°C)	National Lab	Profi Star
Fridge	Liebherr	glass line, Premium, comfort
heating block	Eppendorf	ThermoStat plus
heating block	Liebisch	
Homogenizer	IKA Labortechnik	Eurostar digital
Homogenizer	Janke/Kunkel	Ultra Turrax T25
Incubator	Heraeus	Function line
Incubator	Forma Scientific	Steri-Cult 200
light table	Uni Würzburg	
Luminometer	Berthold Industries	Lumat LB9501
magnetic stirrer	IKA Labortechnik	RH basic2, Ikamag RET, Ikamag RCT
magnetic stirrer	Hartenstein	Hotplate Stirrer L81
Microscope	Leitz	Labovert

Photometer	Eppendorf	Bio-Photometer
power supply	Biometra	P25, PP4000
power supply	Hoefer	SX250
Scale	Sartorius	BP61
Scale	Kern	
sealing machine	Severin	Folio
Shaker	Heidolph	Duomax 1030
Shaker	Hartenstein	L40
Shaker	Braun Biotech	Certomat R
Spectrometer	Perkin Elmer	LS 50 B
supersonic bath	Julabo	USR05
Turner	Heidolph	REAX 2
Vortexer	Heidolph	REAX 1 D R
Vortexer	Scientific Industries	Vortex-Genie2
water bath	Haake	Thermo C10
water bath	Inlabo	SW20C

5.4. Consumable material.

The common consumable material, e.g. tubes and pipette tips, has been obtained from the University of Wuerzburg (Medical Department I; „Zentrallager“), Hartenstein and Noras (both Wuerzburg, Germany). All chemicals have been received from the University of Wuerzburg (Medical Department I, Central pharmacy), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) or Sigma-Aldrich (Munich, Germany).

5.5. Working kits.

Vectastain Elite ABC-peroxidase kit Rabbit IgG ALEXIS (VC-PK-6101).

Vector Hematoxilin QS ALEXIS (VC-H-3404).

Vector labs DAB Substrate kit for peroxidase. VECTOR (SK-4100).

Immpact DAB peroxidise substrate. VECTOR (SK-4105).

Avidin/Biotin blocking kit. VECTOR (SP-2001).

Immpress reagent kit. VECTOR (MP-7401).

5.6. RIA kits.

antigen	species	company	Cat.No.
Progesterone,	coat-A-count	DPC	TPKG1
17-OH-Progesterone	coat-A-count	DPC	TKOP1
Androstenedione	coat-A-count	DPC	TKAN1
Corticosterone	coat-A-count	DPC	TKRC1
Aldosterone	rabbit	ICN	07-108202
Cortisol	coat-A-count	DPC	TKCO1
Estradiol	rabbit	DPC	KE2D1
ACTH	rabbit	Phoenix Pharmaceuticals, Inc.	RK-001-21
Angiotensin II	rabbit	Peninsula Laboratories (Bachem)	S-2012.0001

5.7. Solutions and buffers.

5.7.1. Immunohistochemistry and Immunofluorescence.

5x PBS (Phosphate Buffered Saline) pH 7.4:

component (MW [g/mol])	weight / 5 l	Final concentration (5x)	final concentration (1x)
NaCl [58.44]	40.0 g	685.0 mM	137.0 mM
KCl [74.6]	1.0 g	13.4 mM	2.7 mM
$Na_2HPO_4*2H_2O$ [177.99]	7.2 g	40.5 mM	8.1 mM
KH_2PO_4 [136.09]	1.0 g	7.5 mM	1.5 mM
dH_2O (adjust to pH 7.4)	ad 5000 ml		

0.1 % Triton / 1x PBS: addition of 100.0 µl Triton®X100 to 100.0 ml 1x PBS

4 % PFA / 1x PBS: addition of 4.0 g Paraformaldehyde (PFA) to 100.0 ml 1x PBS

5.7.2. Western blotting.

RIPA (RadioImmunoPrecipitation Assay) Buffer:

component (MW [g/mol])	stock	volume/weight / 100 ml	final concentration
NaCl [58.44]	1.0 M	15.0 ml	150.0 mM
Tris [121.14]	1.0 M	5.0 ml	50.0 mM
PMSF	100.0 mM	1.0 ml	1.0 mM
IGEPAL CA-630		1.0 ml	1.0 %
Sodium deoxycholate (DOC)	10.0 %	5.0 ml	0.5 %
Sodium dodecyl sulfate (SDS)	10.0 %	1.0 ml	0.1 %
dH_2O		ad 100.0 ml	

Low Salt Buffer:

component (MW [g/mol])	stock	volume/weight / 100 ml	final concentration
Tris [121.14]	1.0 M	5.0 ml	50.0 mM
PMSF	100.0 mM	1.0 ml	1.0 mM
IGEPAL CA-630		1.0 ml	1.0 %
dH_2O		ad 100.0 ml	

1.5 M Tris pH 8.8:

component (MW [g/mol])	weight / 100 ml	final concentration
Tris [121.14]	18.15 g	1.5 M
dH_2O (adjust to pH 8.8)	ad 100.0 ml	

0.5 M Tris pH 6.8:

component (MW [g/mol])	weight / 100 ml	final concentration
Tris [121.14]	6.0 g	0.5 M
dH_2O (adjust to pH 6.8)	ad 100.0 ml	

10 % SDS (Sodium dodecyl sulfate):

component (MW [g/mol])	weight / 100 ml	final concentration
Sodium dodecyl sulfate (SDS)	10.0 g	10.0 %
dH_2O (adjust to pH 8.0)	ad 100.0 ml	

5x PBS pH 7.4:

component (MW [g/mol])	weight / 5 l	final concentration (5x)	final concentration (1x)
NaCl [58.44]	20.0 g	342.0 mM	68.4 mM
$NaH_2PO_4*H_2O$ [137.99]	11.73 g	85.0 mM	17.0 mM
Na_2HPO_4 [141.96]	41.17 g	290.0 mM	58.0 mM
dH_2O (adjust to pH 7.4)	ad 5000 ml		

5x Electrophoresis Buffer pH 8.3:

component (MW [g/mol])	weight / 5 l	final concentration (5x)	final concentration (1x)
Tris [121.14]	75.0 g	0.62 M	124.0 mM
Glycin [75.07]	360.0 g	4.8 M	960.0 mM
SDS	25.0 g	0.5 %	0.1 %
dH_2O (adjust to pH 8.3)	ad 5000 ml		

Transfer Buffer pH 8.3:

component	stock	volume / 1 l	final concentration
5x Electrophoresis buffer	5x	200.0 ml	1x
Methanol		200.0 ml	20.0 %
dH_2O		ad 1000 ml	

Washing solution

Component	stock	volume / 1 l	final concentration
5x PBS pH 7.4	5x	200.0 ml	1x
Tween20		0.5 ml	0.05 %
dH_2O		ad 1000 ml	

Blocking solution

Component	stock	volume / 1l	final concentration
5x PBS pH 7.4	5x	20.0 ml	1x
nonfat dried milk powder		5.0 g	5.0 %
dH_2O		ad 100.0 ml	

SDS Gels:

Separating Gels:

Component	5 %	7,5 %	10 %	12 %	15 %
dH_2O	11.39 ml	9.69 ml	8.02 ml	6.69 ml	4.69 ml
1,5 M Tris pH 8,8	5.0 ml	5.0 ml	5.0 ml	5.0 ml	5.0 ml
10 % SDS	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml
Acrylamide/Bis (30 % - Stock)	3.3 ml	5.0 ml	6.67 ml	8.0 ml	10.0 ml
10 % APS	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml
TEMED	0.01 ml	0.01 ml	0.01 ml	0.01 ml	0.01 ml

Stacking Gel (5 %):

Component	2 Gels
H_2O_{dest}	5.65 ml
0,5 M Tris pH 6,8	2.5 ml
10 % SDS	0.1 ml
Acrylamide/Bis (30 % - Stock)	1.7 ml
10 % APS	0.05 ml
TEMED	0.01 ml

5.7.3. Antibodies.

Primary Antibodies:

antibody	species	company	Cat.No.
Rac 1	mouse	BD transduction laboratories	610651
P67phox	mouse	BD transduction laboratories	610912
GAPDH	mouse	Chemicon	MAB374
eNOS	mouse	BD transduction laboratories	610297
eNOS Pser 1177	rabbit	Upstate	07-428
nNOS	mouse	BD transduction laboratories	610309
iNOS	mouse	BD transduction laboratories	610432
MnSOD	rabbit	Upstate	06-984
Pre ANP	rabbit	Chemicon	ab5490
SERCA	rabbit	Abcam	ab3625
PLN	mouse	Alexis Biochemicals	804-093-c100
P-PLN	rabbit	Upstate	07-052
ER A	rabbit	Santa Cruz	sc-542
ER B	rabbit	Molekulare Kardiologie Wuerzburg	_
Cox-2	rabbit	Cayman Chemical company	160106
PR	mouse	Neomarkers	MD-298-P1
MR	goat	Santa Cruz	sc-6860
GR	rabbit	Santa Cruz	sc-1004
AII AT1	rabbit	Santa Cruz	sc-1173
AII AT2	rabbit	Alpha Diagnostics Intl	AT21-A
ENaC	rabbit	Abcam	ab3464
Nitro tyrosin	rabbit	Upstate	06-284
PECAM	goat	Santa Cruz	sc-1505
vWF	goat	Santa Cruz	sc-8068
OPN	rabbit	Abcam	ab8440-200

Secondary Antibodies:

antibody	label	species	company	Cat.No.
mouse	Alexa Fluor 594nm	goat	Molecular Probes; Leiden, Netherlands	A-11005
rabbit	Alexa Fluor 488nm	goat	Molecular Probes; Leiden, Netherlands	A-11008
goat	HRP (Horseradish Peroxidase)	donkey	Abcam; Campbrigde, UK	ab6667
rabbit	HRP (Horseradish Peroxidase)	donkey	GE Healthcare; Munich, Germany	NA9340V
mouse	HRP (Horseradish Peroxidase)	donkey	GE Healthcare; Munich, Germany	NA9310V

6. Methods.

6.1. Animal experiments.

All procedures described here were approved by the institutional animal research committee and kept under conventional conditions in the animal facility in the "Medizinische Universitätsklinik Wuerzburg". Experimental approches were performed in according to the animal care guidelines of the American Physiology Society. Female Wistar K rats were provided by Harlan-Germany (Myocardial infarction model) and Charles Rivers-France (Aldosterone Salt Treatment rat model). All surgical procedures were carried out under isoflurane anesthesia (isoflurane 1.5vol% supplemented by 0.5l oxygen per minute) following pre-treatment with tribromoethanol / amylene hydrate ("Avertin"; 2,5% WT/vol, 6 µl/g BW ip.). One week before the initiation of the protocols the rats were randomized in different groups and were ovariectomized. Two days after ovariectomy hormone supplementation was began.

6.1.1. Hormone supplementation.

Steroids were dissolved in EtOH and administered by daily sc. injections using either peanut oil (17β-estradiol) or castor oil (MPA, drospirenone) as carrier; spironolactone was supplied with tapwater.

6.1.2. Experimental myocardial infarction in rat.

The protocol for left coronary artery ligation has been described previously and is routinely performed in our laboratory (Pelzer T, 2005). Rats were sacrificed twoo months post infarction to analyze chronic responses. Ventricles were isolated and preserved in tissue tec.

6.1.2.1. Surgery.

After an adequate depth anesthesia the rat was fixed in a supine position with tape. A 5-0 ligature was placed behind the front upper incisors and pulled taut so that the neck is slightly extended. The tongue was retracted, held with forceps and a 20-G i/v catheter was inserted

into the trachea. Afterwards, the catheter was attached to the rat ventilator via the Y-shaped connector. Ventilation was performed with a tidal volume of 200.0 µl and a respiratory rate of 133 per minute. 100 % oxygen was provided to the inflow of the ventilator. Prior to the incision, chest was disinfected with Betadine solution and 70 % ethyl alcohol. The chest cavity was opened by an incision of the left fourth intercostal space, whereby the chest retractor was applied to facilitate the view. The heart was exposed, the pericardial sac was opened and pulled apart, and the left anterior descending (LAD) artery was excavated. Ligation was proceeded with a 7-0 silk suture passed with a tapered needle underneath the LAD artery about 1-2 mm lower than the tip of the left auricle. Occlusion was confirmed by pallor of the anterior wall of the left ventricle. A drop of 1 % Lidocaine was placed on the apex of the heart to prevent arrhythmia. Finally, lungs were overinflated and the chest cavity, muscles and skin were closed.

6.1.3. AST rat model.

The model of mineralocorticoid hypertension used in the present study followed the protocol previously described by Brilla and Weber (1992). In this model, animals are uninephrectomized and given a 1% NaCl drinking solution to exacerbate, without qualitatively modifying, the hypertension and end-organ damage induced by mineralocorticoids. At the time of surgery, an Alzet 2004 osmotic minipump (Charles Rivers-Alza Corp., Palo Alto, CA, USA) containing either vehicle (9% ethanol/ 0,9%NaCl in dH2O) or 2.9 mg/mL d-aldosterone (Sigma Chemical Company, St. Louis, MO, USA) was inserted subcutaneously between the shoulder blades. The dose of aldosterone administered was calculated according to the delivery time of the minipump in order to release 0.75 µg of aldosterone per hour. Minipumps were changed after 4 weeks and the final evaluation of the morphological parameters as well as organ collection was done after 8 weeks of treatment.

6.1.4. Transthoracic echocardiography. (M-mode: echocardiography, measurements).

Echocardiography was performed 3 days and 8 weeks following myocardial infarction using a Toshiba Power Vision 6000 system and a 15MHz ultrasound probe under with ketamine / xylasine anesthesia. 2D left-parasternal short-axis and transversal M-mode tracing were recorded according to published protocols (Pelzer T, 2005). Animals in groups 3 to 6 without detectable or with minor infarcts were excluded from further analysis upon echocardiography at day 3.

6.1.5. Hemodynamic measurements and left ventricular volume.

Hemodynamic measurements were performed as reported previously under light isoflurane anesthesia and spontaneous respiration (isoflurane 1.5vol%; 0.5l oxygen/minute) (Pelzer T, 2005). Pressure curves were obtained via fluid-filled PE 50 tubing connected to a microtip manometer (Millar Instruments) inserted via the right carotid artery and calibrated to mid-chest level. Left ventricular pressure curves were recorded from the LV cavity, systolic and diastolic blood pressure measurements were obtained upon catheter placement in the thoracic aorta. Measurements were performed by a single, trained observer blinded for treatment groups.

6.1.6.Morphological measurements.

After hemodynamic measurements a PE50 plastic tube was inserted in the right carotid artery and used to collect blood. Still anesthetized rats were decapitated and the toraxic cavity was opened to remove heart, lungs and thoraxic aorta. Then a medial insition was followed to expose the abdominal cavity and to remove liver, right kidney and uteri. Organs were weighted, prepared for histology and preserved at -80C. Uteri were fixed in buffered formaldehyde, cut into 4 µm transverse sections and stained with HE for quantitative evaluation of luminal epithelial cell height using an Axiophot 2 microscope and a KS 400 imaging system (Carl Zeiss Vision GmbH, Germany). The right kidney from each rat were embedded in TissueTec OCT and divided in interpolar axis sections. Mid-kidney sections

were consecutively fixed in 10% buffered formalin and processed for conventional paraffin sectioning. Multiple sections were prepared from each animal for hematoxylin / eosin and Masson trichrome staining. The remaining sections were immediately snap-frozen in liquid nitrogen. Frozen samples were stored at -80°C for molecular analysis.

6.1.7. Cardiac histology.

Hearts were arrested in diastole using 1mol/L KCl and dissected into three consecutive slices consisting of apex, mid-ventricle and base. The mid-ventricular section was embedded into TissueTec OCT (Sakura) and cut into 5 μm sections for hematoxylin-eosin (HE) and 6 μm sections for picro sirius red (PSR) staining.

6.1.7.1. Cardiomyocyte Cross Sectional Area evaluation.

Measurements were performed according to published protocols on mid-ventricular sections (6μM) stained with HE from manual tracings of 80 randomly selected cardiac myocytes from each animal using the "Image J" software (NIH).

6.1.7.2. Perivascular collagen quantification.

Perivascular collagen accumulation was quantified around 6 randomly selected coronary arteries in 3 non-adjacent sections stained with Picro Sirius Red, from each animal using the image analysis system "Scion" (NIH).

6.1.7.3. Infarct size measurement.

Infarct sizes were determined according to previously published protocols using mid-ventricular sections (6μM) that were fixed in Tissue-TEK OCT and stained with hematoxylin-eosin (HE) or picrosirius red (PSR) (Pelzer T, 2005). Infarct size was calculated from manual tracings of HE and PSR stained sections and are expressed as the percentage of infarcted vs. total LV circumference (n=3 sections / animal).

6.1.8. Aorta Intima to media Area.

Media and intima areas and intima to media ratios were measured in 3 non-adjacent aortic sections from each animal following incubation in 1mol/L KCl, fixation in TissueTec OCT

and HE or picro sirius red staining. Intima and media areas were calculated from manual tracings of the intima-media border and media-externa border using the "Image J" software (NIH).

6.1.9. Metabolic cages.

Water consumption and urinary excretion were analyzed by placing rats in metabolic cages for 24 hours with liquids and food ad libitum, control animals received normal tap water and AST treated rats 1%NaCl solution to drink. To reduce stress and related alterations in behavior the animals were previously placed in the metabolic cages without recording the measurements (Cordaillat M, 2005).

6.2. Western Blotting.

Cardiac gene expression was analyzed by Western blotting of crude cardiac extracts generated from the remote myocardium (septum), kidney protein extracts were prepared from a complete transversal to the interpolar axis section.Tissues were lyzed in RIPA buffer, then the samples were centrifugated at 1500 rpm for ten minutes at 4°C to remove nuclei and the insoluble fraction. Afterwards the samples were stored at -80^{o}C. The protein concentrations were determined by the Bradford Protein Assay using a BSA standard curve. Laemmli buffer was added to each sample (v/v 1:1) and boiled at 95°C for ten minutes to denaturate the proteins. Protein samples were separated on 7.5 - 15 % SDS polyacrylamide gels, depending on the size of each target protein, and electrophoretically transferred to nitrocellulose membranes. After transfer membranes were blocked for one hour using blocking solution (5 % nonfat dried milk powder in PBS/Tween20), following incubation with the primary antibody (diverse incubation times; regularly ON at 4°C). After washing with 1x PBS (six times each ten minutes) membranes were incubated with species specific horseradish peroxidase labelled secondary antibodies in PBS/Tween20 buffer for one hour at room temperature. Visualization was done by ECL kit accordining to the manufacturer's manual (GE Healthcare, Amersham; Munich, Germany). The ImageQuant software (Biometra) was

used for densitometric analysis based on peak area. GAPDH was employed as internal standard.

6.3. Immunohistochemistry.

Fresh frozen rat heart, kidney and arota tissue sections (5 μm) were mounted on object slides. After fixation in 4 % Paraformaldehyde/PBS for twenty minutes the tissues were permeabilized in 1 % Triton X-100/PBS for twenty minutes. Sections were incubated in PBS 1% H2O2 to block endogenous peroxidase activity and then washed in PBS. Nonspecific protein binding was blocked by incubation with Immpress Horse or Goat blocking solution (species specific respecting to the second antibody, Vector Laboratories), for sections marked with the ABC kit an extra step of blocking endogenous avidin/biotin was done before proceding with the primary antibody incubation. Sections were incubated with primary antibodies ON at 4°C. Next day, sections were washed (six times each ten minutes with PBS) and incubated with diverse Avidin labeled secondary antibodies (species specific to the first antibody). Antibodies were diluted in 1x PBS containing 5 % serum (species specific respecting to the second antibody). After 5 washes (each ten minutes with PBS) the signal was detected using the Immpress kit and 3´3´-diaminobenzidine in addition Hematoxylin QS was used as counterstaining (Vector laboratories). For section marked with the avidin/biotin system (ABC) a previous step of ABC labeling preceeded the development. Negative controls consisted in primary antibodies pre-incubated 1 h at room temperature with non relevant IgG, for the nitro-tyrosin antibody the antigenic binding site was blocked with 3-nitrotyrosin (10mM). Each slide was compared to an adjacent section in which primary antibodies were blocked.

6.3.1. Quantitative immunohistochemistry.

Sections were visualized in the Axioscop 2 microscope and image acquisition was done using the software Methamorph. DAB positive signal was quantified using the Scion software (NIH), taking at least three sections per specimen with three fields of view per section in at

least 4 animals per group Immediately adjacent sections, in which primary antibodies were blocked, served as negative controls.

6.4. In situ detection of reactive oxygen species (ROS) generation.

The fluorescent dye APF (3´-(p-aminophenyl) fluorescein, Molecular Probes-Invitrogen) was employed to visualize ROS generating enzyme activities in 25 μm cardiac cross sections, mounted on horizontal chamber slides. Slides were incubated for 1 h in a buffer containing $CaCl_2$ (10mM), MgCl2 (1.1mM), KCl (5.4mM), NaCl (140mM), NaH_2PO_4 (4.2mM), glucose (10mM), $NaHCO_3$ (23 mM) and APF (5 μM). Negative control slides were pre-treated for 2 h with superoxide dismutase (SOD; 1 μg/ml) and catalase (1 μg/ml SIGMA). The reactions were stopped and samples were fixed by adding 4% paraformaldehyde in 100mM/L PBS (pH 7,4). Digital images were obtained using a Nikon ACT 1 imaging system.

6.5. Statistics

All data are expressed as mean $\pm$ SEM. Multi-group comparisons were done by ANOVA tests followed by Students-Newman-Keuls post-hoc pair wise testing, using the programm Sigma Stat. A p-value of <0.05 was considered as significant.

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Abbreviations.

A II	Angiotensin II
ACE	Angiotensin Converting Enzyme
Ach	Acetylcholine
ACTH	Adreno-Corticotrophin Hormone
ADH	Anti Diuretic Hormone
ANP	Atrial Natriuretic Peptide
APF	3´-(p-aminophenylfluorescein)
Apoe	Apolipoprotein E
AR	Androgen Receptor
AST	Aldosterone Salt Treatment
AT 1	Angiotensin Type 1 receptor
AT 2	Angiotensin Type 2 receptor
AW	Anterior Wall
BP	Blood Pressure
CAD	Coronary Artery Disease
CCSA	Cardiomyocyte Cross Sectional Area
CEE	Conjugate Equine Estrogens
CHF	Congestive Heart Failure
CVD	Cardio-Vascular Disease
DCM	Dilated Cardio-Myopathy
DRO	Drospirenone
E2	17-β estradiol
ENaC	Epithelial Sodium Channel
eNOS	endothelial Nitric Oxide Synthase
ER	Estrogen Receptor
ER-α	Estrogen Receptor alpha
ER-β	Estrogen Receptor beta
FS	Fractional Shortening
GR	Glucocorticoid Receptor
HE	Hematoxylin-Eosin
HERS	Heart and Estrogens/Progestins Replacement Study
HF	Heart Failure
HRT	Hormone Replacement Therapy
HUVEC	Human Umbilical Vein Endothelial Cells
iNOS	inducible Nitric Oxide Synthase
JG	Juxtaglomerular
KO	Knock Out
LAD	Left Anterior Descending
LTCC	L-Type Ca+2 Channels
LVEDA	Left Ventricle End Diastolic Area
LVEDP	Left Ventricle End Diastolic Pressure
LVESA	Left Ventricle End Systolic Area
MPA	Medroxy Progesterone Acetate
MR	Mineralocorticoid Receptor
NCX	Na+/Ca+2 exchanger
nNOS	neuronal Nitric Oxide Synthase

NO	Nitric Oxide
NR	Nuclear Receptor
OPN	Osteopontin
PLN	Phospholamban
P-PLN	Phospho-Phospholamban
PR	Progesterone Receptor
PRAKO	Progesterone Receptore A Knock Out
PRBKO	Progesterone Receptor B Knock Out
PRKO	Progesterone Receptor Knock Out
PSR	Picro Sirius Red
RAAS	Renin Angiotensin Aldosterone System
RIA	Radio Immuno Assay
ROS	Reactive Oxygen Species
RyR	Ryanodine Receptor
SERCA2a	Sarcoplasmic Reticulum Ca+2 ATPase
SNP	Sodium Nitroprusside
SOD	Super Oxide Dismutase
SPIRO	Spironolactone
SR	Sarcoplasmic Reticulum
VSMC	Vascular Smooth Muscle Cells
WHI	Women´s Health Initiative

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- Heart Failure in ER β knock out mice following myocardial infarction. 4. Bad Brückemauer Wintertagung. Feb, 2005 (Medizinische Klinik und Poliklinik I, Universität Würzburg-Germany).

Curriculum vitae.

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Academic Education.

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1982-1993.	School and High-School education in the Santa Teresa School. La Paz-Bolivia.

Professional Experience.

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Scholarships.

2003-2005.	PhD Scholarship (Promotionstipendium), German Academical Exchange Service (DAAD).
2002; 2006-2007.	Research Scholarship from the Bayerischen Julius-Maximilians-University of Wuerzburg.

Awards.

1998.	Winner of the second prize in Pure and Natural Sciences in the II Scientific Investigation Contest EXPO-UMSA 1998. Topic: Preliminary study for the development of a diagnotic kit of Rotavirus. CIPGIS-UMSA. La Paz-Bolivia.
1998-1997.	Guido Capra Jemio Excellence Award as the best student of the Biology career of UMSA. La Paz-Bolivia.
1998-1995.	Honor diploma for a Distinguish Academic Efficiency in Biology. Given by the UMSA Honorable University Council. (Award for the best student), La Paz-Bolivia.