Stress and Cardiovascular diseases

More insights into a possible role of RAAS

Master report
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Abstract

Cardiovascular diseases (CVDs) are a major cause of all deaths worldwide. Endothelial dysfunction and its initiation to atherosclerosis are key factors in the development of CVD. Stress has an important role as well in the progression of CVD, although the knowledge about the exact underlying mechanism is not complete yet. Similarly, while the role of Renin-Angiotensin-Aldosterone-System (RAAS) in cardiovascular homeostasis is well described, there is not much known about the effects of stress on endothelial dysfunction through RAAS. Therefore, the present study aimed to investigate changes in RAAS activity as a consequence of stress. To evaluate this, rats from the resident-intruder paradigm were used. Intruders, which are rats that were repeatedly exposed to a social defeat interaction were compared to control rats. The two groups of animals were housed socially (group-wise) or solitary. Several parameters and measurements, including body weight, relative organ weights, tissue ACE activity, aorta vasodilatation/-constriction responses and plasma creatinine levels and ROS production were assessed in all rats. Relative organ weights were increased in solitary housed rats with respect to socially housed animals. Analysis of tissue ACE activity showed that intruders had increased ACE activity in the heart ventricle as compared to their respective controls. Absolute contraction to a high dosage of KCl was reduced in aortic rings of the group of intruders, accompanied by lack of LNMMA-mediated constriction at baseline. Furthermore, Ang2-stimulated constriction in the presence of LNMMA was significantly smaller in the latter group. These results demonstrated a remarkable impact of social stress on RAAS activity, in particular in cardiovascular tissue. Social stress by means of social defeat enhances ACE activity locally in the heart, leading to alterations in vascular function, impaired responsiveness to Ang2 and reduced basal NO-release. These observations require further experiments to understand the exact interplay between stress, RAAS and the development of CVDs.

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Introduction

Cardiovascular disease (CVD) belongs to one of the most common causes of mortality and morbidity worldwide. In Westerns countries 30 to 50% of all deaths are the result of CVD. Globally, 17.3 million people die every year from the consequences of CVDs. Further predictions indicated that this number will reach 25 million in 2030 if effective therapy is not found (*Mahmood D et al, 2014*). CVD affects blood vessels and the heart leading to hypertension, coronary heart disease and stroke (*Aleman BM et al, 2014*, *Nasser Z et al, 2015*).

A key player in CVD is the endothelium (*Sampson UK et al, 2015*). The endothelium consist of lined cells that form a monolayer on the luminal side of vessels and thereby segregates tissue from the circulating blood (*Hahn CS et al, 2015*). The endothelium regulates angiogenesis, inflammation and vascular tone. The latter is accomplished by the production of vasodilatation and vasoconstriction mediators, particularly nitric oxide (NO) and endothelin-1. The endothelium has its main task of maintaining a physiological balance between the secretion of relaxing and contracting factors (*Kong BW et al, 2015*). In healthy conditions endothelial nitric oxide synthase (eNOS) produces NO as a vaso-protective agent. The hydrolyzation of L-arginine by eNOS results in the production of NO (*Hwang HM et al, 2015*). NO diffuses to vascular smooth muscle cells, where it activates soluble guanylate cyclase leading to enhanced levels of cyclic guanosine monophosphate (cGMP). Subsequently, stimulated cGMP-dependent kinases induce a reduction in calcium levels in the cells resulting in relaxation (Santillo M et al, 2015). Oppositely, when L-arginine is converted into urea and L-ornithine the activity of eNOS will decline, accompanied by insufficient cofactors, reduced NO release and enhanced NO degradation, resulting in less NO bioavailability (*Hwang HM et al, 2015, Boa BC et al, 2015, Radenković M et al, 2014*). This leads to endothelial dysfunction which is the initiating pathophysiologic state of atherosclerosis (*Hwang HM et al, 2015, Boa BC et al, 2015, Badran M et al, 2015*).

Atherosclerosis is a complex disorder associated with the recruitment of inflammatory cells and the release of inflammation-inducing cytokines (*Alfaidi M et al, 2015*). The early stage of atherosclerosis is characterized by the inflammation of the endothelium which induces the expression of adhesion molecules. This is followed by the attachment of leukocytes to the endothelium, which then invade the intima (*Frostegard J et al, 2013*). This leads to further accumulation of lipids, white blood cells, other cellular debris and plaque formation, consequently promoting arterial wall thickening (*Shen Z et al, 2015*). Atherosclerosis could reduce blood flow by stenosis. Damaged plaques and rupture facilitate the exposure of prothrombotic material to the coagulation system, imbedding the blood flow (*Frostegard J et al, 2013*).

An important feedback mechanism involved in the regulation of blood pressure is the Renin-anigiotensin-aldosterone-system (RAAS). The activation of RAAS starts with the release of renin to the circulation by juxtaglomerular cells in the kidney. As a result, renin induces the conversion of angiotensinogen into angiotensin-1 (Ang1), followed by the hydrolyzation of Ang1 into Ang2 by angiotensin-converting-enzyme (ACE). In the normal physiological condition, Ang2 binds to angiotensin-1 receptor (AT1) to cause vasoconstriction and salt-retention. However, when the RAAS is hyperactive, Ang2 contributes to cardiac hypertrophy, oxidative stress and inflammatory responses (*Raven PB et al, 2014*). Besides, several vascular pathologies including hypertension and atherogenesis are associated with Ang2-stimulatd structural changes in the vessel wall (*Santana AB et al, 2014*). Next to the effects of Ang2 on cardiovascular system, RAAS has several local functions in tissues such as the kidneys, adrenals, vasculature, heart and nervous system. Depending on the tissue, local RAAS exerts its actions independently and dependently of the circulating RAAS. RAAS has a very significant contribution on cellular level through autocrine and paracrine effects, which can

mediate cell specific actions involved in cell proliferation, growth, apoptosis and metabolism (*Paul M et al, 2006*).

Important risk factors for CVD such as smoking, elevated blood pressure and cholesterol level trigger CVD via endothelial dysfunction (*Aleman BM et al, 2014*, *Nasser Z et al, 2015, Sampson UK et al, 2015*). Another important but less well investigated risk factor for CVD is stress. The link between stress and cardio-related diseases was already proposed more than 40 years ago. For instance, Wiliam Osler proposed 'the wear and tear of life' as an important contributor to myocardial infarction. Robert Karasek, who introduced the 'job strain model' suggested that when there is demand for high physiological effort while there is just low individual control available, this will induce physiological strain leading to CVD (*Kivimäki M et al, 2015*). Stress is also found to be a major cause of mortality in heart failure patients. Patients suffering from CVD were shown to respond adversely to psychological stress. Psychological stress induced declined blood supply of coronary artery, increased progress of heart disease, worse prognosis and decreased cardiac function (*Alhurani AS et al, 2014*).

Stress responses can be divided into acute and long term responses. The autonomic nervous system mediates acute responses through parasympathetic and sympathetic nervous systems. The activity of the autonomic nervous system by the release of epinephrine, norepinephrine and acetylcholine induces changes in heart contractility, vascular tone, heart rate and blood pressure. The SNS innervates the inner part of the adrenal gland, which results in the secretion of norepinephrine and epinephrine. Long-term physiological responses are driven by the release of corticotrophine releasing hormone, adrenocorticotrophic hormone and cortisol via the hypothalamic-pituitary-adrenal (HPA) pathway. Therefore, cortisol is regarded as the hormone that is directly associated with stress (*Roemmich JN et al, 2014*).

Taken together, RAAS is involved in normal regulation of blood pressure, but also in processes which trigger CVD. Stress is also suggested to contribute to CVD development, although the underlying mechanisms herein remain largely unknown. In particular, the link between stress and RAAS in CVD is relatively unstudied. Therefore, this study aimed to investigate changes in RAAS activity following stress conditions. On the basis of available literature and the interpretation hereof we hypothesize that stress induces 'hyperactivity' of RAAS.

To address this hypothesis we employed the rat resident-intruder model. The resident-intruder paradigm is widely used in the research field of aggression as a model to investigate offensive and defensive behaviour. Essentially, the placement of an intruding rat in the home cage of the resident triggers a social conflict, characterized by a social defeat of the intruder (*Koolhaas JM et al, 2013*). Hence, this model allows to investigate the impact of social stress by studying intruders repeatedly exposed to a defeat situation. Since, the housing of intruding animals is a very important factor in the research of social stress, the social conflict is always performed in a separate room to avoid control animals witnessing the conflict. Therefore, to additionally investigate the buffering effects of social environment on social stress, intruder and control animals were housed either socially or solitary (*Koolhaas JM et al, 2013*). To further define the impact of stress by social defeat on RAAS activity, blood plasma and organ tissues were collected at the end of the study and assayed for (tissue) ACE-activity. In addition, isolated aorta preparations were studied *in vitro* for contractile responses to Ang2. Isolated aorta preparations were also studied for acetylcholine-induced endothelium-dependent relaxation as an indices for the impact of stress on endothelial function. In the latter studies, the NOS-inhibitor N^G-Monomethyl-L-arginine (LNMMA) was used to further investigate possible alterations in NO. Comparisons were made to control rats which were similarly housed and handled but without the exposure to a resident.

Materials and methods

Animals

In this study 36 Wild-type Groningen (WTG) rats were used. WTG rats are originally wild-trapped and have been bred for more than 25 years at the laboratories of the department of Behavioural Physiology at the University of Groningen. Until the start of the study, rats were housed in group cages (n=5-6) under a 12:12-h light-dark cycle with free access to (tap-) water and standard food. During the study period each resident was housed with a sterilized female (*Coppens CM et al, 2014*). Groups of intruder and control rats were housed either socially (in group cages, n=4-6) or solitary.

The resident-intruder model

Five groups of adult male animals (6-7 months old) were studied, namely male residential animals (n=6), male intruding animals housed either socially (n=9) or solitary (n=11), and male control animals housed either socially (n=5) or solitary (n=5). One hour prior to the conflict situation the female was removed from the home cage. Subsequently, an unfamiliar male intruder (a little smaller and younger (1-2 months) compared to the resident) was placed in the residential cage and the behavioural patterns of the animals were recorded by a video camera. When the test was completed (after about 10 minutes) the intruder returned to its home cage and was replaced by the female (*Koolhaas JM et al, 2013*). In this setup the intruders are always the ones who experience a social defeat, hence those animals were exposed to repeated stress. On average each resident experienced n=50 interactions (i.e. wins) and each intruder n=25 interactions (i.e. defeats).

Sacrifice and tissue collection

At the end of the study period, rats were shortly exposed to CO_2 on dry ice, followed by decapitation. Immediately after that, blood was collected in plastic K2E (EDTA) 4,0 mL blood collection tubes and centrifuged at 4000 rpm for 10 minutes at 4 °C. The obtained plasma was transferred in new eppendorf tubes and stored at -80 °C until assessment of malondialdehyde (MDA) and creatinine. Heart, right lung, left kidney and left adrenal were excised, cleaned, rinsed free of blood and weighed before being stored at -80 °C. In addition, the thoracic aorta was removed and used for *in vitro* studies of vascular reactivity.

Fluorometric measurement of ACE activity

Tissue lung, kidney and heart ventricle ACE activity was assayed fluorimetrically using the substrate hippuryl-L-histidyl-L-leucine (HHL). First the tissues were diluted (100x) in cold potassium phosphate buffer and homogenized using ultrathorax. Based on the type of tissue, further dilutions were made as followed: heart: 200x; lung: 1500x; kidney: 200x. The tissue homogenates were immediately used in the ACE activity assay. Subsequently, 50 µL of diluted homogenized heart, lung or kidney tissues with 25 µL demineralized water and 50 µL substrate HHL were incubated at 37 °C. After 15 minutes the enzyme-substrate reaction was stopped by adding 750 µL 270 mM NaOH. The product His-Leu generated by the cleavage reaction was labelled with 50 µL o-phathaldialdehyde (OPA) dissolved in methanol, followed by 10 minutes incubation in the dark. As the last step, all samples were incubated with 100 µL 3 M HCL for 30 minutes in the dark. Fluoroscentric measurement has been performed using Costar 96-well polystyrene plate. The fluorescence was read at 355 nm excitation and 460 nm emission by Synergy H4. The amount of ACE activity in the samples was calculated by comparison with a predetermined His-Leu standard curve.

In vitro assessment of vascular reactivity

Aortic ring preparation

The aorta was kept in a Krebs buffer solution containing (in mM) 120.4 NaCl, 5.9 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 11.5 glucose and 25 $NaHCO_3$, continuously aerated with 95% O_2 – 5% CO_2 at pH 7.4. The vessel was cleaned of fat and adhering tissue and cut into 8 ring segments (of approximately

2 mm in width).The rings were mounted into an organ bath system filled with Krebs solution at 37 °C and connected to a transducer for assessment of isotonic displacements. Rings were given a preload of 1.4 g and allowed to equilibrate for 45-60 min. After that, the rings were primed and checked for viability by repeated exposure (3 times) to 60 mM potassium chloride (KCl) with intermediate washing and renewed stabilisation. The last contractile response to KCl was set to a 100% and used as a reference response.

Experimental Protocols

Four different protocols were used in duplicate rings. In order to evaluate receptor-independent contractility KCl-induced vasoconstriction was studied by cumulative administration of KCl (10-100 mM). α1-Adrenergic receptor-mediated contractility was assessed by cumulative addition of phenylephrine (PE, 1 nM – 10 µM) and AT-receptor mediated contraction was studied by cumulative administration of angiotensine-2 (Ang2, 1 nM - 1 µM). Contractions to PE and Ang2 were expressed as a % of the above mentioned KCl reference response. Finally, endothelium-dependent relaxation was assessed by cumulative administration of acetylcholine (ACh, 1 nM – 10 µM) to rings pre-constricted with (1 µM) PE. After administration of the final concentration of ACh, a single high concentration of sodium nitropruside (SNP, 10 µM) was administered to assess maximal endothelium-independent relaxation. Here, PE-induced pre-contraction was considered as 100% and used to express the relaxation responses. All protocols were assayed under basal conditions (saline) as well as after pre-incubation with the NOS inhibitor N^G-Monomethyl-L-arginine (LNMMA, 100 µM).

Plasma creatinine measurement

Creatinine concentration was determined in 150 µL plasma at the Central laboratory in the UMCG, Groningen, Netherlands.

Quantification of MDA in plasma

The amount of reactive oxygen species was measured indirectly by assessing the levels of malondialdehyde (MDA) in plasma using the thiobarbituric acid reactive substances (TBARS) assay kit (Cell Biolabs, Inc, Netherlands). To minimize the interference by hemoglobine, 1 µL 100x butylated hydroxytoluene (BHT) was added to 99 µL plasma sample. Then 100 µL of SDS lysis Solution was added to 100 µL supernatant and MDA standards. After 5 minutes incubation at room temperature, 250 µL TBA Reagent was added to each supernatants and standards as well, followed by 60 minutes incubation at 95 °C. Next, all samples and standards were cooled to room temperature on ice for 5 minutes and centrifuged at 3000 rpm for 15 minutes. As an additional step, 300 µL of 2-Butanol was added to 300 µL of sample supernatant, vortexed vigorously and centrifuged at 10000 g for 5 minutes. MDA level in tissue was determined by reading fluorescence at 540 nm excitation and 590 nm emission in 150 µL butanol fraction using Synergy H4. The amount of MDA in plasma was calculated using the MDA standard curve.

Statistical analysis

All statistical analysis were carried out using IBM SPSS Statistics 22 software. Outliers (two times standard deviation) were excluded where appropriate. For primary analysis of groups we focussed on the impact of social defeat and solitary housing as two forms of 'stress'. Therefore a two-way analysis of variance was performed using social defeat and housing as separate factors. In addition, the data for the group of resident rats are given; when of interest, this group was compared to other groups using a one-way ANOVA followed by LSD post-hoc analysis. Data are shown as mean ± SEM and a p-value of <0.05 was considered as statistically significant.

Results

Rat characteristics

Body weight and relative organ weights are presented in Table 1. Two-way analysis suggested a marked effect of housing rather than of repeated experience of a social defeat. Indeed, intruder rats had a slightly larger body weight compared to their respective controls but this reached no statistical significance. Similarly, relative organ weights were not significantly different between controls and intruders, except for the adrenal gland weight which was significantly larger in the latter group. However, both ventricular heart weight and kidney weight as well as adrenal gland weight were significantly increased in rats housed solitary as compared to those housed group-wise - i.e. independent of their experience of a social defeat or not. As for the group of residents, these rats had a significant higher body weight as compared to the group of control rats. Vice versa, relative organ weights of residents more resembled that of socially housed rats.

ACE activity in tissues

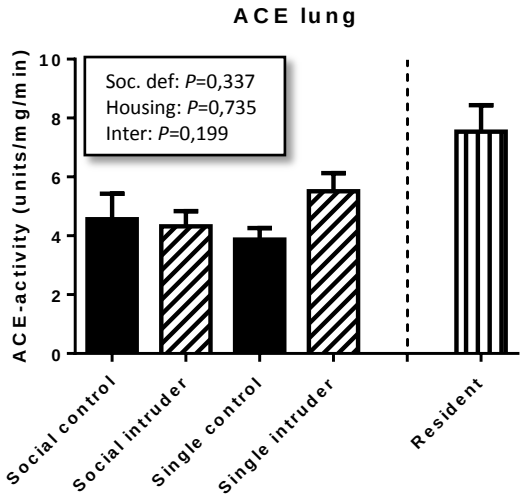
To explore the effect of 'stress' on the RAAS system, ACE activity was measured in lung, kidney and heart tissue of all rats, the data of which are shown in Figure 1. On average, lung tissue ACE activity was the highest in the group of intruder rats housed singly but this reached no statistically significant difference as compared to the other groups (Figure 1A). Indeed, there were no significant group differences in lung tissue ACE activity between intruders and their respective controls. Two-way ANOVA analysis also suggested that lung tissue ACE activity was not significantly influenced by the type of housing. Similarly, renal tissue ACE activity did not significantly differ between controls and intruders, nor was it influenced by the type of housing (Figure 1B). In contrast, ventricular heart tissue ACE activity was profoundly increased by 3- to 8-fold in the groups of intruder rats, as compared to their respective controls (Figure 1C). Moreover, this effect appeared independent of the type of housing. As for the group of residents, these rats clearly showed the highest levels of tissue ACE-activity in the lung ($p<0.05$ vs all other groups) and particularly in the kidney ($p<0.05$ vs all other groups) (Figure 1A/B). This, while heart ventricular tissue ACE activity in these rats appeared most in line with that observed in intruder rats (Figure 1C).

Table 1. Effect of social defeat and housing on rat body weight and relative organ weights

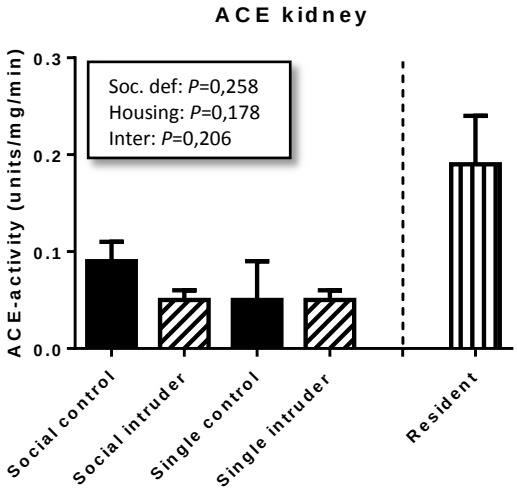
	Social		Single		Two-way ANOVA (p-values)			
Variables	Control	Intruder	Control	Intruder	Social defeat	Housing	Interaction	Resident
BW (g)	401 ± 16 (5)	418 ± 18 (9)	380 ± 26 (5)	400 ± 6 (9)	0,285	0,263	0,935	453±13 (6) *
VHW/BW (mg/g)	2,62±0,16 (5)	2,83±0,07 (9)	3,14±0,31 (5)	3,09±0,06 (9)	0,584	0,011	0,366	2,71±0,06 (6) #
LKW/BW (mg/g)	2,77±0,24 (5)	3,05±0,06 (9)	3,40±0,42 (5)	3,26±0,10 (9)	0,724	0,04	0,274	3,05±0,10 (6)
LAdrW/BW (mg/ g)	0,05±0,003 (4)	0,06±0,003 (8)	0,06±0,006 (4)	0,07±0,002 (5)	0,014	0,024	0,988	0,04±0,006 (6) **
SNP saline (µm)	*-2,9±11,2 (5)*	*1,1±5,9 (8)*	*1,2±8,6 (5)*	*-14,1±5,5 (10)*	*0,459*	*0,47*	*0,486*	*7,8±2,9 (6)*
SNP LNMMA (µm)	*-20,0±16,1 (5)*	*0,8±6,6 (8)*	*5,9±4,8 (5)*	*-23,5±11,0 (10)*	*0,703*	*0,944*	*0,034*	*7,5±6,0 (6)*

Abbreviations: body weight, BW; ventricular heart weight, VHW; left kidney weight, LKW; left adrenal weight, LAdrW. Data are mean ± SEM (n=number of valid observations). P-values indicate the result of two-way ANOVA for social defeat and housing. Hence, data for resident rats are shown for reasons of completeness but were not included in the two-way ANOVA analysis. Where appropriate, results for resident rats were compared using one-way ANOVA followed by LSD post-hoc analysis to each group. Both social defeat and solitary housing did not affect body weight. However, solitary housing significantly increased relative organ weights. * indicates p<0,05 versus control animals; # indicates p<0,05 versus singly housed animals. ** indicates p<0,01 versus intruder animals.

1A



1B



1C

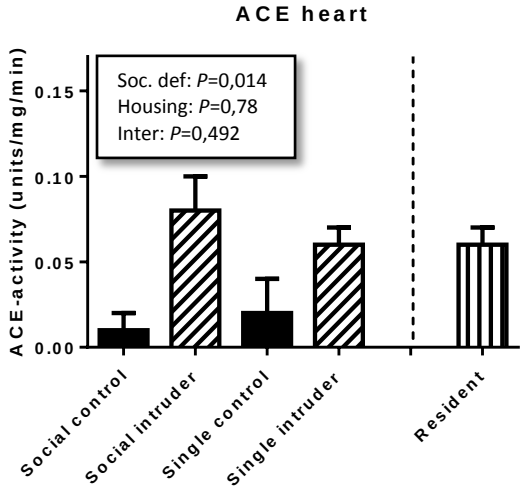


Figure 1: The impact of a social defeat interaction and solitary housing on tissue ACE activity in the lung (1A), kidney (1B) and heart ventricle (1C). Social and single in the x-legend refer to the type of housing - i.e. male rats housed either group-wise or singly. Control and intruder in the x-legend refer to the repeated exposure of a social defeat interaction - i.e. male rats exposed either to a social defeat (intruder; black bars) or not (control; hatched diagonal bars). Results for residents - i.e. male rats housed together with a female but regularly confronted with an intruder and win-situation - are shown separately in the individual panels on the right-side (hatched vertical bars). Data are mean ± SEM, p-values shown in the boxes indicate the result of the two-way ANOVA analysis for social defeat, housing and interaction. No significant differences were found for lung and kidney ACE activity between the groups (1A-B). Two-way analysis suggested a significant increase in heart ACE activity in the group of intruders as compared to their respective controls (1C).

Aorta studies of vascular reactivity

Contraction to KCL

The (third) response to a high concentration of the receptor-independent agonist KCl at the start of the aorta studies was taken as an indices for absolute contractility (i.e. in µm). Two-way analysis suggested a significant effect of stress by social defeat - but not solitary housing - such that absolute contractility was smaller in intruders as compared to control rats (Figure 2A). Full concentration-response (CR) curves to KCl were also established. These responses were expressed as a percentage of the maximum response to KCl to correct for absolute differences in contractility (Figure 2B). This figure shows that CR-curves seemed to differ significantly from intruders and controls – such that the group of control animals were more sensitive to KCL stimulation compared to intruder rats. This suggests that vascular smooth muscle sensitivity (EC_{50}-values) to calcium-increases after KCl was altered by a social defeat interaction.

2A 2B

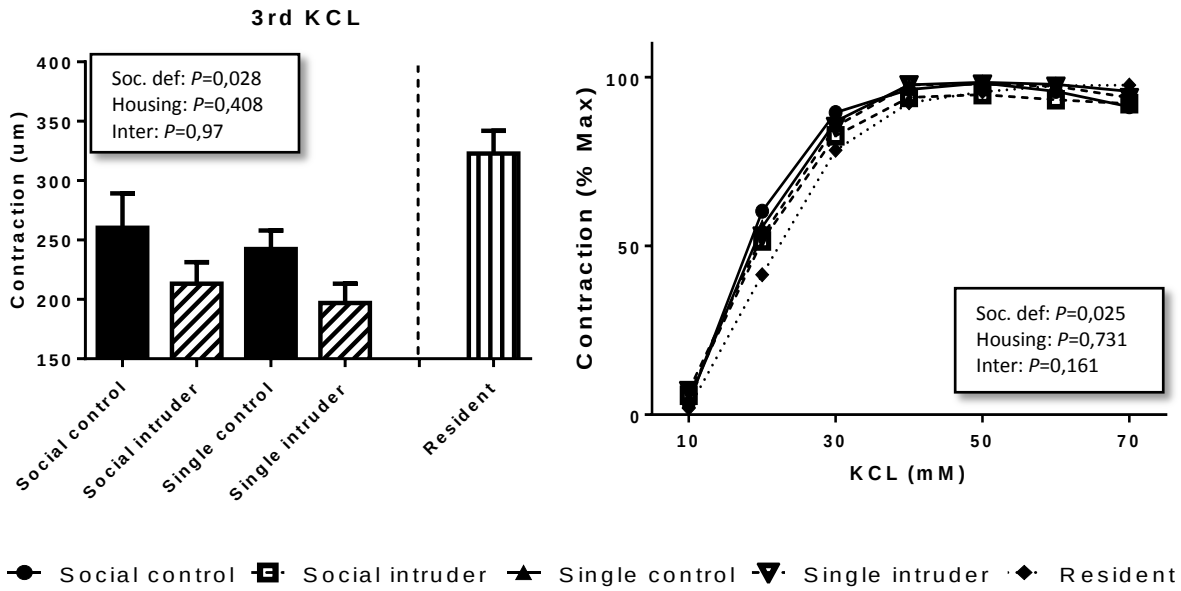


Figure 2: The effect of a social defeat interaction and solitary housing on aorta contractility to a high concentration (60 mM) of KCl (expressed as absolute contraction in µm) (2A) and full CR curves (expressed as a percentage of maximum) (2B). Solid curves composed of filled black symbols represent male control rats, dashed curves with unfilled symbols refer to male intruder rats and dotted line represent the group of male residential rats. Data are mean ± SEM or mean. Absolute contraction to KCl was significantly smaller in the group of intruders as compared to their respective controls (2A). Sensitivity of vascular smooth muscle, as assessed by cumulative administration of KCl seemed to differ significantly between intruders and controls (2B).

Effect of LNMMA on baseline

The hereafter described responses to PE, Ang2 and ACh were all studied both in the absence and presence of NO-inhibition. To this end, parallel rings were pre- incubated either with saline (vehicle) or LNMMA for 20 min. Rings incubated with saline showed a small vasodilation at baseline during the incubation period (see Figure 3A). Although two-way analysis suggested a significant effect of social defeat, the observed dilations were negligible and differences of no relevance. In contrast, administration of LNMMA to inhibit NOS caused a gradual, yet pronounced constriction in control, but not in intruder rats. Two-way analysis supported a marked significant effect of social defeat - but not of individual housing – in blunting the constriction to LNMMA (Figure 3B).

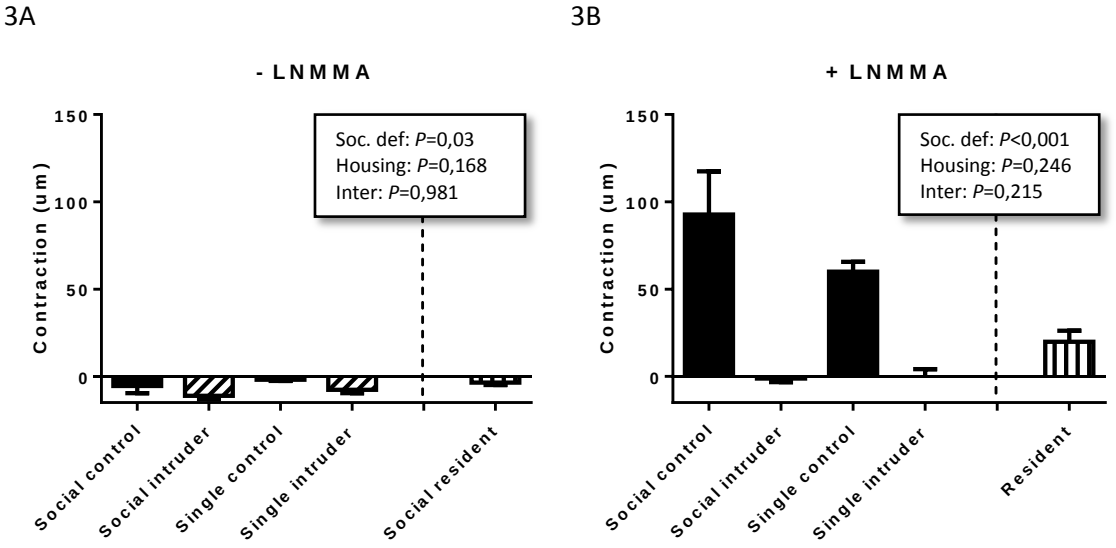


Figure 3: The impact of a social defeat interaction and solitary housing on aorta baseline in the absence and presence of LNMMA. Data are expressed as mean ± SEM. Absolute contraction (in µm) to saline was significantly different between intruders and their respective controls, such that baseline vasodilatation was smaller in controls than in intruders (3A). Same response in the presence of a single concentration of LNMMA (100 µM) gave a very significantly pronounced constriction in controls with respect to intruders (3B).

Contraction to PE and Ang2

PE and Ang2-induced contraction responses were expressed as a percentage of the KCl reference response and are shown in Figure 4. Contractions to PE did not differ significantly between the groups (Figure 4A). The presence of LNMMA generally increased the sensitivity to PE, as compared to saline, but this occurred similarly in all groups (Figure 4B). Contractions to Ang2 were small and did not differ statistically between the groups (Figure 4C). Interestingly, the presence of LNMMA increased the sensitivity and particularly maximal contraction to Ang2 only in controls, but not in intruder rats. Two-way analysis suggested a significant effect of social defeat – but not of solitary housing – in blunting the enhancing effect of LNMMA on Ang2 contraction (Figure 4D).

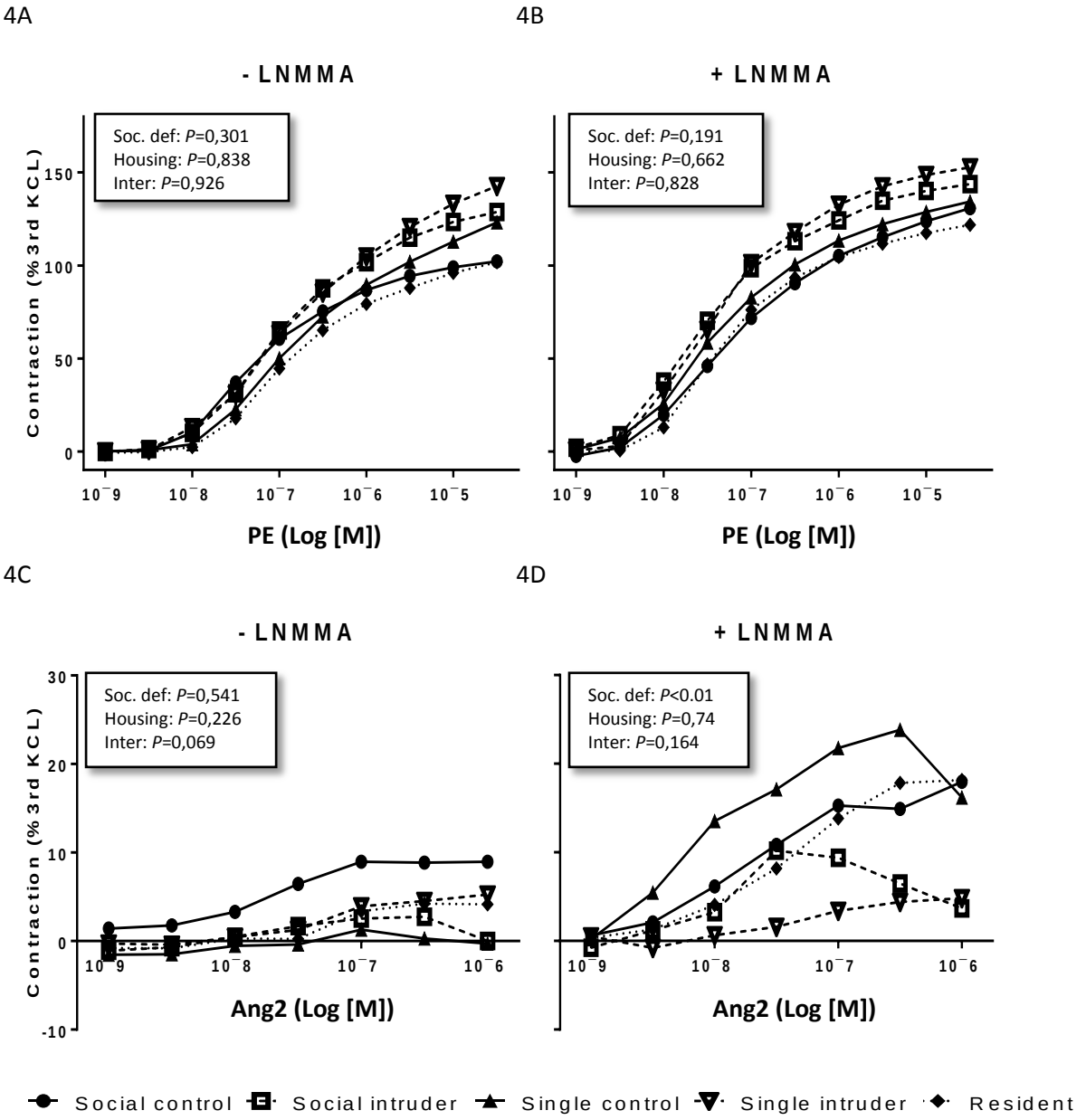


Figure 4: The effect of a social defeat interaction and solitary housing on aorta constriction in response to cumulative doses of PE (top panels) and Ang2 (bottom panels) in the absence (left panels) and presence (right panels) of LNMMA. Data are expressed as mean. PE-mediated contraction (expressed as a percentage of the 3rd KCl response) was not significantly different between intruders and their respective controls in the absence of LNMMA (4A). LNMMA increased the sensitivity to PE in all groups similarly (4B). Ang2-mediated contraction was very small and did not differ significantly between the groups (4C), while LNMMA increased the contraction response and sensitivity to Ang2 significantly in the groups of control rats compared to intruder rats (4D).

Relaxation to Ach

Endothelium-dependent relaxation to ACh was studied in rings pre-constricted with PE, and expressed as a percentage of PE pre-constriction. Cumulative addition of ACh resulted in concentration-dependent responses which maximized at approximately 50% of pre-constriction (Figure 5A). The presence of LNMMA profoundly inhibited the response to ACh, indicating that the relaxations were largely mediated by NO (Figure 5B). Nevertheless, two-way analysis suggested that relaxations to ACh – whether in the absence or presence of LNMMA – were not significantly influenced by either form of stress. Similarly, maximal endothelium-independent relaxation to SNP did not differ between the groups (see Table 1). However, two-way analysis suggested a significant effect for social defeat – housing interaction in the presence of LNMMA, in a way that social defeat interaction reduces SNP-mediated relaxation only in animals housed group-wise, while a social defeat interaction increased SNP-mediated relaxation in singly housed animals.

5A 5B

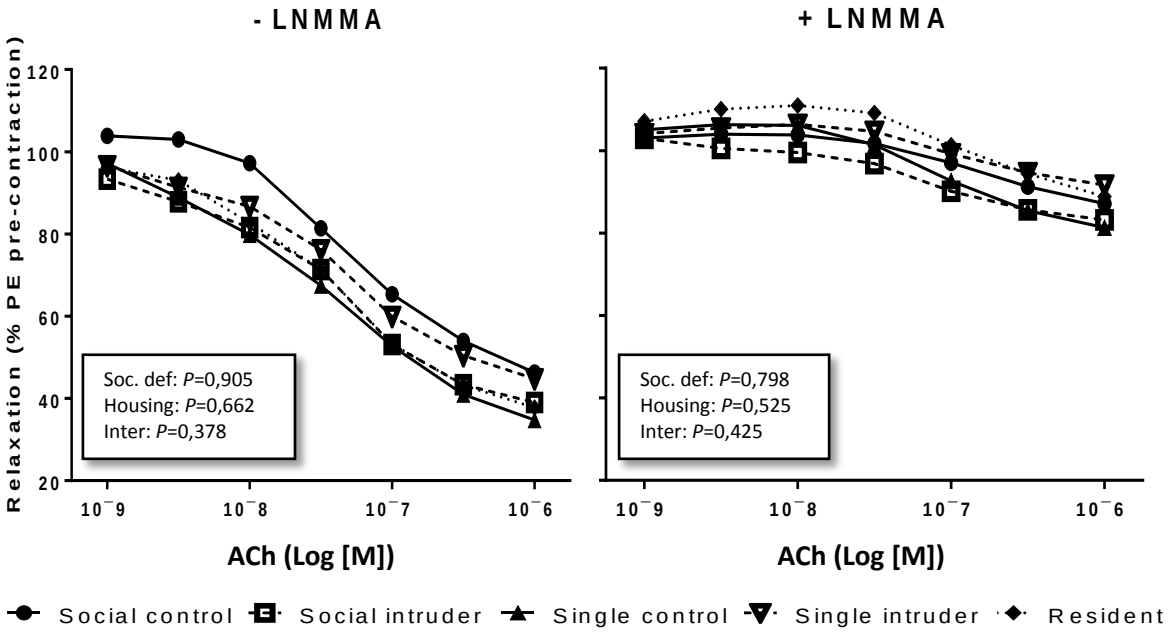


Figure 5: The impact of a social defeat interaction and solitary housing on endothelium-dependent relaxation in response to ACh. Data are expressed as mean. Cumulative doses of ACh induced about 50% relaxation in all groups (5A). This relaxation response was inhibited by LNMMA (5B). No significant differences were found between the groups in the absence and presence of LNMMA.

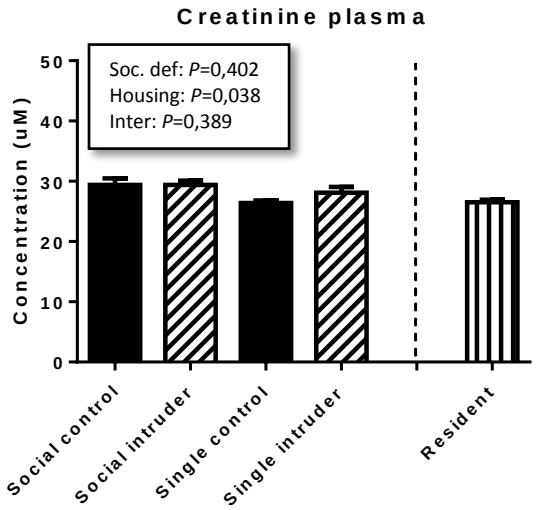
As for the group of resident rats, these animals showed a significant higher constriction to the (third) KCL response as compared to the other groups (Figure 2A). However, vascular smooth muscle sensitivity (EC_{50}-values) in residents as measured by cumulative doses of KCl did not differ significantly from the group of controls and intruders (Figure 2B). Incubation with saline resulted in negligible vasodilation at baseline (Figure 3A). Addition of LNMMA to aorta rings of resident rats induced a gradual contraction at baseline which was slightly larger compared to that in intruder aortas but significantly smaller compared to control aortas (Figure 3B). Contraction to PE in the absence and presence of LNMMA was not significantly different in the group of residential rats compared to the other groups (Figure 4A-B). Similarly, Ang2-mediated contraction responses were not different between the groups. Ang2 administration after LNMMA incubation induced slightly larger contractions in residential rats with respect to intruder animals (Figure 4C). LNMMA increased the sensitivity and maximal contraction to Ang2 in this group as well (Figure 4D). Endothelium-

dependent relaxation to ACh did not differ significantly between the group of residential rats and the other groups neither in the absence nor in the presence of LNMMA (Figure 5A-B)

Plasma creatinine and MDA

To gain more insight into potential consequences of stress on renal function and oxidative stress, plasma creatinine and MDA levels were determined in all rats. In Figure 6A the average plasma creatinine concentration (µM) of each group is shown. Two-way ANOVA analysis suggested that plasma creatinine level was influenced by the type of housing, such that socially housed animals had higher creatinine levels in their plasma as compared to singly housed animals. This effect was independent of the exposure of a social defeat interaction (Figure 6A). MDA levels were found the highest in the group of controls, however this did not reach statistical significance. MDA level in plasma was not affected by any form of stress (Figure 6B). The group of residential animals had quite similar creatinine and MDA levels as the group of singly housed animals.

6A



6B

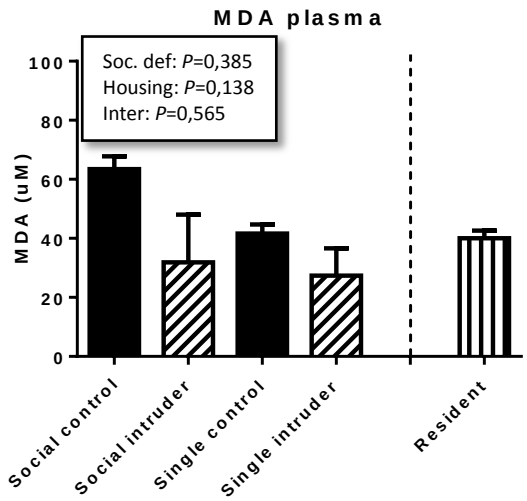


Figure 6: The effect of a social defeat interaction and solitary housing on plasma creatinine (6A) and MDA (6B) levels. Creatinine levels were significantly increased in socially housed animals with respect to singly housed animals, independent of the exposure to a social defeat interaction. No significant differences of MDA levels were found between the different groups.

Discussion

The renin-angiotensin-aldosterone system (RAAS) has emerged as one of the essential links in the pathophysiology of CVD. Here we explored the impact of stress by individual housing and/or social defeat on RAAS activity in the rat resident-intruder model. Long-term solitary-housed animals had increased relative organ weight. Furthermore, intruder rats - after experiencing ~20-times social defeat - showed increased heart tissue ACE activity. In addition, isolated aorta preparations of intruder rats showed a decreased response to Ang2, but not PE, as well as a decreased vasoconstriction response after inhibition of basal NO-release. The data demonstrate that stress induces alterations in cardiovascular RAAS (re-)activity and NO homeostasis which might facilitate CVD development.

Solitary housed animals had larger ventricular and kidney weight. The former might be ventricular hypertrophy which is a common risk factor for CVD (*Gerdts E et al, 2015*). Left ventricular mass can be altered by blood pressure and haemodynamic changes (*Ogah OS et al, 2010*). A previous study on social isolation and autonomic regulation in prairie vole has shown that social isolation increases heart weight (*Grippo AJ et al, 2007*). Whang W and colleagues have reported that depression is associated with left ventricular hypertrophy due to elevated cardiac sympathetic activity (*Whang W et al, 2015*). Even though, blood pressure was not measured in the current study, solitary housing could resemble a form of stress and depression that possibly affects blood pressure, hence future studies with similar experimental settings should include blood pressure. Sympathetic hyperactivity induces elevated RAAS activity leading to kidney injury, probably related to renal hypertrophy (*Blankestijn PJ et al, 2011*). In the present study, only kidney weight was increased and not kidney ACE activity. Possibly, renal hypertrophy is a consequence of increased blood pressure and sympathetic activity. The exact effects of RAAS hyperactivity on the kidney should be investigated in next studies. Chronic stress and depression impairs the HPA stress axis. Adrenal weight was increased in solitary animals and in defeated animals as well. This is in line with findings of a previous study on depressive and CVD comorbidity, where it was reported that social stress involves adrenal hypertrophy (*Wood SK et al, 2012*). According to the existing literature, prolonged social isolation in rats has been associated with greater adrenal weights and adrenocorticotropic hormone-induced corticosteroids production, which is in the same line with our finding (*Hatch AM et al, 1965*).

In accordance with the fact that ACE is produced mainly in the lungs, the highest ACE activity was found in the lung tissue. Both social stress factors (social defeat and solitary housing) did not influence lung ACE activity. Although, the group exposed to the so called worst condition which is solitary housing and experiencing a social defeat interaction did show the highest lung ACE activity among the four groups, this reached no significance. Similarly, ACE activity in the kidney was not changed by both stressors. Since RAAS and one of its component ACE are mainly involved in the regulation of blood pressure which relies in the vascular system, changes in ACE activity might be less obvious in systemic tissues such as lungs and kidneys. In contrast, the heart is crucial in the systemic circulation and also in the vascular system, hence remarkable changes in ACE activity were found in the heart. Heart ACE activity was largely increased in animals exposed to repeated social defeat interaction. A previous study on RAAS and the combination of living alone and depression has reported that renin and aldosterone levels were remarkably increased in solitary living human who had depressive symptomatology (*Häfner S et al, 2012*). However, solitary housing did not affect ACE activity in the heart and also not in the lungs and kidneys. Previously, individually and socially housed mice showed the same plasma renin concentration (*Bing J et al, 1979*). More studies should focus on the effects of housing conditions on the heart.

KCl-mediated absolute contractility was affected by social stress such that defeaters had smaller absolute contractility. This is in line with findings of a previous study in which aortic rings of

chronically stressed rats (exposed to immobilization for 1 hour per day, 5 days a week, lasted for 15 weeks) showed reduced maximal response to KCL stimulation (*Bruder-Nascimento T et al, 2015*). A high concentration of KCL induces membrane depolarization followed by an increase of cytosolic Ca^{2+} from the extracellular space (*Smith L et al, 2003*). The obtained observation that absolute contraction to a high dosage of KCL was small in defeated animals may suggest that the entry of Ca^{2+} via Ca^{2+} channels was decreased in stress conditions. Additionally, EC_{50}-value was higher in control animals compared to defeated rats, suggesting that stress by a social defeat interaction reduces vascular smooth muscle sensitivity to cytosolic free Ca^{2+}. Baseline contractility in the presence of NOS-inhibitor differed extremely between defeated animals and control animals, in the way that control animals had pronounced contractions while defeaters did not. The presence of elevated plasma levels of LNMMA has been found in cardiovascular-related disorders such as stroke and coronary artery disease (*Zwemer CF et al, 2015*). Interestingly, Taddei S and colleagues demonstrated that LNMMA-mediated vasoconstriction was reduced in patients with hypertension, indicating decreased release of basal NO and endothelial dysfunction (*Taddei S et al, 2001*). Stress likely affects basal NO release and thereby it induces endothelial dysfunction, probably leading to hypertension. This could serve as an underlying mechanism for decreased LNMMA-mediated constriction responses found in intruders in the present study. However, endothelium-dependent relaxation in response to ACh was not altered by the repeated exposure of a social defeat interaction and/or solitary housing. Social stress did not affect aortic receptor-dependent contractility to PE. An early study on vascular effects of polyphenols in stressed Wistar-Kyoto rats has reported that stress induced by crowding did not affect PE-induced maximal contractions in femoral artery (*Púzserová A et al, 2006*). Interestingly, Ang2-mediated contraction response was very small in all groups of animals. The addition of LNMMA increased the sensitivity to Ang2 in controls, but not in defeated animals. Since, social defeat stress only had an impact on Ang2-stimulated contractility, it might be that stress induced by social defeat interaction selectively affected vascular reactivity via Ang2-related receptors. Ang2 is known as a strong vasoconstrictor that mediates contraction through angiotensin-2 type-1 receptor (At1R) (*Shinohara K et al, 2015*). Ang2 has been implicated to increase in response to stress (*Armando I et al, 2001*). The increased levels of Ang2 could mediate down regulation of At1R activity, which might account for the smaller vasoconstriction after social defeat in the present study. Future studies need to investigate more about stress and plasma or tissue Ang2 levels.

Since, high creatinine is an indication of decreased renal function, creatinine levels were measured in the plasma of all rats. Although, creatinine assessment is a widely used technique to measure glomerular filtration rate, there are several other factors that can affect the blood levels of creatinine, such as food intake and exercise, irrespective of the glomerular filtration rate (*Scarfe L et al, 2015, Wochyński Z et al, 2015*). In the present study, plasma creatinine levels were not that much different between the groups. This is in line with the findings concerning kidney ACE activity, which showed no differences between the different groups. However, two-way analysis gave very small but significant differences between group-wise housed animals and individually housed animals. Animals from the latter groups had lower plasma creatinine levels. Animals from the same group also showed larger relative organ weights. It could be that solitary housed animals have a different energy or activity pattern, such that those animals are less active. This could lead to decreased muscle growth and less creatinine concentrations, leading to larger relative organ weights. This possible link can be better clarified by getting more insights on the differences of behavioural patterns between socially and solitary housed animals. Ang2, the main effector of RAAS does not only have an important function in inflammatory events, but also involves the induction of oxidative stress (*Qiu Y et al, 2015*). Therefore, MDA was measured in plasma as a marker of oxidative stress. Control animals had slightly more MDA in their plasma in comparison with defeated animals. Besides, group-wise housed controls showed little more MDA than solitary housed control animals. But nevertheless, these differences were not statistically significant. Next to MDA, other inflammatory and endothelial dysfunction biomarkers should be assessed to draw reliable conclusions on it.

Residents were slightly older than intruders and controls and they were housed with a female. Although, residents are not completely comparable with the other groups, they also participated in the social interaction and experienced social stress as well. Taking this into account, the residents were considered as a distinctive group from controls and intruders. The most striking difference found between residential rats and rats from the other groups is the increased ACE activity profiles in tissues. Lung and kidney ACE activity of the group of residents were largely elevated compared to all other groups. The elevated ACE activity patterns found in residential animals might be related to the behavioural characteristics of residential animals. Residential rats used in the resident-intruder paradigm are very aggressive. Interestingly, Poulsen and colleagues have reported the link between aggressive behaviour and RAAS through renin. Aggressive behaviour increased plasma renin levels in mice. This renin was secreted in particular by the kidneys and the submaxillary gland (*Poulsen K et al, 1986*). This might be a possible explanation why the aggressive residents have such enhanced ACE activity in the kidney and lungs. However heart ACE activity was not extremely increased in this group of rats. Previously, Bing and Poulsen showed that the increase in renin was not accompanied by an increase in blood pressure. Possibly, the cardiovascular system has a compensatory feedback mechanism that has the capacity to maintain blood pressure (*Bing J et al, 1979*). Presumably, the latter could be associated with the fact that heart ACE activity did not increase that much in the group of residents. All resident animals were housed with a female rat, which is form of social environment. Interestingly, concerning the relative organ weights, characteristics of residents look most similar to the group of socially housed animals. Vascular reactivity curves of residents resemble most with the group of control animals. This might suggest that although the repeated exposure to stress through a social interaction, in this case a winning, the end result and the social environment of the housing could buffer the prolonged effects of stress, such as increased organ weights, altered vascular tone and NO homeostasis.

Due to the limited time period, only a small part of the RAAS has been investigated. To get more insights other involved biomarkers should be studied as well, such as renin, aldosterone, Ang2, At1R, At2R, NO, and eNOS. Another essential factor that needs to be included as well is blood pressure. Blood pressure is closely regulated by the sympathetic activity and RAAS and therefore it might give more answers in the cause of ventricular, renal and adrenal hypertrophy found in the current study.
The animals used in the resident-intruder paradigm are rats from Groninger wild-type strain. Rats from this strain differ largely in behavioural traits in response to stressors when comparing with Wistar rats. Also, cardiovascular response to social defeat is different among these strains. Wistar rats are much less resilient to a social defeat compared to Wild-type Groningen rats (*Vidal J et al, 2011*). Before translating knowledge from findings in these animals to human, more research is required to investigate the most suitable animal model representing human model. Although, the contractions responses from cumulative doses of Ang2 were small, the sample size was quite large (not smaller than five animals). All experiments were carried out in duplicates and the obtained significance values were all highly significant, indicating consistent findings. However, the experiments should be repeated to confirm current finding's reproducibility.

A human study has reported that depression under chronic stress condition involves the activation of RAAS, by means of increased plasma levels of renin and aldosterone. This implicates a possible underlying mechanism of stress and depression in CVD (*Häfner S et al, 2012*). In same line with that and in accordance with our hypothesis which stated that RAAS activity will be increased in socially stressed animals, we demonstrated locally enhanced ACE activity in the heart tissue. In addition, we showed that Ang2-mediated contractility is impaired in stressed animals. Ang2 is one of the major effector of RAAS. To date, this is one of the few studies that has evaluated the role of RAAS in social stress. As far as we know, findings of the current study has demonstrated for the first time the effect of stress in rats by employing the resident-intruder paradigm on ACE activity, vascular reactivity and endothelial function. The present findings revealed that stress after repeated exposures to a social defeat interaction increases ACE activity locally in the heart, accompanied by decreased baseline

contractility and impaired Ang2-stimulated contractility in the absence of NOS. Stress induced by individually housing increased ventricular heart, left kidney and adrenal weight.

Widely used medicines against CVD target Ang2 receptors and ACE which are components of the RAAS. According to this study's findings, stress induced by a defeat changes ACE activity and Ang2-induced contraction, the latter possibly through Ang2-receptor regulation or sensitivity. Since stress is an important risk factor for CVD and every individual has different level of stress, it might be that the common medicines against CVD would react differently per individuals. This is related to the term of 'personalized medicine' which strives for accurate and specific treatment for the individual. More insight should be gained on this. To conclude, social stress by means of social defeat involves the activation of RAAS particularly in cardiovascular tissues to initiate alterations in vascular function, including a loss in basal NO-release and impaired responsiveness to Ang2. These changes in cardiovascular function in turn could facilitate the development of CVD.

References

Aleman BM, Moser EC, Nuver J, Suter TM, Maraldo MV, Specht L, Vrieling C, Darby SC. Cardiovascular disease after cancer therapy. EJC Suppl. 2014 Jun;12(1):18-28. doi: 10.1016/j.ejcsup.2014.03.002. Epub 2014 May 29. PubMed PMID: 26217163; PubMed Central PMCID: PMC4250533.

Alfaidi M, Wilson H, Daigneault M, Burnett A, Ridger V, Chamberlain J, Francis S. Neutrophil Elastase promotes Interleukin-1β secretion from Human Coronary Endothelium. J Biol Chem. 2015 Aug 12. pii: jbc.M115.659029. [Epub ahead of print] PubMed PMID: 26269588.

Alhurani AS, Dekker R, Tovar E, Bailey A, Lennie TA, Randall DC, Moser DK. Examination of the potential association of stress with morbidity and mortality outcomes in patient with heart failure. SAGE Open Med. 2014 Dec;2. doi: 10.1177/2050312114552093. PubMed PMID: 26246898; PubMed Central PMCID: PMC4523379.

Armando I, Carranza A, Nishimura Y, Hoe KL, Barontini M, Terrón JA, Falcón-Neri A, Ito T, Juorio AV, Saavedra JM. Peripheral administration of an angiotensin II AT(1) receptor antagonist decreases the hypothalamic-pituitary-adrenal response to isolation Stress. Endocrinology. 2001 Sep;142(9):3880-9. PubMed PMID: 11517166.

Badran M, Golbidi S, Ayas N, Laher I. Nitric Oxide Bioavailability in Obstructive Sleep Apnea: Interplay of Asymmetric Dimethylarginine and Free Radicals. Sleep Disord. 2015;2015:387801. doi: 10.1155/2015/387801. Epub 2015 May 6. Review. PubMed PMID: 26064689; PubMed Central PMCID: PMC4438195.

Bing J, Poulsen K. In mice aggressive behavior provokes vast increase in plasma renin concentration, causing only slight, if any, increase in blood pressure. Acta Physiol Scand. 1979 Jan;105(1):64-72. PubMed PMID: 570342.

Blankestijn PJ, Eberhard Ritz. Potential impact on hypertension in kidney disease? Nephrology Dialysis Transplantation. 2011;26(9):2732-2734.

Boa BC, Barros CM, Souza Md, Castiglione RC, Cyrino FZ, Bouskela E. α-Tocopherol Improves Microcirculatory Dysfunction on Fructose Fed Hamsters. PLoS One. 2015 Aug 5;10(8):e0134740. doi: 10.1371/journal.pone.0134740. eCollection 2015. PubMed PMID: 26244369; PubMed Central PMCID: PMC4526657.

Bruder-Nascimento T, Campos DH, Cicogna AC, Cordellini S. Chronic stress improves NO- and Ca2+ flux-dependent vascular function: a pharmacological study. Arq Bras Cardiol. 2015 Mar;104(3):226-33. doi: 10.5935/abc.20140207. Epub 2015 Jan 23. English, Portuguese. PubMed PMID: 25884770; PubMed Central PMCID: PMC4386851.

Coppens CM, de Boer SF, Buwalda B, Koolhaas JM. Aggression and aspects of impulsivity in wild-type rats. Aggress Behav. 2014 Jul-Aug;40(4):300-8. doi: 10.1002/ab.21527. Epub 2014 Jan 24. PubMed PMID: 24464354.

Frostegard J. Immunity, atherosclerosis and cardiovascular diseases. Biomedicine. 2013 May;1. doi:10.1186/1741-7015-11-117. Review.

Gerdts E, Rossebø AB, Pedersen TR, Cioffi G, Lønnebakken MT, Cramariuc D, Rogge BP, Devereux RB. Relation of Left Ventricular Mass to Prognosis in Initially Asymptomatic Mild to Moderate Aortic Valve Stenosis. Circ Cardiovasc Imaging. 2015 Nov;8(11):e003644. doi: 10.1161/CIRCIMAGING.115.003644. PubMed PMID: 26489804.

Grippo AJ, Lamb DG, Carter CS, Porges SW. Social isolation disrupts autonomic regulation of the heart and influences negative affective behaviors. Biol Psychiatry. 2007 Nov 15;62(10):1162-70. Epub 2007 Jul 20. PubMed PMID: 17658486; PubMed Central PMCID: PMC2144909.

Häfner S, Baumert J, Emeny RT, Lacruz ME, Bidlingmaier M, Reincke M, Kuenzel H, Holle R, Rupprecht R, Ladwig KH; MONICA/KORA Study Investigators. To live alone and to be depressed, an alarming combination for the renin-angiotensin-aldosterone-system (RAAS). Psychoneuroendocrinology. 2012 Feb;37(2):230-7. doi: 10.1016/j.psyneuen.2011.06.007. Epub 2011 Jul 13. PubMed PMID: 21742440.

Hahn CS, Scott DW, Xu X, Roda MA, Payne GA, Wells JM, Viera L, Winstead CJ, Bratcher P, Sparidans RW, Redegeld FA, Jackson PL, Folkerts G, Blalock JE, Patel RP, Gaggar A. The matrikine N-α-PGP couples extracellular matrix fragmentation to endothelial permeability. Sci Adv. 2015;1(3). pii: e1500175. PubMed PMID: 26229981; PubMed Central PMCID: PMC4517288.

Hatch AM, Wiberg GS, Zawidzka Z, Cann M, Airth JM, Grice HC. Isolation syndrome in the rat. Toxicol Appl Pharmacol. 1965 Sep;7(5):737-45. PubMed PMID: 4286670.

Hwang HM, Lee JH, Min BS, Jeon BH, Hoe KL, Kim YM, Ryoo S. A Novel arginase inhibitor derived from Scutellavia indica restored endothelial function in ApoE-null mice fed a high cholesterol diet. J Pharmacol Exp Ther. 2015 Aug 11. pii: jpet.115.224592. [Epub ahead of print] PubMed PMID: 26265320.

Kivimäki M, Kawachi I. Work Stress as a Risk Factor for Cardiovascular Disease. Curr Cardiol Rep. 2015 Sep;17(9):630. doi: 10.1007/s11886-015-0630-8. PubMed PMID: 26238744; PubMed Central PMCID: PMC4523692.

Kong BW, Man RY, Gao Y, Vanhoutte PM, Leung SW. Reduced activity of SKC a and Na-K ATPase underlies the accelerated impairment of EDH-type relaxations in mesenteric arteries of aging spontaneously hypertensive rats. Pharmacol Res Perspect. 2015 Jun;3(3):e00150. doi: 10.1002/prp2.150. Epub 2015 May 20. PubMed PMID: 26171229; PubMed Central PMCID: PMC4492766.

Koolhaas JM, Coppens CM, de Boer SF, Buwalda B, Meerlo P, Timmermans PJ. The resident-intruder paradigm: a standardized test for aggression, violence and social stress. J Vis Exp. 2013 Jul 4;(77):e4367. doi: 10.3791/4367. PubMed PMID: 23852258; PubMed Central PMCID: PMC3731199.

Mahmood D, Jahan K, Habibullah K. Primary prevention with statins in cardiovascular diseases: A Saudi Arabian perspective. J Saudi Heart Assoc. 2015 Jul;27(3):179-91. doi: 10.1016/j.jsha.2014.09.004. Epub 2014 Sep 28. Review. PubMed PMID: 26136632; PubMed Central PMCID: PMC4481463.

Nasser Z, Salameh P, Nasser W, Abou Abbas L, Elias E, Leveque A. Outdoor particulate matter (PM) and associated cardiovascular diseases in the Middle East. Int J Occup Med Environ Health. 2015;28(4):641-61. doi: 10.13075/ijomeh.1896.00186. Review. PubMed PMID: 26216305.

Ogah OS, Bamgboye AE. Correlates of left ventricular mass in hypertensive Nigerians: an echocardiographic study. Cardiovasc J Afr. 2010 Mar-Apr;21(2):79-85. PubMed PMID: 20532431; PubMed Central PMCID: PMC3721252.

Paul M, Poyan Mehr A, Kreutz R. Physiology of local renin-angiotensin systems. Physiol Rev. 2006 Jul;86(3):747-803. Review. PubMed PMID: 16816138.

Poulsen K, Jacobsen J. Inhibition of the enzymatic reaction of renin in aggressive mice. J Hypertens. 1986 Apr;4(2):175-80. PubMed PMID: 3519761.

Púzserová A, Csizmadiová Z, Andriantsitohaina R, Bernátová I. Vascular effects of red wine polyphenols in chronic stress-exposed Wistar-Kyoto rats. Physiol Res. 2006;55 Suppl 1:S39-47. PubMed PMID: 17177625.

Qiu Y, Tao L, Lei C, Wang J, Yang P, Li Q, Lei B. Downregulating p22phox ameliorates inflammatory response in Angiotensin II-induced oxidative stress by regulating MAPK and NF-κB pathways in ARPE-19 cells. Sci Rep. 2015 Sep 29;5:14362. doi: 10.1038/srep14362. PubMed PMID: 26415877; PubMed Central PMCID: PMC4586461.

Radenković M. Pioglitazone and Endothelial Dysfunction: Pleiotropic Effects and Possible Therapeutic Implications. Sci Pharm. 2014 Aug 18;82(4):709-21. doi: 10.3797/scipharm.1407-16. eCollection 2014 Dec. Review. PubMed PMID: 26171320; PubMed Central PMCID: PMC4500538.

Raven PB, Chapleau MW. Blood pressure regulation XI: overview and future research directions. Eur J Appl Physiol. 2014 Mar;114(3):579-86. doi: 10.1007/s00421-014-2823-z. Epub 2014 Jan 28. Review. PubMed PMID: 24463603; PubMed Central PMCID: PMC3955090.

Roemmich JN, Lambiase MJ, Balantekin KN, Feda DM, Dorn J. Stress, behavior, and biology: risk factors for cardiovascular diseases in youth. Exerc Sport Sci Rev. 2014 Oct;42(4):145-52. doi: 10.1249/JES.0000000000000027. Review. PubMed PMID: 25061998.

Sampson UK, Engelgau MM, Peprah EK, Mensah GA. Endothelial dysfunction: a unifying hypothesis for the burden of cardiovascular diseases in sub-Saharan Africa. Cardiovasc J Afr. 2015 Mar-Apr;26(2 Suppl 1):S56-60. doi: 10.5830/CVJA-2015-043. PubMed PMID: 25962949.

Santana AB, de Souza Oliveira TC, Bianconi BL, Barauna VG, Santos EW, Alves TP, Silva JC, Fiorino P, Borelli P, Irigoyen MC, Krieger JE, Lacchini S. Effect of high-fat diet upon inflammatory markers and aortic stiffening in mice. Biomed Res Int. 2014;2014:914102. doi: 10.1155/2014/914102. Epub 2014 Jun 11. PubMed PMID: 25013811; PubMed Central PMCID: PMC4071860.

Santillo M, Colantuoni A, Mondola P, Guida B, Damiano S. NOX signaling in molecular cardiovascular mechanisms involved in the blood pressure homeostasis. Front Physiol. 2015 Jul 7;6:194. doi: 10.3389/fphys.2015.00194. eCollection 2015. Review. PubMed PMID: 26217233; PubMed Central PMCID: PMC4493385.

Scarfe L, Rak-Raszewska A, Geraci S, Darssan D, Sharkey J, Huang J, Burton NC, Mason D, Ranjzad P, Kenny S, Gretz N, Lévy R, Kevin Park B, García-Fiñana M, Woolf AS, Murray P, Wilm B. Measures of kidney function by minimally invasive techniques correlate with histological glomerular damage in SCID mice with adriamycin-induced nephropathy. Sci Rep. 2015 Sep 2;5:13601. doi: 10.1038/srep13601. PubMed PMID: 26329825; PubMed Central PMCID: PMC4556979.

Shen Z, Ye C, McCain K, Greenberg ML. The Role of Cardiolipin in Cardiovascular Health. Biomed Res Int. 2015;2015:891707. doi: 10.1155/2015/891707. Epub 2015 Aug 2. Review. PubMed PMID: 26301254; PubMed Central PMCID: PMC4537736.

Shinohara K, Kishi T, Hirooka Y, Sunagawa K. Circulating angiotensin II deteriorates left ventricular function with sympathoexcitation via brain angiotensin II receptor. Physiol Rep. 2015 Aug;3(8). pii: e12514. doi: 10.14814/phy2.12514. PubMed PMID: 26290529.

Smith L, Payne JA, Sedeek MH, Granger JP, Khalil RA. Endothelin-induced increases in Ca2+ entry mechanisms of vascular contraction are enhanced during high-salt diet. Hypertension. 2003 Mar;41(3 Pt 2):787-93. Epub 2002 Dec 30. PubMed PMID: 12623997.

Taddei S, Virdis A, Ghiadoni L, Salvetti G, Bernini G, Magagna A, Salvetti A. Age-related reduction of NO availability and oxidative stress in humans. Hypertension. 2001 Aug;38(2):274-9. PubMed PMID: 11509489.

Vidal J, Buwalda B, Koolhaas JM. Male Wistar rats are more susceptible to lasting social anxiety than Wild-type Groningen rats following social defeat stress during adolescence. Behav Processes. 2011 Oct;88(2):76-80. doi: 10.1016/j.beproc.2011.08.005. Epub 2011 Aug 18. PubMed PMID: 21854839.

Whang W, Davidson KW, Palmeri NO, Bhatt AB, Peacock J, Chaplin WF, Shimbo D, Edmondson DE. Relations among depressive symptoms, electrocardiographic hypertrophy, and cardiac events in non-ST elevation acute coronary syndrome patients. Eur Heart J Acute Cardiovasc Care. 2015 Oct 8. pii: 2048872615610736. [Epub ahead of print] PubMed PMID: 26450780.

Wochyński Z, Sobiech K. Post-exercise proteinuria in the cadets trained on Special Aerial Gymnastics Instruments. Int J Occup Med Environ Health. 2015;28(5):863-73. doi: 10.13075/ijomeh.1896.00454. PubMed PMID: 26224498.

Wood SK, McFadden KV, Grigoriadis D, Bhatnagar S, Valentino RJ. Depressive and cardiovascular disease comorbidity in a rat model of social stress: a putative role for corticotropin-releasing factor. Psychopharmacology (Berl). 2012 Jul;222(2):325-36. doi: 10.1007/s00213-012-2648-6. Epub 2012 Feb 10. PubMed PMID: 22322324; PubMed Central PMCID: PMC3613282.

Zwemer CF, Davenport RD, Gomez-Espina J, Blanco-Gonzalez E, Whitesall SE, D'Alecy LG. Packed red blood cells are an abundant and proximate potential source of nitric oxide synthase inhibition. PLoS One. 2015 Mar 20;10(3):e0119991. doi: 10.1371/journal.pone.0119991. eCollection 2015. PubMed PMID: 25793525; PubMed Central PMCID: PMC4368738.