Sex Differences in Blood Pressure, Free Radicals and Plasma Cholesterol Fractions in Ren-2 Transgenic Rats of Various Ages

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Key words

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Summary

Sex-related cardiovascular differences were observed in humans as well as in experimental animals. Our previous study demonstrated a marked sexual dimorphism in blood pressure (BP) of 9-month-old heterozygous transgenic Ren 2 rats (TGR), in which mouse Ren-2 renin gene was inserted into the genome of normotensive Hannover Sprague-Dawley rats (HanSD). We found significantly elevated BP only in male TGR, whereas BP of TGR females was similar to that of HanSD females. The aim of our present study was to compare BP of 3- and 6-month-old heterozygous TGR with age- and sex-matched HanSD under the same conditions as we measured in 9-month-old rats. We also monitored the amount of oxidative stress marker, thiobarbituric acid-reactive substances (TBARS), and a main intracellular antioxidant, reduced glutathione in the heart, kidneys and liver. We also measured plasma triglycerides and cholesterol levels. We found an increased mean arterial pressure in both female and male 3-month-old TGR (172 ± 17 vs. 187 ± 4 mm Hg, respectively) compared to HanSD (115 ± 5 vs. 133 ± 3 mm Hg, respectively) but there was a marked sexual dimorphism of 6-month-old TGR where only males were hypertensive (145 ± 5 mm Hg) while females became normotensive (123 ± 7 mm Hg). We did not find any correlation/relationship between BP values and concentrations of TBARS or glutathione or plasma lipid levels. Our results demonstrated that 6-month-old TGR exhibited a marked sexual BP dimorphism, which was not dependent on the abnormalities in oxidative stress or cholesterol metabolism.

Introduction

Sexual dimorphism in blood pressure (BP) is well-known not only in humans but also in different experimental animals such as birds, dogs, rabbits, or mouse [1,2]. Several rat models also show such remarkable sex-related differences [3]. Recent review summarizes previous findings regarding the different mechanisms of BP control in female and male genetic spontaneously hypertensive rats (SHR), which is the most commonly used model of primary hypertension [4]. Nevertheless, sex differences were also reported in other models of hypertension – such as deoxycorticosterone acetate (DOCA)-salt treated rats [5], salt-sensitive Dahl rats [6] or rats with NO-deficient hypertension elicited by chronic treatment with N^{ω}-nitro-L-arginine methyl ester (L-NAME) [7].

The renin-angiotensin system (RAS) plays an important role in regulation of body fluid volume, electrolyte balance, systemic vascular resistance and BP control. Ren-2 transgenic rat (TGR) represents a unique model, in which the insertion of the mouse Ren-2 renin gene into the genome of Hannover Sprague Dawley rat (HanSD) is associated with RAS hyperactivity and severe hypertension [8,9]. Since homozygous TGR manifest severe malignant hypertension with the organ damage and death at 7-13 weeks of age, heterozygous TGR, which manifest a less severe hypertension and live markedly longer, are used instead for studies [10]. Our previous study [11] demonstrated a substantial sexual dimorphism in BP of 9-month-old heterozygous TGR. There was a significantly elevated BP only in male but not in female TGR compared to control normotensive HanSD females. Surprisingly, we did not find any corresponding difference in heart or kidney thiobarbituric acid-reactive species (TBARS) concentrations or reduced glutathione levels between the transgene-negative normotensive HanSD and the hypertensive TGR.

The aim of our present study was to search for sex differences in 3- and 6-month-old female and male HanSD and heterozygous TGR concerning their blood pressure, plasma

lipids (triglycerides and cholesterol) and oxidative stress marker (TBARS) and reduced glutathione (as a main intracellular antioxidant) in the heart, kidneys and liver.

Material and Methods

Animals

Adult male and female heterozygous (mRen-2)27 transgenic rats (TGR) aged 3 and 6 months were housed at 23 °C under a 12 h light/dark cycle period, fed a standard rat chow Altromin-1320 (Lage, Germany) containing 0.45 % NaCl and given tap water *ad libitum*. Transgene-negative HanSD served as controls. At the end of the experiment blood pressure was measured. After blood collection, kidneys, heart and liver were excised and used for tissue analysis. Plasma samples (in the EDTA presence) were prepared and stored at -80 °C until further analysis.

All procedures and experimental protocols were performed in accordance with guidelines and practice established by the *Ethical Committee of the Institute of Physiology CAS*, conformed to the *European Convention on Animal Protection and Guidelines on Research Animal Use* (Protocol Nr. 90/2019).

Measurement of blood pressure

Mean arterial (MAP), systolic (SBP) and diastolic blood pressure (DBP) and heart rate (HR) were measured by a direct puncture of carotid artery under the isoflurane anesthesia (2.5 % isoflurane) as well as in awaking animals (switched to 0.5 % isoflurane). BP and HR were recorded using a pressure transducer and a multichannel recorder (ADInstruments, Bella Vista,

Australia). To eliminate the influence of circadian BP variation, the measurements were always performed approximately at the same time of day (between 8:00 and 10:00 a.m.).

Determination of thiol concentration

The intracellular content of reduced glutathione in heart, kidney and liver was determined immediately in fresh tissues according to the methods described earlier [12]. Briefly, the tissue samples were homogenized in 3 % sulfosalicylic acid and 10 % homogenates were centrifuged for 10 min at 3000 g. A portion of the supernatant was mixed with 0.02 M 5, 5'-dithiobis-(2-nitrobenzoic acid) in 0.1 M phosphate buffer (pH 8). The absorbance of a colored product was read at 412 nm, the concentration of glutathione was calculated from the standard curve prepared by a serial dilution of 1 mM stock solution. The results were expressed as μmol glutathione/g tissue.

Measurement of lipid peroxidation

Lipid peroxidation in the samples was monitored by measuring TBARS formation [13]. The frozen-thawed 10 % homogenates were incubated with thiobarbituric and acetic acid at 95 °C for 45 min. The absorbance was measured at 535 nm using Tecan Infinite M200 multimode microplate spectrofluorometer. The results were expressed as nmol of TBARS/mg of protein.

Biochemical parameters

Folin method was used for the determination of protein concentration using bovine serum albumin as standard [14].

The concentrations of plasma triglycerides (TG), total cholesterol (TC), and high-density lipoprotein-cholesterol (HDL) were measured using appropriate commercial kits

(Pliva-Lachema Diagnostika, Brno, Czech Republic) following the protocol provided by the manufacturer. Low-density lipoprotein-cholesterol (LDL) was estimated indirectly using the formula: LDL = TC - (TG/5) - HDL.

Statistical analysis

The results are expressed as the means ± SEM. The statistical differences were evaluated by a paired Student's t-test. Values of $P \leq 0.05$ were considered to be statistically significant.

Results

Figure 1 shows MAP and HR in awaking rats. 3-month-old TGR females and males had significantly higher MAP as compared to sex-matched HanSD rats. MAP values of HanSD females were significantly lower in comparison to males but MAP values of TGR females were not significantly different from males (Fig. 1). On the other hand, 6-month-old females did not show significantly higher MAP values in TGR, while 6-month-old males still had significantly higher values of MAP compared to age-matched HanSD rats. Similar results on SBP and DBP were observed in both 3- and 6-month-old TGR (Fig. 1). Resting values of HR in awaking male or female rats did not differ between HanSD and TGR rats compared to age-matched rats (data not shown).

Body weights were similar in 3- and 6-month-old HanSD and TGR females as compared to age-matched females. However, males were significantly heavier than females in all studied groups. Moreover, 6-month-old TGR males were significantly heavier than 6-month-old HanSD (Table 1). The absolute weights of heart and left ventricle were significantly greater in TGR than in HanSD, and in males than in females of both genotypes.

The relative weights of heart and left ventricle were significantly higher in TGR females of both ages. Relative weights of heart and left ventricle were significantly higher only in 3-month-old males TGR but not significant in 6-month-old males. The absolute and relative kidney weights were similar in females of both ages and genotypes, whereas they were higher in males in comparison to females. The absolute and relative kidney weights of 6-month-old males were significantly greater in TGR than HanSD (Table 1).

We evaluated the degree of organ damage by lipid peroxidation via measuring the TBARS concentrations as an indirect marker of lipid peroxidation in the heart, kidneys and liver (Table 2). We found no significant differences between HanSD and TGR animals except for the TBARS concentrations in the liver of 6-month-old TGR males which were higher in comparison with age-matched females and sex-matched HanSD males.

The lowest concentrations of reduced glutathione were observed in the heart and the highest in the liver (Table 3). Significantly higher concentrations of thiol groups were seen in the liver of TGR as compared to the age-matched HanSD. Moreover, we disclosed significantly higher values of the reduced glutathione in the 3-month-old female liver compared to those of the age-matched males. Concerning the kidney glutathione content, 3-month-old HanSD females as well as 6-month-old HanSD and TGR females had significantly higher values compared to the age-matched males (Table 3).

Table 4 shows lipid profile. Females of both ages did not show significant differences between genotypes with the exception of lower total cholesterol/high-density lipoprotein-cholesterol (TC/HDL) ratio in the 3-month-old female TGR. Male TGR of both ages had significantly higher values of serum triglycerides as compared to the age-matched HanSD and these values were also significantly higher than in age-matched female TGR. Three-month-old TGR and 6-month-old males of both genotypes had significantly higher TC/HDL ratio as compared to the age-matched females.

Discussion

Our present study shows increased BP of both female and male 3-month-old TGR, a well-defined genetic model of hypertension with the overexpression of a mouse renin gene, as compared to normotensive HanSD rats but later a marked sexual dimorphism appears since only male but not female 6-month-old TGR stay hypertensive.

A substantial sexual dimorphism in BP was also seen in 9-month-old TGR in which BP was significantly elevated in males but not females, which were normotensive [11]. Our results correspond well with the earlier studies in heterozygous TGR. Springate *et al.* [15] described a significantly higher SBP of conscious male TGR than that of HanSD rats at the age of 2, 4 and 8 months. Nevertheless, the severity of their hypertension diminished over time: 8-month-old TGR (169 ± 5 mm Hg) had significantly lower systolic BP than the 2- and 4-month-old counterparts (225 ± 8 and 195 ± 6 mm Hg, respectively). These findings indicated a gradual age-related decline in BP [15]. Cargnelli *et al.* [16] followed this study measuring the systolic BP in 5-, 11- and 35-week-old female TGR and sex- and age-matched normotensive HanSD counterparts. Significantly higher values of Sc BP were observed in 11-week-old TGR as compared to the 5-week and 35-week-old animals, while the SBP in HanSD was unaffected by ageing [16]. In the study of Lee *et al.* [17], hypertension was evident already at 4-5-week-old TGR with maximum values of up to 240 mm Hg in males and up to 200 mm Hg in females at 8-9-week-old while BP values in HanSD ranged between 115 - 140 mm Hg. The phase of established hypertension in TGR was followed by a decrease in BP by 20-30 mm Hg in male and by 40-60 mm Hg in female TGR aged 20-24 weeks [17]. Vaněčková *et al.* [18] demonstrated that female and male TGR developed fulminant hypertension within 2–3 weeks after weaning. At the age of 5 months, a stepwise decline of

the BP to control levels was observed in female TGR, whereas the BP of male TGR remained elevated until the end of the study which lasted one year [18]. These results demonstrated an age-dependent decline of BP in TGR, although the values remained higher in male TGR.

The imbalance between the production and elimination of oxidative and nitrosative species can cause oxidative or nitrosative stress, which is often consider as an important factor contributing to the pathogenesis of different forms of hypertension [19,20]. However, we did not find significant links between BP values and concentrations of TBARS in three different organs: the heart, kidneys and liver. Our previous study in 9-month-old female and male TGR and HanSD did not also reveal significant differences [11]. On the other hand, Kopkan *et al.* [21] found increased levels of tissue malondialdehyde (the end product of lipid peroxidation) in the kidney and left heart ventricle of 3-month-old male TGR compared with HanSD. They also found that chronic administration of tempol (a superoxide scavenger) or apocynin (an inhibitor of NADPH oxidase activity preventing the superoxide production) significantly reduced levels of malondialdehyde in TGR. However, the reduced production of the superoxide had no effect on the systolic BP. Therefore, the authors concluded that the contribution of oxidative stress to the development of hypertension in TGR appeared to be negligible [21]. Similarly, Liu *et al.* [22] reported no effect of apocynin on the elevated systolic BP in the transgenic mice overexpressing rat angiotensinogen in their renal proximal tubular cells. This study demonstrated that systemic hypertension is independent of reactive species generation [22]. On the other hand, blockade of NADPH oxidase-mediated superoxide production with apocynin resulted in a decrease of BP in different mouse or rat models, such as angiotensin II-infused mice [23], Sprague-Dawley rats with dexamethasone-induced hypertension [24], fructose-fed Sprague-Dawley rats [25] or 3-week-old spontaneously hypertensive rats [26].

Glutathione is a small molecular weight thiol-containing tripeptide produced in the cell, which is found in all animal tissues. The main function of glutathione is linked to its antioxidant and redox buffering properties [27,28]. For example, it plays important roles in peroxide detoxification, recycling of vitamins C and E or cysteine storage. We found different glutathione concentrations in particular examined tissues (heart, kidney, liver) in 3-month-old [29] or 9-month-old HanSD and TGR [11]. Nine-month-old HanSD and TGR showed sex-dependent glutathione concentrations in all three examined tissues: males had higher glutathione concentrations in the heart and liver but lower in the kidneys. However, glutathione concentrations were not influenced by rat genotype [11]. In present study, we confirmed significantly lower glutathione concentration in male kidney in 3-month-old HanSD and 6-month-old HanSD and TGR. In contrast, liver showed lower glutathione concentrations in 3-month-old males and genotype differences with significantly higher glutathione concentrations in male and female TGR of both ages.

Our previous study with 9-month-old HanSD and TGR revealed lipid parameters that were significantly higher in males than in females (with the exception of HDL cholesterol) and no differences between the genotypes [11]. Our present study with 3- and 6-month-old rats showed significantly higher plasma triglycerides (TG) in males (with the exception of 3-month-old HanSD) and higher TG levels in TGR than HanSD in both ages. Vettor *et al.* [30] reported similar fasting levels of plasma TG in 5-month-old HanSD and TGR.

Plasma total cholesterol (TC) did not differ significantly depending on sex, genotype and age in our 3- and 6-month-old rats. Lee *et al.* [31] reported that plasma cholesterol level in females was twice higher than in 2-month-old male Sprague-Dawley rats. Similarly, Borbélyová *et al.* [32] demonstrated that 18-month-old female Lewis rats had cholesterol in plasma by 32 % higher than males. He *et al.* [33] also reported higher total cholesterol in female rats. Their sex-specific reference intervals of total cholesterol were indicated being

0.81-2.03 mmol/l for females and 0.68-1.77 mmol/l for males. Thus, our TC values of males (1.67-2.05 mmol/l) are in the upper limit of the reference interval. Interestingly, our earlier data for 9-month-old males also showed higher TC values than in females [11].

There are several limitations in the current study. First, we did not measure circulating angiotensin levels in male or female TGR at different ages. Consequently, it remains to be determined whether the dimorphism of BP and/or the differences in biochemical markers in TGR are the outcomes of different plasma levels of angiotensin. It is also unclear if the changes in plasma levels of TBARS, glutathione, and lipid profile are the cause or consequence of the elevated BP in TGR. Similarly, we do not know their roles in gender dimorphism of the hypertensive phenotype in TGR.

In conclusion, the present study confirmed a sexual dimorphism in blood pressure of 6-month-old heterozygous TGR where only males stay hypertensive but not females. Additionally, we did not find any corresponding link between blood pressure values and differences in TBARS or glutathione concentrations or plasma lipid levels. Thus, it seems that a sexual blood pressure dimorphism did not rely on oxidative stress or abnormal cholesterol metabolism. It would be desirable to examine which vasoactive systems contribute to the age-dependent decrease of blood pressure in female TGR.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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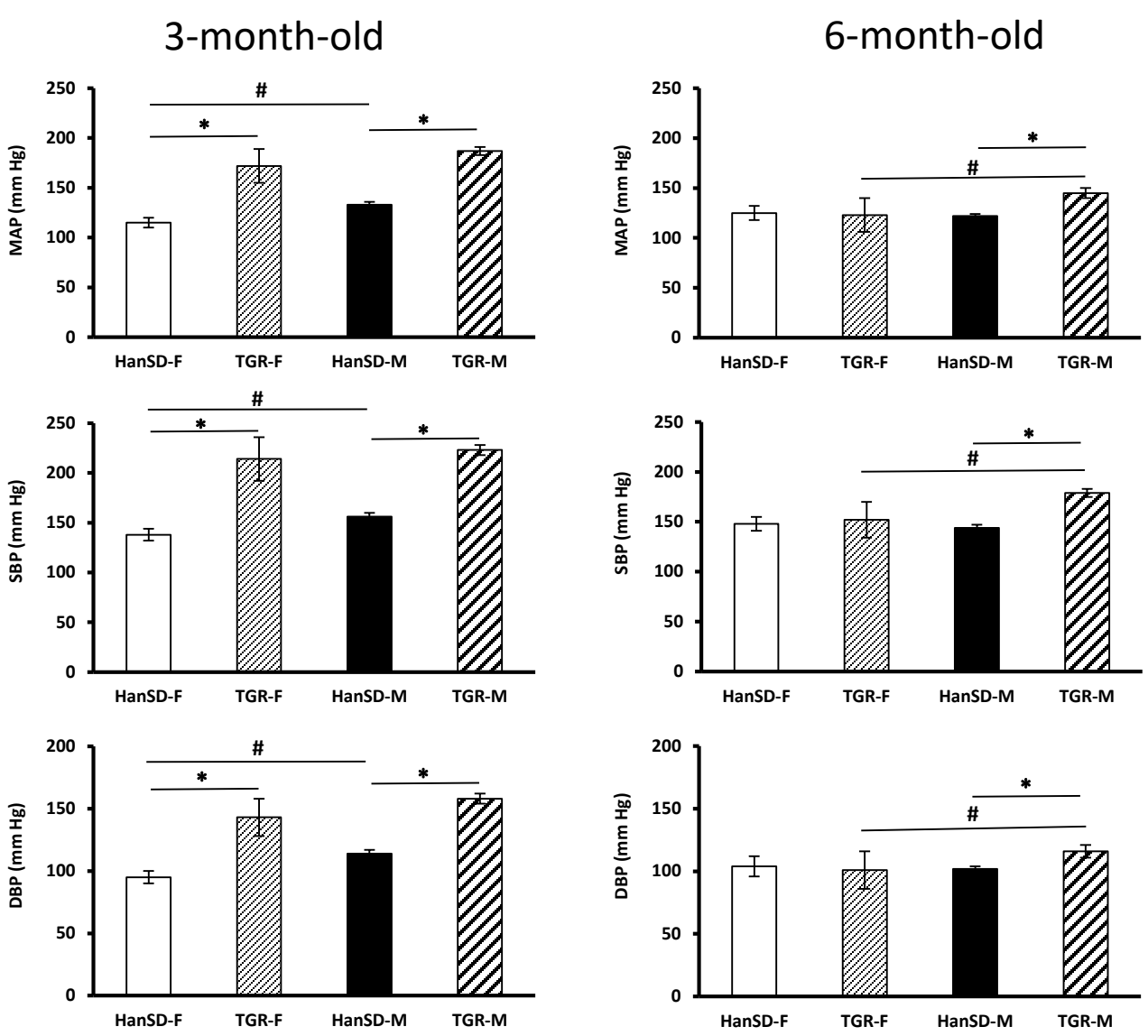
Legends to Figures

Fig. 1. Mean (MAP), systolic (SBP) and diastolic (DBP) blood pressure in awaking 3- and 6-month-old females (F) and males (M) Hannover Sprague Dawley (HanSD) and Ren-2 transgenic (TGR) rats. All values are mean ± SEM. Significantly different: $^{*}P \leq 0.5$ *vs.* HanSD, $^{\#}$ $P \leq 0.5$ *vs.* female


3-month-old
6-month-old
MAP (mm Hg)
SBP (mm Hg)
DBP (mm Hg)
HanSD-F
TGR-F
HanSD-M
TGR-M
#
*

T**able 1.** Body and organ weights in experimental 3- and 6-month-old female and male Hannover Sprague-Dawley (HanSD) and Ren-2 transgenic (TGR) rats.

Females	**3-month-old**		**6-month-old**	
	HanSD (n=5)	**TGR** (n=5)	**HanSD** (n=5)	**TGR** (n=5)
Body weight (BW) (g)	294 ± 7	280 ± 7	320 ± 10	328 ± 10
Heart weight (HW) (mg)	919 ± 35	1049 ± 33*	964 ± 17	1192 ± 49*
Relative heart weight (HW/BW) (g/mg)	3.12 ± 0.06	3.74 ± 0.06*	3.02 ± 0.09	3.64 ± 0.18*
Left ventricular weight (LVW) (mg)	730 ± 22	864 ± 25*	790 ± 18	1007 ± 46*
Relative left ventricular weight (LVW/BW) (mg/g)	2.48 ± 0.03	3.08 ± 0.05*	2.48 ± 0.09	3.07 ± 0.16*
Kidney weight (KW) (mg)	2022 ± 140	1939 ± 83	2072 ± 88	2117 ± 41
Relative kidney weight (KW/BW) (mg/g)	6.86 ± 0.42	6.91 ± 0.19	6.50 ± 0.36	6.46 ± 0.13
Males	**3-month-old**		**6-month-old**	
	HanSD (n=8)	**TGR** (n=6)	**HanSD** (n=8)	**TGR** (n=6)
Body weight (BW) (g)	489 ± 10$^{\#}$	504 ± 9$^{\#}$	534 ± 21$^{\#}$	775 ± 39$^{*\#}$
Heart weight (HW) (mg)	1333 ± 60$^{\#}$	1547 ± 46$^{*\#}$	1413 ± 32$^{\#}$	2110 ± 43$^{*\#}$
Relative heart weight (HW/BW) (g/mg)	2.72 ± 0.10$^{\#}$	3.08 ± 0.10$^{*\#}$	2.67 ± 0.10$^{\#}$	2.75 ± 0.13$^{\#}$
Left ventricular weight (LVW) (mg)	1066 ± 48$^{\#}$	1268 ± 38$^{*\#}$	1135 ± 27$^{\#}$	1765 ± 32$^{*\#}$
Relative left ventricular weight (LVW/BW) (mg/g)	2.18 ± 0.12$^{\#}$	2.46 ± 0.08$^{*\#}$	2.14 ± 0.07$^{\#}$	2.30 ± 0.11$^{\#}$
Kidney weight (KW) (mg)	2955 ± 108$^{\#}$	3152 ± 69$^{\#}$	2398 ± 79$^{\#}$	4455 ± 131$^{*\#}$
Relative kidney weight (KW/BW) (mg/g)	6.04 ± 0.16$^{\#}$	6.32 ± 0.15	4.51 ± 0.12$^{\#}$	5.85 ± 0.38*

All values are mean ± SEM. Significantly different: * $P \leq 0.05$ *vs.* age-matched HanSD, $^{\#}$ $P \leq 0.05$ *vs.* age-matched females; n, number of animals is given in parentheses.

T**able 2.** Thiobarbituric acid-reactive substances of 3- and 6-month-old female and male Hannover Sprague-Dawley (HanSD) and Ren-2 transgenic (TGR) rats.

Females	**3-month-old**		**6-month-old**	
	HanSD (n=5)	**TGR** (n=5)	**HanSD** (n=5)	**TGR** (n=5)
Heart (µmol/mg protein)	33.91 ± 6.78	33.81 ± 8.84	28.82 ± 2.62	34.72 ± 5.73
Kidney (µmol/mg protein)	38.59 ± 2.20	41.00 ± 1.33	34.60 ± 1.11	34.40 ± 1.54
Liver (µmol/mg protein)	24.09 ± 4.15	26.58 ± 5.29	25.66 ± 1.93	23.06 ± 2.35
Males	**3-month-old**		**6-month-old**	
	HanSD (n=8)	**TGR** (n=6)	**HanSD** (n=8)	**TGR** (n=6)
Heart (µmol/mg protein)	46.40±7.72	34.60±7.98	30.43 ± 5.08	27.29 ± 0.98
Kidney (µmol/mg protein)	45.02 ± 5.99	42.20 ± 3.67	32.16 ± 2.00	30.28 ± 2.34
Liver (µmol/mg protein)	24.37±1.96	24.91±1.35	26.94 ± 2.89	35.28 ± 2.14$^{*\#}$

All values are mean ± SEM. Significantly different: * $P \leq 0.05$ *vs.* age-matched HanSD, $^{\#}$ $P \leq 0.05$ *vs.* age-matched females; n, number of animals is given in parentheses.

Table 3. Concentration of thiol (-SH) groups in heart, kidney and liver of 3- and 6-month-old female and male Hannover Sprague-Dawley (HanSD) and Ren-2 transgenic (TGR) rats.

Females	**3-month-old**		**6-month-old**	
	HanSD (n=5)	**TGR** (n=5)	**HanSD** (n=5)	**TGR** (n=5)
-SH in the heart (µmol/g)	2.42 ± 0.20	2.21 ± 0.09	2.57 ± 0.18	2.24 ± 0.14
-SH in the kidney (µmol/g)	4.06 ± 0.16	4.08 ± 0.13	4.59 ± 0.19	4.62 ± 0.14
-SH in the liver (µmol/g)	6.81 ± 0.19	8.04 ± 0.16*	5.11 ± 0.48	7.20 ± 0.27*
Males	**3-month-old**		**6-month-old**	
	HanSD (n=8)	**TGR** (n=6)	**HanSD** (n=8)	**TGR** (n=6)
-SH in the heart (µmol/g)	2.26 ± 0.05	2.36 ± 0.11	2.43 ± 0.08	2.20 ± 0.03*
-SH in the kidney (µmol/g)	3.16 ± 0.17$^{\#}$	4.08 ± 0.19*	3.44 ± 0.12$^{\#}$	3.46 ± 0.08$^{\#}$
-SH in the liver (µmol/g)	4.41 ± 0.41$^{\#}$	6.44 ± 0.51$^{*\#}$	5.39 ± 0.31	6.55 ± 0.28*

All values are means ± SEM. Significantly different: * $P \leq 0.05$ *vs.* age-matched HanSD, $^{\#}$ $P \leq 0.05$ *vs.* age-matched females; n, number of animals is given in parentheses.

T**able 4.** Lipid profile of 3- and 6-month-old female and male Hannover Sprague Dawley (HanSD) and Ren-2 transgenic (TGR) rats.

Females	**3-month-old**		**6-month-old**	
	HanSD (n=5)	**TGR** (n=5)	**HanSD** (n=5)	**TGR** (n=5)
Serum TG *(mmol/l)*	0.32±0.03	0.26±0.02	0.18±0.01	0.29±0.04
Total TC *(mmol/l)*	1.61±0.09	1.70±0.07	1.55±0.27	1.68±0.11
HDL *(mmol/l)*	1.11±0.04	1.29±0.06	1.17±0.18	1.39±0.11
LDL *(mmol/l)*	0.44±0.02	0.39±0.03	0.30±0.07	0.21±0.03
TC/HDL ratio	1.50±0.04	1.32±0.04*	1.31±0.05	1.21±0.03
Males	**3-month-old**		**6-month-old**	
	HanSD (n=8)	**TGR** (n=6)	**HanSD** (n=8)	**TGR** (n=6)
Serum TG *(mmol/l)*	0.35±0.06	0.75±0.06$^{*\#}$	0.36±0.06$^{\#}$	0.63±0.04$^{*\#}$
Total TC *(mmol/l)*	2.05±0.25	1.97±0.17	2.03±0.17	1.78±0.11
HDL *(mmol/l)*	1.41±0.32$^{\#}$	1.32±0.08	1.23±0.10	1.10±0.05$^{\#}$
LDL *(mmol/l)*	0.60±0.18	0.51±0.07	0.71±0.07$^{\#}$	0.54±0.07$^{\#}$
TC/HDL ratio	1.45±0.12	1.49±0.04$^{\#}$	1.65±0.04$^{\#}$	1.61±0.05$^{\#}$

All values are mean ± SEM. Significantly different: * $P \leq 0.05$ *vs.* age-matched HanSD, $^{\#}$ $P \leq 0.05$ *vs.* age-matched females; n, number of animals is given in parentheses; TG, triglycerides; TC, total cholesterol; HDL, high-density lipoprotein-cholesterol; LDL, low-density lipoprotein-cholesterol.